UNIVERSITY OF SOUTHAMPTON

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES
Biological Sciences

INVESTIGATION OF THE EFFECTS OF DIFFERENT mTOR INHIBITORS ON PROTEIN SYNTHESIS

by

Yilin Huo

Thesis for the degree of Doctor of Philosophy
December 2011

UNIVERSITY OF SOUTHAMPTON

ABSTRACT

FACULTY OF NATURAL AND ENVIRONMENTAL SCIENCES
Biological Sciences
Doctor of Philosophy

INVESTIGATION OF THE EFFECTS OF DIFFERENT mTOR INHIBITORS ON PROTEIN SYNTHESIS

by Yilin Huo

The mammalian target of rapamycin (mTOR), which controls diverse cellular processes, is regulated by the integration of many signals. Rapamycin strongly inhibits the proliferation of many cancer cell lines and there is a high level of interest in its potential use as an anti-cancer agent. However, some tumours and cancer cells are resistant to rapamycin. This has prompted the development of mTOR kinase inhibitors (mTOR-KIs), such as PP242 and AZD8055, which compete with ATP for binding to the kinase domain in mTOR. In this research, I have studied whether the effects of mTOR-KIs on cell signalling and protein synthesis differed in comparison to those of rapamycin. My data shows that mTOR-KIs have strikingly different effects on proteins (including formation of the eIF4F translation factor complex) that control mRNA translation. Furthermore, while rapamycin only has a very small inhibitory effect on the rate of protein synthesis, mTOR-KIs have a much bigger effect. A new mass spectrometric approach, 'pSILAC', was applied to explore the effects of rapamycin and mTOR-KIs on the synthesis of specific proteins. The data from pSILAC reveal (i) mTOR-KIs impair synthesis of many proteins; (ii) rapamycin always inhibits less than mTOR-KIs; (iii) their effects are strongest for proteins encoded by 5'-TOP mRNAs, but mTOR-KIs again inhibit more strongly; (iv) synthesis of some other proteins which are not encoded by known 5'-TOP mRNAs shows a similar pattern of inhibition to 5'-TOP mRNAs. These data show that pSILAC is a valuable tool for studying the control of the synthesis of specific proteins. I have also investigated the effects of disruption of eukaryotic translation initiation factor 4E (eIF4E) phosphorylation on (i) its modification by SUMO-1 (ii) TNFα biosynthesis in macrophages and (iii) the interaction with specific mRNAs encoding protumourigenic factors.

List of contents

List of Figures

List of tables

DECLARATION OF AUTHORSHIP

I, Yilin Huo, declare that the thesis entitled Investigation of the effects of different on mTOR inhibitors on protein synthesis and the work presented in the thesis are both my own otherwise stated, and have been generated by me as the result of my own original research. I confirm that:

- this work was done wholly or mainly while in candidature for a research degree at this University;
- where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
- where I have consulted the published work of others, this is always clearly attributed;
- where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
- I have acknowledged all main sources of help;
- where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
- parts of this work have been published as: *HUO, Y., IADEVAIA, V. & PROUD, C. G. 2011. Differing effects of rapamycin and mTOR-KIs on protein synthesis. Biochem Soc Trans, 39, 446-50*

Signed: ……

Date:……

ACKNOWLEDGEMENTS

First and foremost, I would like to thank my supervisor, Professor Chris Proud for giving me the opportunity to study for this PhD and his expert advice and support throughout my PhD study. I would also like to thank European Union and AstraZeneca for their funding on this project, and the Secretary of State for Education and Science and University of Southampton for their funding on my study.

I would like to thank all the previous and present members of the lab for their support and company over the past three years. A special thanks to Dr. Xuemin Wang and Dr. Valentina Iadevaia for guiding me through the difficult steps of my research.

I would like to thank those who proofread this thesis, Dr. Noel Wortham, Dr. Justin Kenney, Dr. Valentina Iadevaia, and Dr Joanne Cowan. Without your kind help, this thesis could not be submitted on time.

I would like to thank my parents for their unconditional support all the time. Finally, I would like to dedicate this thesis to Chuan Wang and give him a big thank you for giving me all the support and help.

ABBREVIATIONS

β-GP: β-glycerolphosphate

β-ME: β-mercaptoethanol

2D: 2-dimensional

2-DG: 2-Deoxyglucose

4E-BP: eIF4E-binding protein

5'-TOP: 5'-terminal tract of oligopyrimidines

AA: amino acid

AICAR: 5'-aminoimidazole-4-carboxamide riboside

AMP: adenosine monophosphate

AMPK: AMP-activated protein kinase

APS: ammonium persulphate

ARNT: aryl hydrocarbon receptor nuclear translocator

ATG: autophagy-specific gene

ATM: ataxia telangiectasia mutated

ATP: adenosine triphosphate

BAD: Bcl2-associated agonist of cell death

Bcl-2: B-cell CLL/lymphoma 2

BCA: bicinchoninic acid

BH3: Bcl-2 homology domain 3

BSA: bovine serum albumin

cPLA2: cytoplasmic phospholipase A2

DEPTOR: DEP domain containing mTOR-interacting protein

DMEM: Dulbecco's modified Eagle's medium

DMSO: dimethyl sulphoxide

dNTPs: deoxynucleoside triphosphates

DTT: dithiothreitol

eEF: eukaryotic elongation factor

eIF: eukaryotic initiation factor

eIF4F: eukaryotic initiation factor 4F complex

EDTA: ethylenediaminetetraacetic acid

EGTA: ethylene glycol tetraacetic acid

ER: endoplasmic reticulum

eRF: eukaryotic release factor

Erk: extracellular signal-regulated protein kinase

FBS: fetal bovine serum

FOXO: forkhead box protein O

GAP: GTPase-activating protein

GAPDH: glyceraldehyde-3-phosphate-dehydrogenase

GCN2: general control nonderepressible-2

GDP: guanosine diphosphate

GSK3: glycogen synthase kinase 3

GTP: guanosine triphosphate

h: hours

HEAT domain: huntingtin, elongation factor 3 (EF3), protein phosphatase 2A (PP2A), and the yeast PI3-kinase TOR1 domain

HEK 293 cells: Human Embryonic Kidney 293 cells

HIF: hypoxia-inducible transcription factor

HnRNP: heterogeneous nuclear ribonucleoprotein

HRE: hypoxia-response elements

HRI: haem-regulated inhibitor kinase

HSPA8: heat shock 70kDa protein 8

IGFs: insulin-like growth factors

IRESs: internal ribosome entry sites

IRS: insulin receptor substrate

LDHB: lactate dehydrogenase B

LKB1: liver kinase B1

LPS: lipopolysaccharide

m^7GTP: 7-methyl-GTP

MAPK: mitogen-activated protein kinase

MCF7 cells: Michigan Cancer Foundation -7 cells

MDM2: murine double minute clone 2

MEK: mitogen-activated protein kinase kinase

Met-tRNAi: Methionyl tRNA specialised for initiation

mLST8: mammalian lethal with SEC13 protein 8

min: minutes

Mnk: mitogen-activated protein kinase-interacting kinase

MO25: mouse protein 25

MOPS: 3-(N-morpholino) propanesulfonic acid

mRNA: messenger RNA

MS: mass spectrometry

mTOR: mammalian target of rapamycin

mTOR-KIs: mTOR kinase inhibitors

NACA: nascent polypeptide-associated complex alpha subunit

NAP1L1: nucleosome assembly protein 1-like 1

NEM: N-ethylmaleimide

NES: nuclear export signal

PABP: poly (A)-binding protein

PBS: phosphate-buffered saline

Pdcd4: programmed cell death 4

PDK: phosphatidyl -dependent kinase

PERK: PKR-like endoplasmic reticulum kinase

PH domain: pleckstrin homology domain

PHD: prolyl 4-hydroxylases

pI: Isoelectric point

PI3K: phosphatidylinositol 3-kinase

PIKK: phosphatidylinositol kinase-related kinase

PIP2: phosphatidylinositol -4, 5- bisphosphate

PIP3: phosphatidylinositol -3, 4, 5- trisphosphate

PKB: protein kinase B

PKC: protein kinase C

PKR: RNA-dependent protein kinase

PMA: phorbol myristate acetate

PMSF: phenylmethylsulfonyl fluoride

PRAS40: proline-rich Akt substrate of 40 kDa

PRDX6: peroxiredoxin 6

PTEN: phosphatase and tensin homolog deleted on chromosome 10

QPCR: quantitative real time polymerase chain reaction

Raptor: regulatory associated protein of mTOR

REDD1: regulated in development and DNA damage response 1

Rheb: ras homolog enriched in brain

Rictor: rapamycin-insensitive companion of mTOR

RIPA: RNP immunoprecipitation assay

RSK: p90 ribosomal S6 kinase

RTK: receptor tyrosine kinase

S6K: p70 ribosomal protein S6 kinase

SDS-PAGE: sodium dodecyl sulphate-polyacrylamide gel electrophoresis

sec: seconds

SGK: serum and glucocorticoid-induced protein kinase

SILAC: stable isotope labelling by amino acids in cell culture

SSC: saline-sodium citrate

STRAD: sterile-20 related adaptor

SUMO: small ubiquitin-like modifier

TCA: trichloroacetic acid

TEMED: tetramethylethylenediamine

TIF-I: transcription initiation factor I

TNFα: tumour necrosis factor α

TKT: transketolase

TOS: TOR-signalling

tRNA: aminoacyl-transfer RNA

TCTP: translationally controlled tumour protein

TSC1/2: tuberous sclerosis complex 1/2

TSS: transcription start site

UBF: upstream binding factor

UTR: untranslated region

VEGFC: vascular endothelial growth factor C

Vps34: vacuolar protein sorting protein 34

CHAPTER 1

INTRODUCTION

1.1 mTOR signalling pathway

1.1.1 The structure and composition of TOR

mTOR, which is also known as FRAP (FKBP-rapamycin-associated protein), RAPT1 (rapamycin and FKBP target) or RAFT1 (rapamycin target), is the mammalian homologue of the yeast TOR (the target of rapamycin) proteins (reviewed in Hay and Sonenberg, 2004). The yeast genes, *TOR1* and *TOR2,* were identified in a genetic screen for resistance to the immunosuppressant drug rapamycin in *Saccharomyces cerevisiae* (Kunz et al., 1993, Helliwell et al., 1994). Rapamycin was first discovered as a natural product of the bacterium *Streptomyces hygroscopicus* in a soil sample from Easter Island — an island also known as Rapa Nui (Vezina *et al.*, 1975). Shortly after the discovery of yeast TORs, the mammalian TOR (mTOR) was identified and cloned (Brown *et al.*, 1994, Chiu *et al.*, 1994, Sabatini *et al.*, 1994). mTOR is a large (~289 kDa) atypical kinase belonging to the phosphatidylinositol kinase-related kinase (PIKK) family. Like other members in this family, mTOR contains a C-terminal kinase domain (Fig. 1.1), which has significant homology to the lipid kinase phosphatidylinositol 3-kinase (PI3K). However, unlike PI3K, mTOR and the remaining PIKK members are exclusively protein kinases. The C-terminal end contains a FATC (for FRAP, ATM, TRRAP) domain that might have a role in its redox-dependent regulation and stability (Peterson *et al.*, 2000, Dames *et al.*, 2005). Immediately upstream of the kinase domain is the FRB (FKBP12/rapamycin binding) domain and the relatively large FAT domain. In the N-terminal half of mTOR, there are a large number of tandem HEAT (originally found in Huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast PI3-kinase TOR1) repeat subunits (Andrade and Bork, 1995). Each HEAT repeat consists of two α helices of 37~43 amino acids that form a rod-like helical structure and have been implicated in protein-protein interactions (Andrade and Bork, 1995).

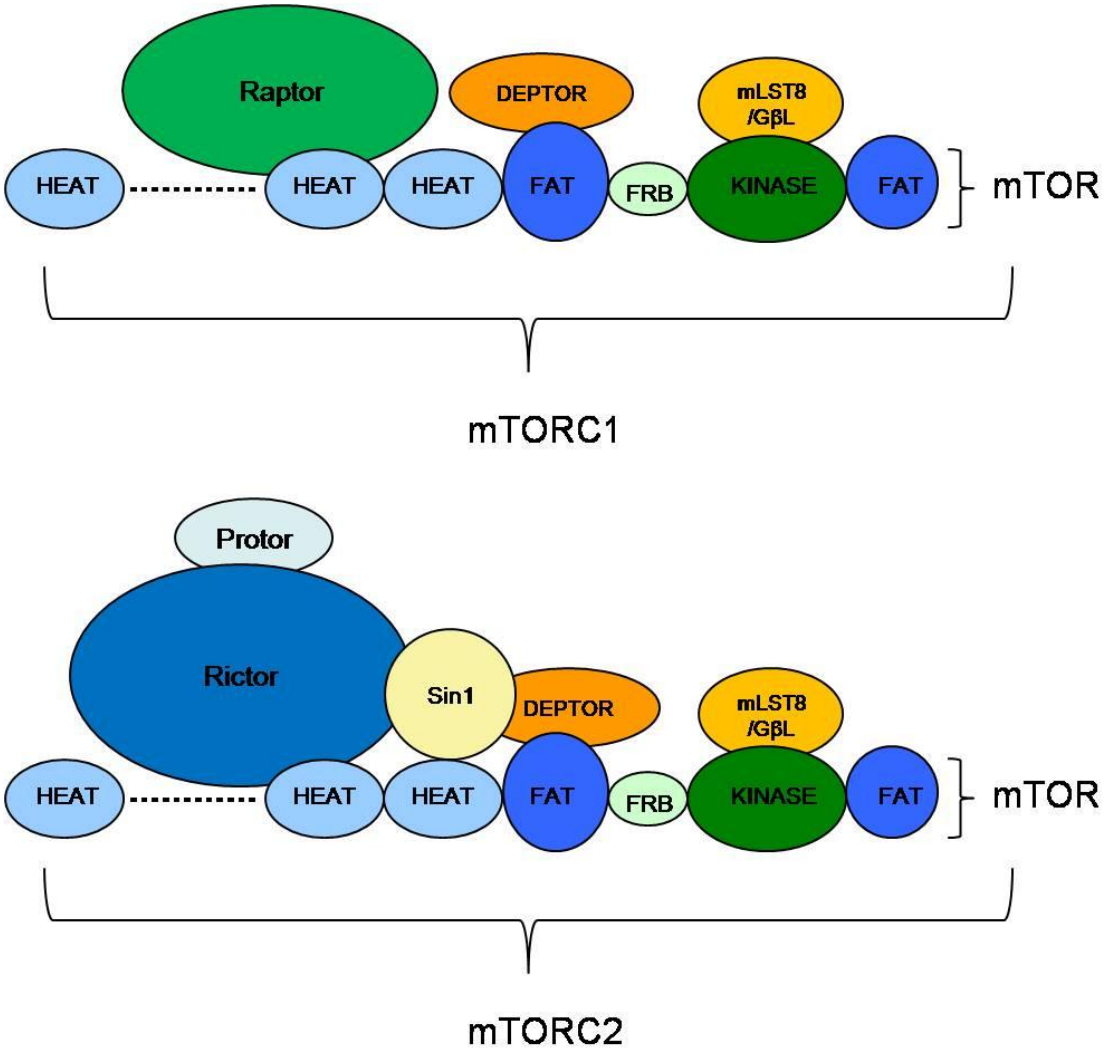


Figure 1.1. Two mTOR complexes.

A large number of tandem HEAT (originally found in Huntingtin, elongation factor 3, protein phosphatase 2A, and the yeast PI3-kinase TOR1) tandem repeats are located in the C-terminal of mTOR. mTORC1 contains the core component mTOR, the large protein Raptor and mLST8/GβL; mTORC2 also contains mTOR and mLST8/GβL, but it associates with several unique subunits: Rictor, Sin1 and Protor. FAT: a domain structure shared by FRAP, ATM, TRRAP; FRB: FKBP12/rapamycin binding domain; Raptor: regulatory associated protein of mTOR; Rictor: rapamycin-insensitive companion of mTOR; mLST8: mammalian lethal with SEC13 protein 8; Sin1: stress-activated protein kinase-interacting protein 1. Protor: protein observed with Rictor-1. DEPTOR: DEP domain containing mTOR-interacting protein.

mTOR exists as at least two heteromeric complexes, mTORC1and mTORC2, which are differentially regulated, have distinct substrate specificities, and are differentially sensitive to rapamycin (Fig. 1.1). mTORC1 contains the core component mTOR, the large protein Raptor (Hara *et al.*, 2002, Kim *et al.*, 2002) and mLST8/GβL (Kim et al., 2003; Fig. 1.1). Raptor is an evolutionarily-conserved protein which contains a novel N-terminal domain (named RNC), followed by three HEAT repeats and seven WD40 repeats (approximately 40 amino acids with conserved *W* and *D* forming four anti-parallel β strands) in the C-terminal half (Kim et al., 2002, Neer et al., 1994). Raptor has been proposed to interact with mTOR through multiple contact points (multiple regions on Raptor and at least the HEAT repeats of mTOR; Kim et al., 2002, Yang and Guan, 2007). Amino acid withdrawal (mimicked by removing leucine from the culture medium) markedly increased the amount of Raptor bound to mTOR and this effect was reversed by a 10 min addition of leucine (Kim *et al.*, 2002). Kim *et al.* (2002) have reported that increased Raptor binding to mTOR negatively regulated mTOR kinase activity through decreasing S6K1 phosphorylation and increasing the amount of 4E-BP1 bound to eIF4E. However, Raptor has also been reported to be a scaffolding protein to recruit substrates for mTOR and positively modulates the mTOR kinase reaction *in vivo* (Hara *et al.*, 2002)

mLST8/GβL was identified later than Raptor and possibly associates with the kinase domain of mTOR (independently of Raptor; Kim et al., 2003). Like Raptor, the 36-kDa mLST8/GβL is conserved among all eukaryotes. Because of the possible interaction with the kinase domain of mTOR, mLST8/GβL was initially proposed to regulate mTOR kinase activity. For example, knocking down mLST8/GβL in cell culture resulted in decreased phosphorylation of S6K1 and 4E-BP1 (Kim *et al.*, 2003). However, more recent studies indicate that the precise role of mLST8/GβL remains ambiguous. Guertin *et al.* (2006) found that basal phosphorylation of S6K1, ribosomal protein S6 or 4E-BP1 was normal in mLST8 null mouse embryo fibroblasts (MEFs). Also, mLST8/GβL was found not necessary for S6K1 phosphorylation in response to insulin or serum stimulation. Furthermore, the loss of mLST8/GβL does not alter the regulation of mTORC1 signalling by rapamycin or nutrients (Guertin et al., 2006). Further studies are needed to elucidate the exact role of mLST8/GβL in mTORC1 signalling.

mTORC2 was discovered more recently and its function is not yet fully understood because of the lack of an effective mTORC2 inhibitor. Compared to mTORC1,

mTORC2 has a distinctive composition and physiological role. mTORC2 also contains mTOR and mLST8/GβL, but it associates with several unique subunits: rapamycin-insensitive companion of mTOR (Rictor, also known as mAVO3), Sin1 (also named as Mip1) and Protor. Rictor is a large protein (~190 kDa) and was first identified as a unique component of mTORC2 (Jacinto *et al.*, 2004, Sarbassov *et al.*, 2004). It shares regions of homology with pianissimo from *D. discoidieum*, STE20p from *S. pombe*, and AVO3p from *S. cerevisiae* (Sarbassov *et al.*, 2004). The interaction between Rictor and mTOR is not affected by short-term rapamycin treatment (Sarbassov et al., 2004). Rictor has also been implicated in regulating the actin cytoskeleton (Sarbassov *et al.*, 2004). The finding that Rictor null mouse embryos were developmentally delayed and died around e10.5 (embryonic day 10.5) suggests a possible role of Rictor in fetal vascular development (Guertin *et al.*, 2006).

Sin1 is required for mTORC2 assembly and function. Knocking down Sin1 decreases the interaction between mTOR and Rictor in MEFs, supporting the idea that Sin1 is an essential component of mTORC2 (Jacinto et al., 2006). Moreover, loss of Sin1 in MEFs abolishes the phosphorylation of Akt (also known as PKB, serine/threonine protein kinase B) at Ser473 (mTORC2 is the kinase for AKT Ser473 phosphorylation in the hydrophobic motif; Jacinto et al., 2006, Sarbassov et al., 2005). mLST8/GβL was first identified in mTORC1 and later also found in mTORC2 (Kim *et al.*, 2003). The loss of mLST8/GβL completely eliminates the interaction between mTOR and Rictor in MEFs, indicating that mLST8/GβL is essential to maintain mTORC2 integrity (Guertin *et al.*, 2006). mLST8/GβL-deficient embryos lost mTORC2-dependent Akt phosphorylation at Ser473 and also died around e10.5 like Rictor null mice (Guertin *et al.*, 2006). Protor was identified as a novel Rictor-binding subunit of mTORC2 (Pearce *et al.*, 2007). Protor-1 and Protor-2 are two closely related isoforms which are encoded by different genes and interact with Rictor through a conserved N-terminal region (Pearce *et al.*, 2007, Woo *et al.*, 2007, Thedieck *et al.*, 2007). Pearce *et al*. (2011) have recently suggested that Protor-1 may play a role in enabling mTORC2 to efficiently activate SGK1, at least in the kidney.

DEPTOR (DEP domain containing mTOR-interacting protein) is described as an mTOR-interacting protein which normally inhibits the mTORC1 and mTORC2 pathways (Peterson et al., 2009). Unlike mTORC1, mTORC2 is resistant to inhibition by short-term treatment of rapamycin. However, prolonged treatment of rapamycin can inhibit mTORC2 assembly and function in some cell lines (Sarbassov *et al.*, 2006).

1.1.2 mTOR signalling network

1.1.2.1 Signalling through mTORC1

mTORC1 activity is regulated by various inputs including growth factors, nutrients and energy metabolism. Multiple upstream effectors are involved in the regulation. The following gives a detailed description.

1.1.2.1.1 Regulation of mTORC1 by growth factors

The IRS proteins

Insulin or insulin-like growth factors (IGFs) initiate signalling at the insulin receptor (IR) or IGF-receptor (IGFR), which are transmembrane glycoproteins with intrinsic protein tyrosine kinase activities (reviewed in Corradetti and Guan, 2006). Tyrosine phosphorylation mediates many processes by directly controlling the activity of receptors or enzymes at an early stage in signalling pathways, or by organising the assembly of signalling complexes around activated receptors or docking proteins (White, 1998). Binding of insulin or IGFs to their receptors leads to recruitment and phosphorylation of insulin receptor substrate (IRS). IRS-1 was initially identified as a docking protein (White *et al.*, 1985) and other identified members in this family include IRS-2, IRS-3, IRS4, IRS-5 and IRS-6 (reviewed in Mardilovich et al., 2009). This family shares several common domains (Fig. 1.3). IRS-1, which exists in most cell types, has a calculated molecular weight of 132 kDa (White, 1998). IRS-2 is about 10 kDa larger than IRS-1 and is also ubiquitously expressed. These two proteins are primary mediators in the cellular response to microenvironmental stimuli. The N-terminus of IRS-1 and IRS-2 has a well conserved pleckstrin homology (PH) domain that is followed by a phosphotyrosine binding (PTB) domain. Many studies suggest that IRS-1 and IRS-2 may mediate distinct signalling cascades (Withers *et al.*, 1998, Sun *et al.*, 1997, Schubert *et al.*, 2003, Withers *et al.*, 1999). A recent study found that IRS-1 and IRS-2 played opposite roles in breast cancer metastasis (Nagle *et al.*, 2004, Sachdev *et al.*, 2004). IRS-3 also contains a PH domain followed by a PTB domain in its N-terminus. It was recently reported that IRS-3 was located in both the nucleus and cytosol (Kabuta *et al.*, 2010). IRS-4 is only expressed in human and found in brain, kidney, thymus and liver (Lavan *et al.*, 1997). Although possessing conserved PH and PTB domains, IRS-5 and IRS-6 have truncated C-termini and are more distantly

related IRS family members (Mardilovich *et al.*, 2009). A schematic diagram of IRS1-6 domain structures is shown in Fig. 1.2.

The tumour suppressor: PTEN

Once the IRS proteins are bound to cell surface receptors through their PH/PTB domains, they are phosphorylated on tyrosine residues. Subsequently, PI3K binds to IRS and converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3). The accumulation of PIP3 is antagonized by PTEN (phosphatase and tensin homolog on chromosome 10), which removes phosphates from PIP3 to generate PIP2. Discovered by Li *et al.* (1997), PTEN is a 47 kDa protein that contains an N-terminal phosphatase domain with a catalytic core motif. Five phosphorylation sites (Ser370, Ser380, Thr382, Thr383 and Ser385) are located in the C-terminal non-catalytic domain and the phosphorylation of these sites can lead to the degradation of PTEN (Raftopoulou *et al.*, 2004, Torres and Pulido, 2001). The significance of PTEN as a phosphatase and a regulator of the PI3-K signalling pathway has been established over the past few years (Leslie and Downes, 2002, Salmena *et al.*, 2008, Sulis and Parsons, 2003). However, while it is clear that PTEN has many diverse biological effects, some of these effects are not obviously linked to its lipid phosphatase activity, and a number of studies have supported an additional role of PTEN as a protein phosphatase (Gildea *et al.*, 2004, Mahimainathan and Choudhury, 2004, Leslie *et al.*, 2007). *PTEN* was first observed to be mutated in cell lines derived from several cell cancers (Lynch *et al.*, 1997, Risinger *et al.*, 1997, Rhei *et al.*, 1997). Now a wide range of cancers have been found to be accompanied by the deregulation of PTEN, including lung cancer, gastric cancer, thyroid cancer, colorectal cancer and prostate cancer (reviewed in Steelman et al., 2004). Studying the mechanisms of PTEN stimulation or reintroducing functional PTEN may provide novel approaches for cancer therapy.

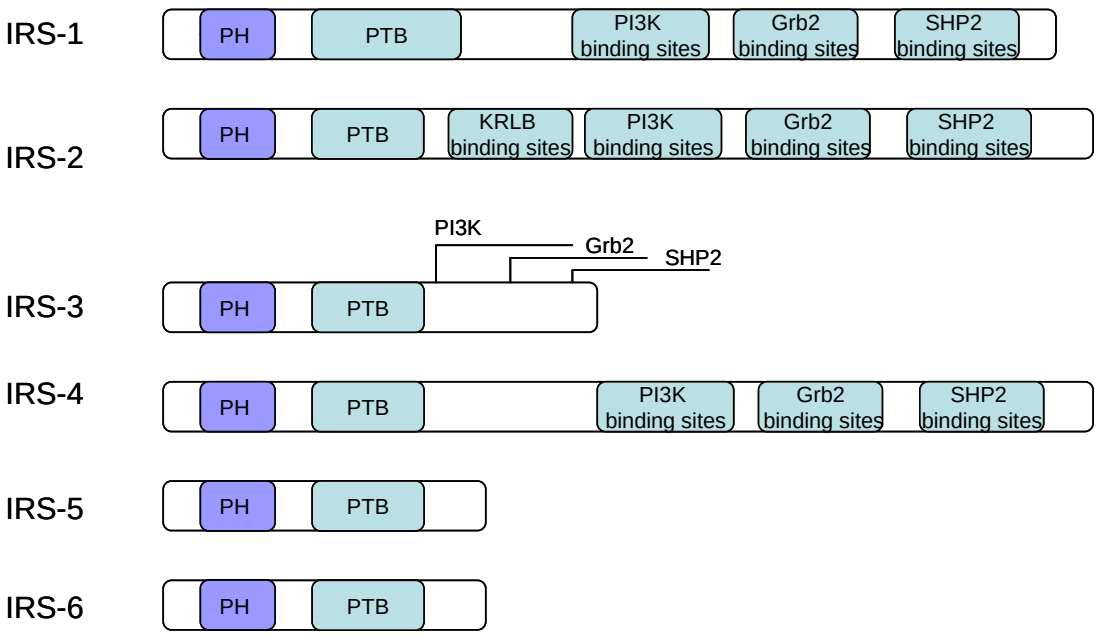


Figure 1.2. IRS protein family members.

Interaction domains of the IRS proteins are indicated. The IRS proteins share their homology in their N-termini, which containing two conserved domians, PH and PTB domian. IRS-5 and IRS-6 have truncated C-termini. PH: pleckstrin homology domain; PTB: phosphotyrosine binding domain; KRLB: kinase regulatory loop binding sites; Grb2: growth factor receptor-binding protein-2 binding site; SHP2: SH2-containing tyrosine phosphatase binding site.

PDK1

PIP3, the lipid product of PI3K, recruits Akt to the plasma membrane through its PIP3 binding PH domain (Corradetti and Guan, 2006). Whether PDK1 also translocates to the plasma membrane is controversial (McManus *et al.*, 2004, Currie *et al.*, 1999). The binding of Akt to PIP3 can induce a conformational change that either generates a PDK1 interaction site and/or exposes Thr308 in Akt. Both PDK1 and Akt belong to the AGC (protein kinase A/protein kinase G/protein kinase C) kinase family, which also includes PKA, PKG, PKC, S6Ks, serum and glucocorticoid-induced protein kinase (SGK) and p90 RSKs. PDK1 is a 556-amino acid containing enzyme possessing a kinase domain at its N-terminus (residue 70-539) and a PH domain at its C-terminus (residues 459-550) (Alessi et al., 1997a). PDK1 also possesses another regulatory hydrophobic PIF pocket (Biondi, 2004).

PDK1 knockout mice are embryonic lethal, suggesting the importance of PDK1 in controlling cellular responses, especially in regulating AGC family signal transduction (Lawlor *et al.*, 2002). Because it is not possible to identify the downstream effectors of PDK1 using the PDK1 knockout mice, mice with a mutant PH domain (Lys465Glu) were generated (Bayascas *et al.*, 2008), in which the non-PIP3 binding form of PDK1 was expressed at physiological levels. Embryonic stem (ES) cells from these mutant mice were stimulated with IGF1, resulting in weak activation of Akt. This reveals that the binding of PDK1 to PIP3 is essential to activate Akt effectively. However, the activation of Akt in these mutant mice was not abolished, and was still detected at approximately 25% of the wild type levels. The crystal structure of the isolated PDK1 PH domain from these mutant mice revealed that the phosphoinostide binding sites were disrupted completely, suggesting that a pool of PDK1 constitutively associated with the plasma membrane may exist (Bayascas, 2008, Bayascas *et al.*, 2008).

The central regulator: Akt

Once activated, Akt is transiently localized to the plasma membrane and phosphorylates numerous targets to promote cell growth and survival. The Akt family is comprised of three highly homologous isoforms: Akt1 (PKBα), Akt2 (PKBβ) and Akt3 (PKBγ) (Jones *et al.*, 1991b, Jones *et al.*, 1991a, Konishi *et al.*, 1995, Brodbeck *et al.*, 1999). These proteins are products of three separate genes located on distinct chromosomes and are widely expressed with a few isoform-specific features. All the isoforms have a conserved PH domain in their N-terminus, a central-located kinase

domain, and a C-terminal regulatory domain. As discussed above, Akt interacts with the lipid products of PIP3 via its PH domain, demonstrating the important function of the PH domain in recognition by upstream kinases. The central kinase domain shares high homology with other members of AGC family. In the region of C-terminus, all isoforms possesses the F-X-X-F/Y-S/T-Y/F hydrophobic motif (where X is any amino acid; Song et al., 2005). In mammals, this motif is identical (FPQFSY) and deletion of this motif abolishes the enzymatic activity of Akt (Andjelkovic *et al.*, 1997).

Indeed, many studies have shown that agonists activate AGC kinases by introducing their phosphorylation at three highly conserved residues. The three sites are located in the activation loop in the kinase domain, in the middle of a tail/linker region C terminal to the kinase domain, and within a hydrophobic motif (HM) at the end of the tail region (Hauge *et al.*, 2007). In the case of Akt, it can be phosphorylated at Thr308 in the activation loop (also known as T-loop) by PDK1 (Alessi et al., 1997b, Stokoe et al., 1997) and Ser473 in the hydrophobic motif (HM) by PDK2 or mTORC2 (Sarbassov et al., 2005). Akt has another threonine phosphorylation site in the turn motif (TM) (Hiraoka *et al.*, 2011).

The opinions on the interplay between the two sites Ser473 and Thr308 appear diverse. Transient disruption of *Rictor* in mice ablates Akt phosphorylation of both Ser473 and Thr308 (Yang *et al.*, 2006) while in the MEFs derived from Rictor null mice, there was little effect on the phosphorylation of Akt at Thr308. Furthermore, regulation of Akt Thr308 phosphorylation by insulin remains normal in mTORC2-deficient MEFs (Guertin *et al.*, 2006). These results indicate two different models. One is that Ser473 phosphorylation is important for Thr308 phosphorylation (perhaps as a docking site). The other is that these two phosphorylation sites are independent of each other. Although the interplay between Ser473 and Thr308 still needs further investigation, Akt does need the phosphorylation at both sites to reach maximum activity (Alessi *et al.*, 1996). It has been suggested that Thr308 is indispensable for kinase activity, whereas Ser473 enhances Akt activity by around 5-fold (Andjelkovic *et al.*, 1997).

The studies in Akt isoform-specific knockout mice indicate that the diverse effects of Akt might be due to the different functions among the isoforms. Disruption of the *Akt1* gene in mice led to growth retardation and the cells displayed higher rates of apoptosis, indicating an essential role for Akt1 in cell normal growth (Chen *et al.*, 2001). Male *Akt2* null mice exhibited insulin resistance and progressed to a severe

form of type II diabetes accompanied by pancreatic β cell failure (Garofalo *et al.*, 2003), which suggests a critical role for *Akt2* in the maintenance of glucose homeostasis. In contrast to *Akt1* or *Akt2* null mice, *Akt3* null mice are viable and have normal growth and glucose metalism, however, they display impaired brain development (a reduction in brain size and weight in AKT3 null mice at the age of 1 month; Garofalo et al., 2003). Although these studies strongly demonstrate the distinct roles of Akt isoforms in growth regulation, glucose homeostasis and brain development, they do possess some overlapping functions. For example, like *Akt1* null mice, mice lacking *Akt2* exhibited slow growth rate, showing that Akt2 also participates in the regulation of growth.

Manning *et al.* (2007) have done a careful search of the literature examining reported Akt substrates and summarized the characteristics and experimental evidence of 18 substrates. The reported substrates include Bcl2-associated agonist of cell death (BAD), forkhead box protein O (FOXO), glycogen synthase kinase 3 (GSK3), proline-rich Akt substrate of 40 kDa (PRAS40), tuberous sclerosis complex 2 (TSC2), and murine double minute clone 2 (MDM2; HDM2 in humans) which are implicated in diverse cellular functions. For example, Akt promotes cell survival by blocking the function of proapoptotic effectors like the BH3 (Bcl-2 homology 3)-only proteins such as BAD, which exert their proapoptotic effects by binding to and inactivating prosurvival Bcl-2 family members (Datta *et al.*, 1997, del Peso *et al.*, 1997, Datta *et al.*, 2000). Akt can also inhibit the expression of the BH3-only proteins through effects on transcription factors, such as FOXO1, FOXO3a and FOXO4. The phosphorylation of FOXO displaces it from its targets and induces export from the nucleus. Through this mechanism, Akt blocks FOXO-induced transcription of various proapoptotic genes and finally enhances cell survival (reviewed in Tran et al., 2003). MDM2 is an E3 ubiquitin ligase which is a key regulator of the transcription factor P53. Akt also phosphorylates MDM2 and promotes its translocation to the nucleus, which triggers P53 degradation (Zhou *et al.*, 2001). GSK3 (glycogen synthase kinase 3) is another important target of Akt. GSK3 has two isoforms, GSK3α and GSK3β, which have extensive homology, especially at the kinase domain. Akt phosphorylates GSK3α at Ser21 and GSK3β at Ser9 and this phosphorylation results in inactivation of GSK3 (Cross *et al.*, 1995). Inactive GSK3 relieves glycogen synthase (GS) inhibition and eIF2B phosphorylation, leading to active forms of GS and eIF2B, which results in increased glycogen and protein synthesis (reviewed in Rayasam et al., 2009) .

Another important effect of Akt is its role in promoting cell growth. The predominant mechanism appears to be through mTORC1. The tuberous sclerosis proteins, TSC1 (hamartin) and TSC2 (tuberin), have been shown to be the critical negative regulators of mTORC1 signalling (Gao and Pan, 2001, Tapon *et al.*, 2001). Tuberous sclerosis is an autosomal dominant genetic disease which frequently causes non-malignant tumours (termed harmatomas) in multiple organ systems like the brain (Ridler *et al.*, 2004), kidney (O'Hagan *et al.*, 1996), heart (Webb *et al.*, 1993) and skin (Hamilton and Tonkovic-Capin, 2011). A combination of symptoms may include developmental delay, learning difficulties, behavioural problems and skin abnormalities. This disease is caused by a mutation in genes encoding either TSC1 or TSC2. About 400 different mutations of the *TSC* genes have been described (Cheadle *et al.*, 2000). TSC1 and TSC2 interact through their N-termini and form a complex in cells. In this complex, TSC1 stabilizes TSC2 by preventing its degradation (Chong-Kopera *et al.*, 2006), whereas TSC2 works as a GTPase-activating protein for the small GTPase Rheb (Ras homolog enriched in brain). By promoting the conversion of the active form Rheb-GTP into the inactive form Rheb-GDP, the TSC1/2 complex inhibits mTORC1 and its downstream signalling (reviewed in Manning and Cantley, 2003). Therefore, TSC1/2 is a critical negative regulator of mTOR. Although functions for either individual TSC1 or TSC2 have been proposed, whether they can work independently from the complex is still controversial. Overexpression of TSC1 or TSC2 alone in *Drosophila* is not able to induce a phenotype, but overexpression of both genes can dramatically reduce cell growth (Potter *et al.*, 2001). The phenotype induced by loss of TSC1 is identical to that due to loss of TSC2 (Zhang *et al.*, 2003a). TSC2 is dramatically reduced in TSC1-null cells (Zhang *et al.*, 2003a).

Inoki *et al.* (2002) and and Manning *et al.* (2002) found that Akt directly phosphorylated two sites (Ser939 and Thr1462) on TSC2 in mammalian cells, which are conserved and phosphorylated in *Drosophila* TSC2. A mutant of TSC2, in which Ser939 and Thr1462 residues are substituted by alanine, can block growth factor-induced S6K1 activation (Manning *et al.*, 2002), suggesting the phosphorylation of TSC2 mediated by Akt is required to activate mTORC1. Moreover, in TSC2 null cells, both S6K and 4E-BP1 are constitutively phosphorylated, which is sensitive to the inhibition of rapamycin. Despite the abundance of data implying that Akt functions by relieving the inhibitory effect of TSC1/2 on mTOR, the mechanism by which Akt phosphorylation affects the function of TSC1/2 is not clear. Some studies suggest that

the phosphorylation on TSC2 disrupts TSC1/TSC2 heterodimer formation (Inoki *et al.*, 2002, Potter *et al.*, 2002). However, other studies do not support this model (Dan *et al.*, 2002). Notably, whether TSC2 phosphorylation by Akt is sufficient to fully activate mTORC1 also remains unresolved.

Apart from TSC2, a second Akt substrate has been found to be involved in the regulation of cell growth by mTORC1. The proline-rich Akt substrate of 40 kDa (PRAS40), which is a 14-3-3 binding protein, was detected using the Akt phospho-substrate antibody (Kovacina *et al.*, 2003). Kovacina *et al.* (2003) showed that (1) PRAS40 was phosphorylated *in vitro* by purified Akt on the same site which was phosphorylated in insulin-stimulated cells; (2) activation of an inducible Akt was sufficient to stimulate the phosphorylation of PRAS40; and (3) cells without Akt exhibit a diminished ability to phosphorylate this protein. Akt directly phosphorylates PRAS40 at Thr246, which leads the binding of this protein to 14-3-3 protein (Kovacina *et al.*, 2003). More importantly, PRAS40 has been found to associate with mTORC1 and inhibit the signalling from mTORC1 to its physiological substrates (Sancak *et al.*, 2007, Vander Haar *et al.*, 2007). These studies place PRAS40 upstream of mTORC1. On the other hand, phorbol esters, which can activate mTORC1, do not induce phosphorylation of PRAS40 at Thr246 (Fonseca *et al.*, 2008). The binding of PRAS40 to 14-3-3 protein, which requires both amino acids and insulin, is inhibited by TSC1/2 and is stimulated by Rheb in a rapamycin-sensitive manner (Fonseca *et al.*, 2008). Knockdown of PRAS40 impairs both the amino acid- and insulin-stimulated phosphorylation of 4E-BP1 and phosphorylation of rpS6, without inhibiting the phosphorylation of Akt or TSC2 (Fonseca *et al.*, 2007). These data indicate that PRAS40 may not be involved in controlling mTORC1, but rather be a downstream target of mTORC1.

Rheb: an activator of mTORC1

Another important finding during genetic screens in *Drosophila* is the identification of Rheb (Ras homolog enriched in brain) as a positive regulator of mTORC1 (Saucedo *et al.*, 2003, Stocker *et al.*, 2003). Rheb GTPases represent a unique family of the Ras superfamily of G-proteins. TSC2 was shown to weakly accelerate the intrinsic rate of GTP hydrolysis of Rheb, converting Rheb from the GTP-bound to the GDP-bound form, which suggests that Rheb is a directly target of TSC2 GAP activity (Tee *et al.*, 2003, Garami *et al.*, 2003, Inoki *et al.*, 2003a). Knocking down Rheb in *Drosophila* S2

cells inhibits S6K1 phosphorylation (Zhang *et al.*, 2003b), while overexpression of Rheb increases S6K1 and 4E-BP1 phosphorylation in the absence of growth factors or in the presence of wortmannin (Inoki *et al.*, 2002, Castro *et al.*, 2003, Garami *et al.*, 2003, Tee *et al.*, 2003). Furthermore, overexpression of a dominant-negative form of Rheb blocks activation of mTOR by growth factors and insulin as measured by p70-S6K activity (Tabancay *et al.*, 2003). In TSC2 null cells, the level of GTP-bound form of Rheb is increased (Garami *et al.*, 2003). All these data strongly suggest that (1) Rheb is a downstream effector of the TSC1/TSC2 heterodimer and that TSC2 negatively regulates its activity as a GAP for Rheb; (2) Rheb is an upstream positive regulator of mTOR.

Strikingly, Akt activity is increased in Rheb-deficient *Drosophila* cells in which TOR activity is decreased (Stocker *et al.*, 2003). Consistent with this finding, Akt activity is decreased in Rheb-overexpressed *Drosophila* cells in which TOR is activated (Stocker *et al.*, 2003). It is possible that the interplay between Rheb and Akt activities is mediated by a feedback loop mechanism through S6K that appears to be conserved in both mammals and *Drosophila.*

MEK/Erk and p90 RSK

The Ras/Raf/MEK/Erk pathway, which is also stimulated upon the binding of extracellular growth factors to their respective transmembrane RTKs (receptor tyrosine kinases), plays important roles in the transfer of proliferative signals from membrane bound receptors. In this pathway, both Erks and the Erk-activated 90-kDa ribosomal S6 kinases (p90 RSKs) have been proposed to control mTORC1 signalling. One report suggests that this involves the direct phosphorylation of TSC2 by Erks (Ma *et al.*, 2005). This report showed that Erk-dependent phosphorylation led to the dissociation of TSC1/2 complex and markedly impaired TSC2 inhibition of S6K. Other studies indicate that TSC2 is phosphorylated and inactivated by p90 RSK (Roux *et al.*, 2004, Rolfe *et al.*, 2005, Ballif *et al.*, 2005). p90 RSK interacts with and phosphorylates TSC2, resulting in increased mTOR signalling to S6K. Besides TSC2 phosphorylation, TSC1 has been indicated as an additional point of regulation in the control of MEK/Erk over mTORC1 (Fonseca *et al.*, 2011). Fonseca *et al.* also showed that MEK/Erk are likely required (whereas p90 RSK are dispensable) for mTORC1 activation in human embryonic kidney (HEK) 293 cells by phorbol esters.

Activation of p90 RSKs is reportedly required for rpS6 phosphorylation downstream of the Ras/Erk signalling cascade. Roux *et al.* (2007) demonstrated that while S6K1 phosphorylates rpS6 at all sites, p90 RSK exclusively phosphorylates rpS6 at Ser235/236 *in vitro* and *in vivo* using an mTOR-independent mechanism. It has also been reported that p90 RSK phosphorylates Raptor and thereby activates mTORC1 signalling (Carriere *et al.*, 2008). Moreover, mTORC1 inhibition leads to Erk activation through a negative feedback loop stemming from S6K1 in solid tumours, indicating the potential of a combined therapeutic approach of combined mTORC1 and MAPK inhibition (Carracedo *et al.*, 2008).

1.1.2.1.2 Regulation of mTORC1 by amino acids.

Amino acids, fundamental nutrients for all cells, are also essential for mTORC1 signalling. In response to amino acid depletion, mTORC1 activity is rapidly abolished (Hara *et al.*, 1998). Among amino acids, leucine and glutamine are two most important for mTORC1 activity (Hara *et al.*, 1998, Nakajo *et al.*, 2005). Although both insulin and amino acids activate mTORC1, amino acids do not activate Akt/PKB signalling (Campbell *et al.*, 1999). Since TSC2 lies downstream of Akt, it was conceivable that the effects of amino acids on mTORC1 were mediated through TSC2. However, it is evident that amino acid depletion still impairs mTORC1 signalling in TSC2 null cells (Roccio *et al.*, 2006, Smith *et al.*, 2005). Nobukuni and colleagues (2005) have observed similar results in TSC2 null MEFs. These findings support the idea that amino acids actually do not signal to mTORC1 through TSC2. Therefore, how do amino acids affect mTORC1?

Role of hVps34 in the amino acid-mediated activation of mTORC1.

Vps34 (vacuolar protein sorting protein 34) is a Class III PI 3-kinase. Vps34 was first identified in a *Saccharomyces cerevisiae* (budding yeast) screen for proteins involved in vesicle-mediated vacuolar protein sorting (Herman and Emr, 1990). Vps34 and its homologue human Vps34 (hVps34; which is encoded by the *PIK3C3* gene), play a positive role in protein breakdown (autophagy) (Kihara *et al.*, 2001). However, Nobukuni *et al.* (2005) have shown that hVps34 positively regulates mTORC1 signalling. They found that overexpression of hVps34 in mammalian cells increased the phosphorylation of S6K1 and 4E-BP1 while knocking down hVps34 by RNA

interference impaired the ability of amino acids to activate mTORC1 (Nobukuni *et al.*, 2005). The two findings concerning the functions of hVps34 in controlling autophagy and mTORC1 appear at odds. Nobukuni *et al.* (2005) suggested one possibility, which is that hVps34 operates through distinct complexes and differentially regulates autophagy and mTORC1. The above observations also suggest that hVps34 functions upstream of mTORC1 in amino acid sensing. Interestingly, ablation of dVps34 has no effect on cell growth mediated by dTORC1 in *Drosophila* fat body (Juhasz *et al.*, 2008). These observations place Vps34 downstream of dTORC1-dependent nutrient signalling in *Drosophila*. Therefore, future studies using *in vivo* mammalian system will be necessary to clarify the role of hVps34 in amino acid-sensitive mTORC1 regulation.

Role of MAP4K3 in the amino acid-mediated activation of mTORC1.

A study using a genome-wide RNA interference approach in *Drosophila* cells identified the homologue of the mammalian protein kinase MAP4K3 (mitogen-activated protein kinase kinase kinase kinase 3) as a positive regulator of TOR signalling (Findlay *et al.*, 2007). Overexpression of MAP4K3 activates phosphorylation of S6K1 at Thr389 and 4E-BP1 at Thr70 without affecting the phosphorylation of Akt at Ser473 in serum-deprived HEK-293T cells. These data demonstrate that MAP4K3 is a specific positive regulator of mTORC1 signalling. Amino acid withdrawal rapidly decreases phosphorylation of S6K1 at Thr389 (Hara *et al.*, 1998), whereas overexpression of MAP4K3 significantly delays the amino acid withdrawal-induced inactivation of S6K1, indicating that S6K1 is partially relieved from the inhibitory effect of amino acid depletion (Findlay *et al.*, 2007). Moreover, MAP4K3 activity is impaired by starving cells of amino acids and enhanced by adding them back. A more recent study has shown that PR61ε, a PP2A-targeting subunit, plays an important role in the regulation of MAP4K3 phosphorylation in response to amino acids (Yan *et al.*, 2010). Together these data indicate that MAP4K3 is involved in the control of mTORC1 signalling by amino acids.

Roles of Rag GTPases in the control of mTORC1 by amino acids

The identification of the Rag small GTPase family is another important development elucidating the molecular mechanism by which amino acids enhance mTORC1 signalling. The mammalian Rag subfamily of GTPases consists of Rag A, B, C, and D,

and belongs to the super-family of Ras-related GTPases (Sekiguchi *et al.*, 2001). RagA or RagB forms a heterodimer with RagC or RagD and functions as a heterodimeric complex. Sancak *et al.* (2008) found that the Rag proteins interacted with Raptor in an amino acid sensitive manner. A RagA/B mutant, which is constitutively bound to GTP, interacted strongly with mTORC1 and its expression within cells made the mTORC1 pathway resistant to amino acid deprivation (Sancak *et al.*, 2008).

How do Rag GTPases regulate mTORC1 activity in response to amino acids? In *in vitro* studies, purified active Rag GTPases are not able to stimulate mTORC1 kinase activity, suggesting that the Rag proteins do not directly stimulate the kinase activity of mTORC1 (Sancak *et al.*, 2008). Moreover, the Rag proteins have been implicated in the movement of mTORC1 to a compartment that also contains its activator Rheb (Sancak *et al.*, 2008). Recently, Sanack *et al.* reported that lysosomal surface is the compartment where the Rag proteins reside (Sancak et al., 2010). Amino acids induce the translocation of mTORC1 to lysosomal membranes, and a complex termed as Ragulator recruits the Rag GTPases to lysosomes. This Rag-Ragulator-mediated translocation of mTORC1 to lysosomal membranes is considered as the key event in amino acid signalling to mTORC1.

1.1.2.1.3 Regulation of mTORC1 by energy signals

The ability of insulin to activate mTOR is impaired by a reduction in cellular ATP levels, suggesting that cellular energy also regulates mTOR activity (Dennis *et al.*, 2001). The AMP-activated protein kinase (AMPK) is a key cellular energy sensor and regulates a large number of cellular responses upon energy starvation. AMPK is activated by increased cellular AMP levels (Hardie *et al.*, 1999). AMPK activation also requires phosphorylation by the tumour suppressor protein LKB1 (Hawley *et al.*, 2003), which forms a complex with sterile-20 related adaptor (STRAD) and mouse protein 25 (MO25), which maintains LKB1 in an active state (Zeqiraj *et al.*, 2009). 5'-aminoimidazole-4-carboxamide riboside (AICAR) can activate AMPK, leading to a dramatic reduction of S6K and 4E-BP1 activity. Also, culturing cells in low glucose caused reduction of mTOR activity (Bolster *et al.*, 2002, Kimura *et al.*, 2003, Krause *et al.*, 2002). It was shown that AMPK regulated mTORC1 via phosphorylation of TSC2 (Inoki *et al.*, 2003b). However, cells lacking TSC2 remain responsive to energy stress, suggesting that additional AMPK substrates may directly or indirectly modulate

mTORC1 activity. AMPK was reported to phosphorylate Raptor on two well-conserved serine residues and the phosphorylation of Raptor by AMPK was required for the inhibition of mTORC1 and cell cycle arrest induced by energy stress (Gwinn *et al.*, 2008). GSK3 has also been implicated in the regulation of mTORC1 by energy signals through its role in TSC2 phosphorylation (Inoki *et al.*, 2006). .

1.1.2.1.4 Regulation of mTORC1 by hypoxia

A reduction in the normal level of tissue oxygen tension (hypoxia) has a profound inhibitory impact on mTORC1 activity (Tinton and Buc-Calderon, 1999, Arsham et al., 2003).

The hypoxia-inducible transcription factor (HIF) plays a central role in oxygen homeostasis. The HIF-1 transcription factor is a heterodimer that consists of the hypoxic response factor HIF-1α and the constitutively expressed aryl hydrocarbon receptor nuclear translocator (ARNT; also known as HIF-1β; Wang and Semenza, 1995). Under hypoxia, HIF-1α becomes stabilized, allowing dimerization with HIF-1β and translocation of the HIF complex into the nucleus, where it associates with promoters bearing hypoxia-response elements (HRE) (Maxwell et al., 1999, Wang and Semenza, 1993). The HIF-1α subunit is regulated by hydroxylation at two proline residues by family members of oxygen-sensitive enzymes, prolyl 4-hydroxylases (PHD) (Cockman *et al.*, 2000). There are also modifications other than hydroxylation that regulate HIF-1α stability and activity, like desumoylation (Cheng *et al.*, 2007). Ataxia telangiectasia mutated (ATM), one of the key transducers of the stress response, has been identified as the product mutated or inactivated in ataxia telangiectasia (AT) patients (Tichy *et al.*, 2010, Ivanov *et al.*, 2009, Lavin, 1999). Interestingly, Cam and colleagues (2010) showed that hypoxia induced ATM kinase activity, and ATM phosphorylated HIF-1α at Ser696 that affected their stabilization.

A 25-kDa protein, regulated in development and DNA damage response 1 (REDD1; also referred to as DDIT4) is also involved in mTORC1 regulation by hypoxia. In response to hypoxia, REDD1 expression is upregulated by HIF-1 (Shoshani *et al.*, 2002). Importantly, REDD1-induced mTORC1 inhibition requires the TSC1/2 complex (Brugarolas *et al.*, 2004, Sofer *et al.*, 2005). TSC2 was previously reported to interact with 14-3-3 proteins (Cai *et al.*, 2006, Shumway *et al.*, 2003). REDD1, which contains a putative 14-3-3 protein binding motif, was proposed directly binding to and

sequestering 14-3-3 proteins away from TSC2 (DeYoung *et al.*, 2008). However, the mechanism by which REDD1 sequesters 14-3-3 proteins is unclear.

1.1.2.1.5 Downstream targets of mTORC1

mTORC1 plays a role in various cellular processes in yeast and in higher eukaryotes. The following mainly describe its effects on translation, transcription, ribosome biogenesis and autophagy.

1.1.2.1.5.1 Translation

Activation of mTORC1 positively stimulates mRNA translation via its downstream substrates: 4E-BPs and S6 kinases (S6Ks). eIF4E is the rate-limiting translation effector which binds to the cap structure, m^7GpppN (where N is any nucleotide), at the 5'-end of mRNA. Phosphorylation of 4E-BP1 by mTORC1 results in its dissociation from eIF4E, promoting the binding of eIF4E to eIF4G. The role of S6Ks in translation has remained controversial.

4E-BPs

While *Drosophila* only expresses one 4E-BP, mammals have been found to contain three 4E-BPs isoforms, namely, 4E-BP1, 4E-BP2 and 4E-BP3, which are encoded by three different genes (Lin *et al.*, 1994, Pause *et al.*, 1994, Poulin *et al.*, 1998, Bernal and Kimbrell, 2000, Miron *et al.*, 2001). 4E-BP3, which exhibits 57% and 59% identity to 4E-BP1 and 4E-BP2, respectively, was the last of the eIF4E-binding proteins to be identified (Poulin *et al.*, 1998). The 4E-BPs compete with eIF4G proteins for an overlapping site on eIF4E, thus blocking the binding of eIF4E to eIF4G and the formation of initiation factor complexes (Haghighat *et al.*, 1995, Mader *et al.*, 1995, Marcotrigiano *et al.*, 1999). The affinities of 4E-BPs to eIF4E are controlled by their phosphorylation state (Pause *et al.*, 1994). Whereas hypophosphorylated 4E-BPs bind with high affinity to eIF4E, the hyperphosphorylation of 4E-BPs prevent this interaction.

Of the three 4E-BPs, 4E-BP1 is by far the best understood. Its phosphorylation occurs at multiple sites. So far, seven phosphorylation sites have been reported in human 4E-BP1 (Thr37, Thr46, Ser65, Thr70, Ser83, Ser101, and Ser112; Fig. 1.3). Phosphorylation of these sites is a complicated hierarchical process, whereby the

phosphorylation at some sites is required for others (Gingras *et al.*, 1999, Gingras *et al.*, 2001, Mothe-Satney *et al.*, 2000, Wang *et al.*, 2003). For example, Thr37/46 phosphorylation is thought to be a priming event, which is required for subsequent phosphorylation of Thr70 followed by Ser65 (Hay and Sonenberg, 2004). Phosphorylation of Thr70 appears to play a major role in the release of 4E-BP1 from eIF4E (Fadden et al., 1997), while phosphorylation of Ser65 may prevent the reassociation of eIF4E and 4E-BP1 (Karim *et al.*, 2001, Lin *et al.*, 1994). Phosphorylation at Ser83 does not significantly contribute to the control of 4E-BP1 *in vivo.* It has been suggested that the phosphorylation of Ser83 is not required for the release of 4E-BP1 from eIF4E (Choi *et al.*, 2003). Ser101 and Ser112 are located in the C-terminus of 4E-BP1, which is far away from the eIF4E-binding sites in the unfolded structure of 4E-BP1 (Wang *et al.*, 2003). These two sites are unique to 4E-BP1, since neither 4E-BP2 nor 4E-BP3 contains residues equivalent to these two sites (Wang *et al.*, 2003). Based on their location in 4E-BP1, the finding that Ser101 and Ser112 are important to the control of 4E-BP1 seems surprising. Ser101 plays a key role in the insulin-induced phosphorylation of Ser65 while Ser112 is required for insulin-induced release of 4E-BP1 from eIF4E (Wang et al., 2003, Heesom et al., 1998). Thus, at least two priming events, phosphorylation of Ser101 as well as of Thr70, are required for phosphorylation of Ser65.

Two regulatory motifs have been identified in the N and C termini of 4E-BP1 (Fig. 1.3). The N terminus contains the "RAIP" motif (Arg–Ala–Ile–Pro, hence RAIP), which lies in the first 24 residues of 4E-BP1 (Tee and Proud, 2002). This motif is found in 4E-BP1 and 4E-BP2, but is absent in 4E-BP3. Studies show that the RAIP motif is required for the phosphorylation of Thr37/46 as well as Ser65 and Thr70 (Beugnet *et al.*, 2003). Because phosphorylation of 4E-BP1 is a rather complicated hierarchical process, it is likely that the impacts of the RAIP motif on phosphorylation of Ser65 and Thr70 are not direct. The extreme C terminus contains a TOR-signalling (TOS) motif that is also found in S6Ks (Schalm and Blenis, 2002), HIF1α (Land and Tee, 2007) and PRAS40 (Wang *et al.*, 2007a). The TOS motif is required for the binding of these proteins to Raptor and therefore regulation by mTORC1. Mutation or deletion of this motif significantly impairs the insulin-stimulated phosphorylation of 4E-BP1 at Ser65 and Thr70 (Wang *et al.*, 2003, Tee and Proud, 2002, Schalm *et al.*, 2003). So far there is no evidence for a direct interaction between Raptor and the RAIP motif.

Figure 1.3. Domain structure of human 4E-BP1.

The RAIP and TOS motifs, the eIF4E-binding sites and seven phosphorylation sites discussed in the text, Thr37, Thr46, Ser65, Thr79, Ser83, Ser101 and Ser112, are presented in this diagram. The N-terminal RAIP motif is required for the phosphorylation of Thr37/46 as well as Ser65 and Thr70. The C-terminal TOS motif is important for the binding of 4E-BP1 to Raptor. RAIP: Arg–Ala–Ile–Pro, hence RAIP; TOS: TOR-signalling motif.

The importance of 4E-BPs in regulating translation is highlighted by their ability to control cell growth, size and proliferation. Expression of a phosphorylation site-defective mutant of 4E-BP1 results in a reduction in cell size whereas coexpression of eIF4E and this mutant of 4E-BP1 blocks the effects of eIF4E on cell size (Fingar *et al.*, 2002). This mutant also inhibits G_1-phase progression (Fingar *et al.*, 2004). Dowling and colleagues (2010) group recently studied the roles of 4E-BPs in mTORC1 signalling. When mTORC1 activity was blocked by Raptor depletion in MEFs, the cells proliferated more slowly than control ones. However, knockout of 4E-BPs rescued the inhibition of cell proliferation caused by Raptor silencing. When Rictor was depleted, knockout of 4E-BPs had no effect on the inhibition of cell proliferation. Their data demonstrate that 4E-BPs mediate cell proliferation through their effects on mTORC1, but not mTORC2.

S6 Kinases

Humans express two similar S6K proteins, which are S6K1, encoded by *RPS6KB1*, and S6K2, encoded by *RPS6KB2* (Gout et al., 1998, Grove et al., 1991, Koh et al., 1999) (Fig. 1.4). Alternative splicing of S6K1 generates two isoforms, a long one, containing 525 amino acids (p85 S6K1) and a short one containing 316 amino acids (p31 S6K1). An alternative translation start site in the N-terminus of p85 S6K1 results in the generation of another protein, the p70 S6K1, which lacks the first 23 amino acids compared to p85 S6K1 and, comprises 502 amino acids (Grove *et al.*, 1991). The amino acid sequence of p85 S6K1 and p70 S6K1 is almost identical except that p70-S6K1 lacks the first N-terminal 23 amino acids which contain an important signal called nuclear localization-signal (NLS). p31 S6K1 is much shorter, only containing the same amino acids from 1-195 but different in the adjacent 121 amino acids. Alternative translation start sites also give rise to two isoforms of S6K2, a long form p56 S6K2 and a short form p54 S6K2 (Fig. 1.4). The long one differs from the short one due to a 13 amino acid extension at its N-terminus (Gout *et al.*, 1998).

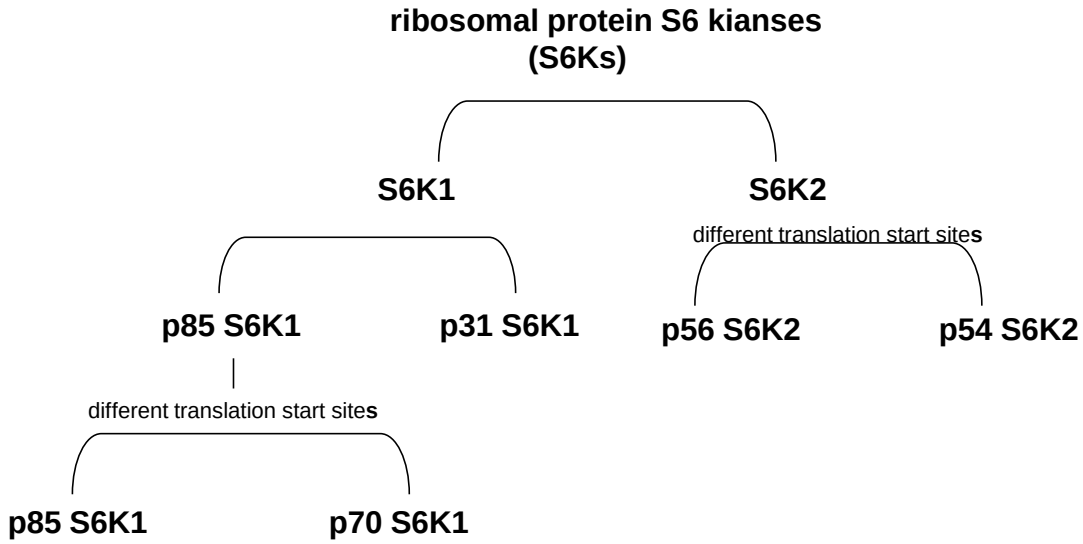


Figure 1.4. The family of S6 kinases.

Humans express S6K1 and S6K2, which are encoded by *RPS6KB1* and *RPS6KB2*, respectively. Alternative splicing of the primary transcript of S6K1 generates two isoforms, p85 S6K1 and p31 S6K1. An alternative translation start site in the N-terminus of P85 S6K1 gives rise to p70 S6K1. S6K2 also has two isoforms, p56 S6K2 and p54 S6K2, due to the alternative translation start sites. Diagram is adapted from an original prepared by Ronser *et al*. (2011a).

Although S6K1 has been widely studied, the nucleocytoplasmic localisation of its isoforms has not been completely clarified. Since p85S6K1 contains a NLS at its N-terminus, it has long been thought of as a nuclear protein. However, using primary non-immortalized, non-transformed human fibroblasts, it has been recently found that p85S6K1 is only present in the cytoplasm (Rosner and Hengstschlager, 2011b). p70-S6K1 was detected in both the cytoplasm and nucleus while p31S6K1 was shown to be nuclear (Rosner and Hengstschlager, 2011a). Both isoforms of S6K2 demonstrate a constitutive nuclear localization (Koh *et al.*, 1999).

S6Ks belong to the AGC family and perform phosphorylation at a series of serine and threonine residues located in different domains. In the case of S6K1, these sites include Thr229, Ser371, Thr389, Ser411, Ser418, Thr421 and Ser424. Among them, Thr229, Ser371, and Thr389 are equivalent to the conserved phosphorylation sites for the AGC family members. T229 is the activation loop (T loop) site and was found to be phosphorylated by PDK1 (Alessi et al., 1997a, Pullen et al., 1998). Ser371 is the turn motif site and Thr389 is the HM (hydrophobic motif) site (Hauge *et al.*, 2007, Davis, 1993). It has been generally accepted that phosphorylation of the HM site primes S6K1 for the phosphorylation of the activation loop, although some data suggest otherwise (Keshwani *et al.*, 2011). As mentioned before, S6Ks also contain a TOS motif (FDIDL in S6K1) like several other mTOR substrates. Raptor binds directly to this motif and mediates the interaction between S6Ks and mTORC1. It is clear that only the HM site of S6Ks (Thr389 in the case of S6K1) is highly sensitive to rapamycin and mTORC1 is responsible for its phosphorylation (Burnett *et al.*, 1998, Isotani *et al.*, 1999).

Many S6K substrates have been reported, such as rpS6, eIF4B, eEF2K, IRS1, Pdcd4 and BAD1 (Raught et al., 2004, Wang et al., 2001, Harrington et al., 2004, Shah et al., 2004, Dorrello et al., 2006, Harada et al., 2001, Ferrari et al., 1991). The best known one is rpS6. There are 5 inducible phosphorylation sites located in the C-terminus of rpS6, which are Ser235, Ser236, Ser240, Ser244 and Ser247 (Bandi *et al.*, 1993, Krieg *et al.*, 1988). While all 5 sites are able to be phosphorylated by p70 S6Ks, Ser235 and Ser236 can also be phosphorylated by p90 RSK (Ferrari *et al.*, 1991, Pende *et al.*, 2004).

The importance of S6Ks in protein synthesis was revealed from genetic studies in mice. The disruption of S6Ks in mice does not affect their viability or fertility, but it significantly slows animal growth and produces a small size phenotype (Shima *et al.*,

1998, Kawasome *et al.*, 1998). The generation of $rpS6^{P}$-/- mice (in which all five phosphorylation sites in S6 were mutated into alanine residues) has provided valuable insight into the physiological roles of S6 phosphorylation. Like S6K knockout mice, $rpS6^{P}$-/- mice display a small cell size phenotype, although they are viable and fertile (Ruvinsky *et al.*, 2005, Ruvinsky *et al.*, 2009). These mice also show a reduction in β-cells and impaired glucose tolerance, suggesting a role for rpS6 in β-cell function and glucose homeostasis. For a long period, the phosphorylation of rpS6 was connected with the translation of a class of mRNA that contain a terminal oligopyrimidine (TOP) stretch at the 5'-end. It was thought that S6 phosphorylation was required to recruit 5'-TOP mRNAs and therefore enhanced a process called "ribosome biogenesis". However, genetic studies demonstrate the lack of association between these events (Tang *et al.*, 2001). More details will be discussed later.

A negative role of S6Ks in insulin signalling was revealed by the finding that mice lacking S6K1 have enhanced insulin sensitivity (Um *et al.*, 2004). Cells with high basal levels of mTOR/S6K activity showed a strong repression of the PI3K/Akt pathway (Shah *et al.*, 2004). It is now known that S6K1 inhibits IRS1 by phosphorylating it (Harrington et al., 2004, Um et al., 2004). Therefore, S6Ks exert a negative feedback loop through IRS1, downregulating the activity of downstream components of the insulin/PI3K/Akt pathway.

1.1.2.1.5.2 Ribosome Biogenesis

Ribosome biogenesis is a complex process that consumes a large proportion of energy within the cell. Research in yeast and mammalian cells demonstrated that ribosome biogenesis is rapamycin-sensitive and thus controlled by TOR (Schmelzle and Hall, 2000). All three nuclear RNA polymerases, Pol I, Pol II and Pol III are required for the synthesis of ribosomes. TOR signalling regulates ribosome biogenesis at several levels, including transcription by Pol I, II and III, pre-rRNA processing and translation. For example, mTOR controls Pol I transcription via TIF-IA. As one of the basal transcription factor, TIF-IA recruits Pol I to rDNA promoter and thereby facilitates the formation of transcription initiation complex (Miller *et al.*, 2001, Yuan *et al.*, 2002). The activity of TIF-IA is modulated by its phosphorylation at two residues, Ser44 and Ser199 in opposite ways. For example, phosphorylation of Ser44 activates while Ser199 inactivates TIF-IA. Inhibition of mTOR signalling inactivates TIF-IA by

regulating the phosphorylation at these sites (Mayer *et al.*, 2004). Furthermore, TIF-IA translocates from the nucleus to the cytoplasm following rapamycin treatment (Mayer *et al.*, 2004). Another basal transcription factor, UBF has also been found to be regulated by mTOR via the involvement of S6K1 (Hannan *et al.*, 2003). S6K1 stimulates the phosphorylation of UBF in its C-terminal region, thus promoting its interaction with the TBP-containing factor TIF-IB. Therefore, mTOR is a key event in the modulation of Pol I activity and the assembly of the transcription complex.

Pol II is responsible for the transcription of ribosomal protein (RP) genes. Studies in yeast have found that TOR controls the expression of RP genes via two factors, SFP1 and FHL1 (Marion *et al.*, 2004, Martin *et al.*, 2004). SFP1 was found to help promote RP gene transcription by binding to the promoters of RP genes. Rapamycin treatment caused the inactivation of SFP1, leading to the downregulation of RP gene transcription (Jorgensen *et al.*, 2004). The forkhead transcription factor, FHL1, has also been identified as a critical regulator of RP gene transcription. FHL1 is regulated by its co-activator, IFH1, and its co-repressor, CRF1 (Martin et al., 2004). TOR maintains the co-repressor CRF1 in the cytoplasm under favourable conditions. If TOR is inactivated due to unfavourable growth conditions, CRF1 translocates into the nucleus and competes with IFH1 to bind to FHL1, inhibiting RP gene transcription (Martin *et al.*, 2004). In addition, histone modifying enzymes are implicated in the control of RP gene transcription and are regulated by TOR signalling (Rohde and Cardenas, 2003, Humphrey *et al.*, 2004). The expression of RPs is also controlled at the translational level via the 5'-TOP sequence. However, the mechanism by which it is regulated directly or indirectly by TOR remains unclear.

Much less is known about the role of TOR in Pol III-dependent transcription (transcription of tRNA and 5S genes) than that in Pol I and Pol II. Studies in yeast show that TOR regulates Pol III transcription by modulating the activity of Pol III and the TBP-containing transcription initiation factor TFIIIB. In mammalian cells, inhibition of mTOR causes repression of Pol III transcription (White, 2005).

1.1.2.1.5.3 Transcription

Besides the role of TOR signalling in the control of transcription by Pol I, Pol II and Pol III, many other genes involved in metabolic and biosynthetic are known to be regulated by mTORC1 at the transcriptional level (Peng *et al.*, 2002). URI

(unconventional prefolding RPB interactor 5) may be involved in the regulation of TOR-dependent transcription programs (Gstaiger *et al.*, 2003). It interacts with Pol I, Pol II and Pol III and is phosphorylated in a TOR-dependent manner. The role of TOR in transcription is further demonstrated by their ability to phosphorylate STAT1 and STAT3 (signal transducer and activator of transcription; Kristof et al., 2003).

1.1.2.1.5.4 Autophagy

Autophagy is the process of degradation of a cell's own components and thereby ensures cell to survival under unfavourable conditions like nutrient starvation, stress, or reduced availability of growth factors. Among numerous regulators, TOR plays a key role in the regulation of this catabolic process.

In yeast, TOR controls autophagy through its effects on autophagy-related (ATG) genes ATG1. ATG1 is perhaps the first component downstream of TOR in the autophagy signalling pathway. It mediates the initial stages in the autophagic process (Noda and Ohsumi, 1998, Kamada *et al.*, 2000). TOR decreases the affinity between ATG1 and its binding proteins, resulting in inhibition of autophagy (Kamada *et al.*, 2000, Kawamata *et al.*, 2008, Kabeya *et al.*, 2009). ATG1 is named ULK1 (UNC-51 like kinase) in mammals. At least three other isoforms of ULK1 have been identified in human cells, which are ULK2, ULK3 and ULK4 (Yan *et al.*, 1999). Following the discovery of ULKs, extensive evidence has shown that mTORC1 negatively regulates autophagy, although the mechanism remains to be established. mTORC1 was reported as the kinase phosphorylating ULK1/2 and ATG13 (Jung *et al.*, 2010, Jung *et al.*, 2009, Ganley *et al.*, 2009). When cells are starved, the phosphorylation of ULK complex by mTORC1 is suppressed. Under this condition, ULK1 may take a ‘‘closed” conformation and trigger downstream events for autophagosome formation (Jung *et al.*, 2010). TORC1 has also been implicated in the regulation of Tap42-associated protein phosphatase type 2A (PP2A) induction of autophagy in yeast (Yorimitsu *et al.*, 2009).

1.1.2.2 Signalling through mTORC2

As mentioned earlier, two TOR complexes have been identified, mTORC1 and mTORC2. Due to the lack of a specific inhibitor for mTORC2, knowledge of mTORC2, especially concerning its regulation, is very limited. In recent years,

exciting progress towards understanding mTORC2 has been made. One promising discovery was made by Zinzalla *et al.* (2011) which established a missing link in the activation of TORC2. Their genetic studies showed that ribosomes are essential for the activation of TORC2 in yeast. In experiments performed in mammalian cells, they further demonstrated the role of ribosome proteins in mTORC2 activation. Knockdown of ribosome subunits decreased the phosphorylation of mTORC2 targets, while inhibition of protein synthesis had no effect on mTORC2 activation. Prior to this report, Oh *et al.* (2010) found that mTORC2 can associate with ribosomes and that this association is important for mTORC2 to phsophorylate Akt.

With respect to the functions of mTORC2, it was suggested as an important regulator of cytoskeleton dynamics (Jacinto *et al.*, 2004). Using small-interfering RNAs for mTOR, mLST8, Sin1 or Raptor in NIH 3T3 cells, Jacinto and colleagues (2004) demonstrated a role for mTORC2 in the regulation of the actin cytoskeleton. As mentioned earlier, mTORC2 phosphorylates Akt on the hydrophobic motif (HM) site Ser473 (Bayascas and Alessi, 2005). Akt is also constitutively phosphorylated at Thr450, which is the TM site (Facchinetti *et al.*, 2008, Ikenoue *et al.*, 2008). Several lines of evidence have established a role for mTORC2 in TM phosphorylation of Akt (Ikenoue et al., 2008, Jacinto and Lorberg, 2008). For example, genetic ablation of components of mTORC2 or inhibition of mTORC2 kinase activity strongly inhibits TM site phosphorylation in Akt.

mTORC2 has also been shown to catalyse the phosphorylation of other AGC kinases, including PKC and SGK. Similarly to Akt, the activity of PKC is controlled by phosphorylation in the T loop, the turn motif (TM) and hydrophobic motif (HM). The importance of mTORC2 in the TM and HM phosphorylation of some PKC isoforms has been demonstrated (Facchinetti *et al.*, 2008, Ikenoue *et al.*, 2008). For PKCα, it seems that mTORC2 is more important for TM phosphorylation than HM phosphorylation, since inactivation of mTORC2 does not abolish HM phosphorylation in PKCα. TM site phosphorylation is responsible for proper C-terminal folding and stability of Akt and PKCs, suggesting a role of mTORC2 in these events (Facchinetti *et al.*, 2008, Bornancin and Parker, 1996). SGK is well known for its ability to regulate diverse cellular processes, one of which is stimulating sodium transport into epithelial cells (Loffing *et al.*, 2006). Like Akt and PKCs, activation of SGKs is under control of its phosphorylation at the T loop and HM site (Ser422 in SGK1). PDK1 phosphorylates SGK1 on its T loop (Kobayashi and Cohen, 1999, Biondi et al., 2001).

Although it was initially thought that mTORC1 was the kinase for the HM site in SGK1, Alessi *et al.* (2008) have provided strong evidence in support of mTORC2 as the HM kinase for SGK1. Loss of mTORC2 components Rictor, Sin1 or mLST8 eliminates Ser422 phosphorylation in SGK1, even though mTORC1 activity is maintained. In various mammalian cells (like HEK293, HeLa or MCF7 cells), rapamycin strongly suppresses the phosphorylation of S6K1 (also an AGC kinase) without affecting SGK1 phosphorylation. The importance of TORC2 in regulating the activity of AGC family members is evident in yeast as well. For instance, YPK (yeast protein kinase) shares the highest homology with SGK and Akt and TORC2 was reported to directly phosphorylate YPK *in vitro* (Kamada *et al.*, 2005). For PKC1 in the budding yeast, phosphorylation of its TM site is regulated by TORC2 as well (Jacinto and Lorberg, 2008).

1.2 Eukaryotic translation factors and regulators.

1.2.1 Overview

Protein synthesis in eukaryotes is a complex process. Over the last few decades, there has been an explosion in the study of eukaryotic post-transcriptional control of gene expression, and in particular of messenger RNA (mRNA) translation. In general, the process of the positioning of aminoacyl-transfer RNAs (tRNAs) with their corresponding amino acids on the mRNAs and ribosomes, and the synthesis of polypeptides is termed mRNA translation. Translation can be divided into three stages: initiation, elongation and termination. In the stage of initiation, the small ribosomal subunit 40S (based on its sedimentation upon centrifugation on density gradients) binds to the 5' end of the mRNA and scans it in a 5'→3' direction until the start codon is identified with the help of initiation factors. The elongation phase involves the delivery of the correct tRNAs to ribosomes, the formation of peptide bonds and the translocation of the ribosomes. The termination of translation occurs when the ribosomes reach one or more STOP codons (UAA, UAG, and UGA). A simplified schematic diagram of the three stages of translation is given below (Fig. 1.5).

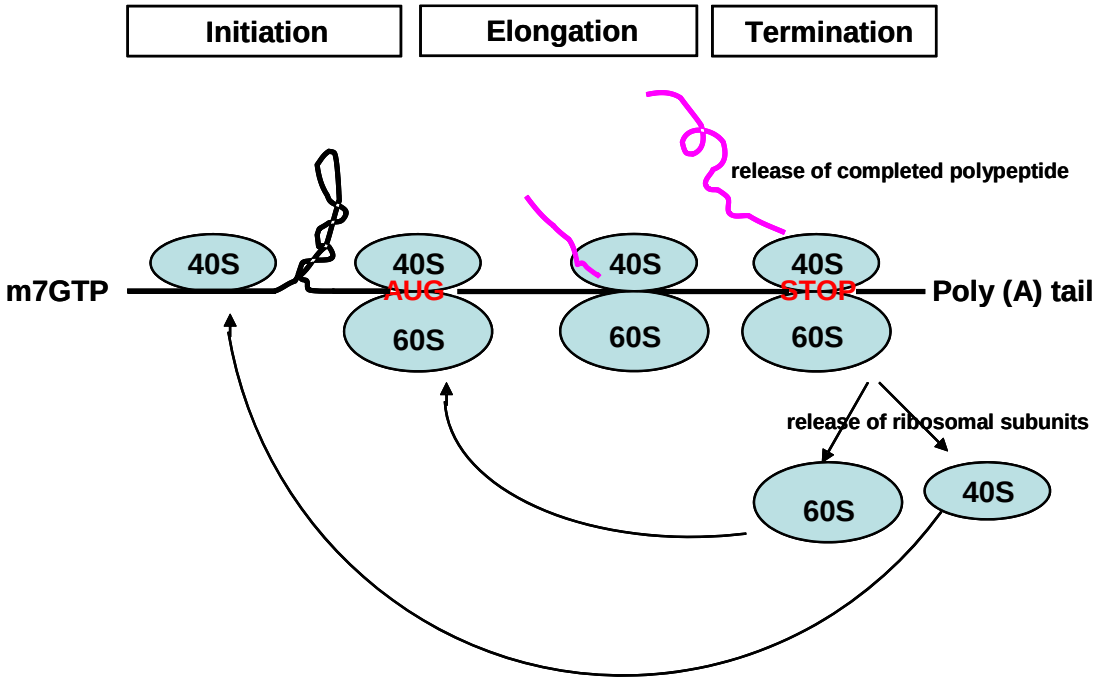


Figure 1.5. A simplified schematic diagram of the three stages of mRNA translation, initiation, elongation and termination.

In the stage of initiation, the small ribosomal subunit 40S, together with other initation factors (eIFs; not shown here, see Fig. 1.6 for more details) binds to the 5'end of the mRNA and begins scanning until it reaches the strat codon. With the release of eIFs, the large ribosomal subunit 60S joins to form an 80S initiation complex, which is ready to accept the correct aminoacyl-tRNA and synthesize the first peptide bond. The elongation stage involves the delivery of the appropriate tRNAs to ribosomes, the formation of peptide bonds and the translocation of the ribosomes. Translation is terminated when ribosomes reach the stop codon. The ribosome complex is disassembled after the release of the peptide chain.

1.2.2 Translation initiation

In eukaryotes, two modes of translation initiation have been found, which are termed as cap-dependent and cap-independent translation. While translation is usually initiated at the 5' end of the mRNA molecule in eukaryotes, a subset of mRNA can circumvent this region by way of specialized sequences, which are called internal ribosome entry sites (IRESs). These sequences were first reported in 1988 (Pelletier and Sonenberg, 1988) and they were described as special regions in RNA that can recruit the ribosome subunits to the mRNA start codon. The use of IRES sequences is more common in viral mRNAs to allow its translation in a cap-independent manner, especially when host translation is inhibited (Kieft, 2008), but some mammals are thought to have IRESs. For example, some cellular IRES elements are located in eukaryotic mRNAs encoding genes involved in stress survival or other key processes (Mokrejs *et al.*, 2006, Komar and Hatzoglou, 2005). Strikingly, some cellular mRNAs that contain IRES elements may promote translation of certain proteins during mitosis and programmed cell death (Komar and Hatzoglou, 2011). Actually, more than 70 eukaryotic mRNAs with IRES elements have been reported in yeast, the fruit fly, and mammals during the past few years, and the number keeps growing (Mokrejs *et al.*, 2006).

The initiation stage of cap-dependent mRNA translation in eukaryotes requires a number of *trans*-acting protein factors called eukaryotic initiation factors (eIFs). It has been accepted that eIF3 is the first factor to bind to the 40S ribosomal subunit followed by eIF2. When bound to GDP, eIF2 is inactive. The guanine nucleotide exchange factor (GEF) eIF2B can facilitate the exchange of GDP to GTP, resulting in the formation of an active ternary complex of eIF2-GTP-Met-tRNAi (Fig. 1.7). The α subunit of eIF2 can be phosphorylated at Ser51 by a number of related protein kinases, called eIF2 α kinases, which are activated in response to cellular stresses. In mammalian cells, there are four different eIF2α kinases, including general control nonderepressible-2 (GCN2), haem-regulated inhibitor kinase (HRI), RNA-dependent protein kinase (PKR), and PKR-like endoplasmic reticulum kinase (PERK). These kinases possess a similar catalytic domain allowing them to phosphorylate a common substrate, but some of them may exert additional effects, due to having additional substrates (reviewed in Proud, 2005). The phosphorylation of eIF2α prevents the recycling of inactive eIF2-GDP to active eIF2-GTP by inhibiting the activity of the guanine nucleotide exchange factor, eIF2B (Sudhakar et al., 2000; Fig. 1.7). Since the

ternary complex cannot form, extensive phosphorylation of eIF2α can result in the global translation repression.

Other eIFs bound to 40S include eIF1, eIF1A and eIF5. Once these factors, and Met-tRNAs, are placed on the 40S subunit, a 43S pre-initiation complex is formed. Formation of the 48S pre-initiation complex occurs after the recruitment of mRNA to the 43S complex, which is facilitated by the cap-binding factor eIF4E and its partners, eIF4G and eIF4A, the so called eIF4F complex. eIF4F is thought to be the key factor in mRNA translation and its activity therefore is regulated by several effectors. 4E-BP1 is the best understood as it competes with eIF4G to bind to eIF4E (Fig. 1.6). The nucleocytoplasmic shuttling protein 4E-T (eIF4E-Transporter) has been identified as another partner for eIF4E (Dostie et al., 2000; Fig. 1.6). In fact, over the past decade, the number of identified eIF4E binding partners has grown rapidly. These novel proteins include cup, bicoid, Vpg and CYFIP1 (Cho *et al.*, 2005, Leonard *et al.*, 2000, Napoli *et al.*, 2008). Via the identification of these new partners, many new roles of eIF4E have been revealed. It is worth noting that mammals express multiple versions of eIF4E and their binding capabilities are different. For example, eIF4E-1 binds both eIF4G and 4E-BP1, but eIF4E-2 binds only 4E-BP1, and eIF4E-3 binds only eIF4G (Joshi *et al.*, 2004). Mammalian eIF4G is also present in various versions, eIF4GI and eIF4GII, which share 46% amino acid identity (Imataka *et al.*, 1998). Although it has been thought that these two paralogs have similar activities and functions, the cleavage of each of them by specific viral proteases reveals distinct effects of their integrity on ongoing translation (Castello *et al.*, 2006). Moreover, Caron *et al.* (2004) reported that eIF4GII is selectively recruited to the cap structure and this event does not correlate with the changes in the phosphorylation status of 4E-BP1 (unlike eIF4GI; Fig. 1.7). Both of eIF4Gs are phospho-proteins, and phosphorylation of eIF4GI is induced by serum stimulation while eIF4GII is phosphorylated at the onset of mitosis (Raught *et al.*, 2000, Pyronnet *et al.*, 2001). It has also been shown that the activities of eIF4GI/II are under differential regulation by cytokines (Caron *et al.*, 2004). Therefore, eIF4GI and eIF4GII are likely differentially regulated and they may play selective roles in mammalian translation.

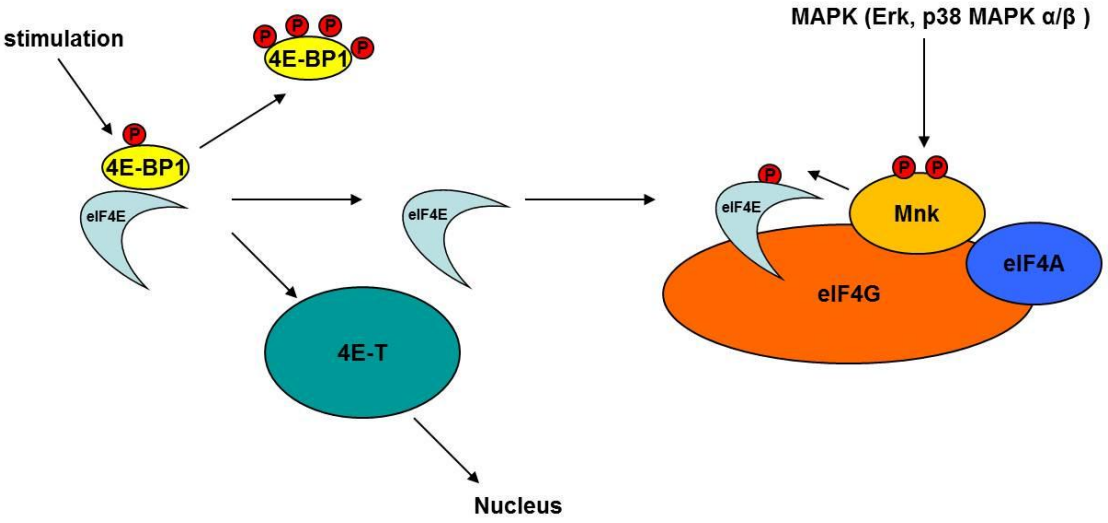


Figure 1.6. Regulation of eIF4F formation.

When cells are stimulated by amino acids or insulin, 4E-BP1 becomes hyperphosphorylated, which decreases the affinity for eIF4E and induces its release from eIF4E. Free eIF4E can then interact with eIF4G, forming a functional eIF4F complex which also contains eIF4A. eIF4E can also bind to 4E-T which is involved in transferring eIF4E to the nucleus. The Mnks (mitogen-activated protein kinase-interacting kinase), which are activated by Erk and p38 MAPK α/β, need to bind to eIF4G to phosphorylate eIF4E. More details about the Mnks will be discussed in *Chapter 5 section 5.1*.

In the eIF4F complex, the subunit eIF4A is an ATP-dependent RNA helicase, which can catalyse RNA unwinding and melt out certain secondary structures in the 5'-proximal region of mRNA to facilitate ribosome binding (Rogers *et al.*, 1999). These base-paired structures can present difficulties to ribosome entry and scanning. It has been found that the translational activities of mRNAs that possess a 5'-UTR with high G-C content are often reliant upon eIF4F (Svitkin *et al.*, 2001). Programmed Cell Death 4 (Pdcd4) is a protein known to bind to eIF4A and eIF4G and inhibit translation initiation (Goke *et al.*, 2002, Yang *et al.*, 2003). That Pdcd4 inhibits translation is likely achieved by preventing the helicase activity of eIF4A and interference with eIF4A binding to eIF4G (Yang *et al.*, 2003). Mitogens stimulate the phosphorylation of Pdcd4 by S6K1 and subsequent degradation by the ubiquitin ligase SCF (Dorrello *et al.*, 2006). As the co-factor of eIF4A, eIF4B interacts with eIF4A and stimulates the ATPase and helicase activities of eIF4A (Rozen *et al.*, 1990, Rogers *et al.*, 1999, Grifo *et al.*, 1984, Rozovsky *et al.*, 2008). eIF4B has also been found to interact with eIF3 subunit and 18S rRNA (Methot *et al.*, 1996b, Methot *et al.*, 1996a). Thus, it has been proposed that eIF4B forms auxiliary bridges between the mRNA and the 40S subunit. In addition, eIF4B is believed important in the assembly of the 48S pre-initiation complex (Dmitriev *et al.*, 2003). eIF4B activity depends on its phosphorylation status, and S6K1 and RSK have been implicated in eIF4B Ser422 phosphorylation (Shahbazian *et al.*, 2006).

Therefore, eIF4F, together with many other initiation factors, mediate the recruitment of the 43S pre-initiation complex (which already contains eIF1, eIF1A, eIF2, eIF3, eIF5 and Met-tRNAs) to the mRNA. There is another protein associated with eIF4G called the poly (A)-binding protein (PABP), which binds the poly-A tail and brings the 3'UTR in close proximity to the 5'end of the mRNA (Wells *et al.*, 1998). Besides eIF4G, PABP also interacts with several other proteins, including eIF4B (Bushell *et al.*, 2001), the termination factor eRF3 (Hoshino *et al.*, 1999) and Paip (poly (A)-binding protein-interacting protein) 1/2 (Craig *et al.*, 1998). The mRNA circulation brought about by the PABP-eIF4G interaction is proposed to promote translation initiation. Take eRF3 for instance, the interaction of eRF3 with the C-terminus of PABP was shown to promote translation by delivering the terminating ribosomes to the 5'end of the mRNA (Sonenberg and Dever, 2003). Recently, it has been reported that Paip1 stabilizes the interaction between PABP and eIF4F (Martineau *et al.*, 2008).

The 43S pre-initiation complex, accompanied by many protein factors, scans along the mRNA towards its 3' end until it reaches the start codon, AUG. When the 43S pre-complex is bound to the start codon, a stable complex called the 48S pre-initiation complex is formed. The start codon selection is critically modulated by eIF1 and eIF1A (Pestova and Kolupaeva, 2002, Pestova *et al.*, 1998). The initiator tRNA is brought to the P site of the small ribosomal subunit by eIF2 and a perfect match with an AUG start codon triggers the hydrolysis of GTP in the eIF2-GTP-Met-tRNA complex and the dissociation of Pi and eIF2-GDP from the small ribosomal subunit. The joining of 60S and release of other initiation factors are catalysed by eIF5B-GTP (Lee *et al.*, 2002). The second GTP hydrolysis reaction causes the release of eIF5B and eIF1A from the initiation complex (Shin *et al.*, 2002), and the 80S initiation complex is finally formed and translation elongation is commenced. A summary of the process of the eukaryotic translation initiation is represented in Fig. 1.7.

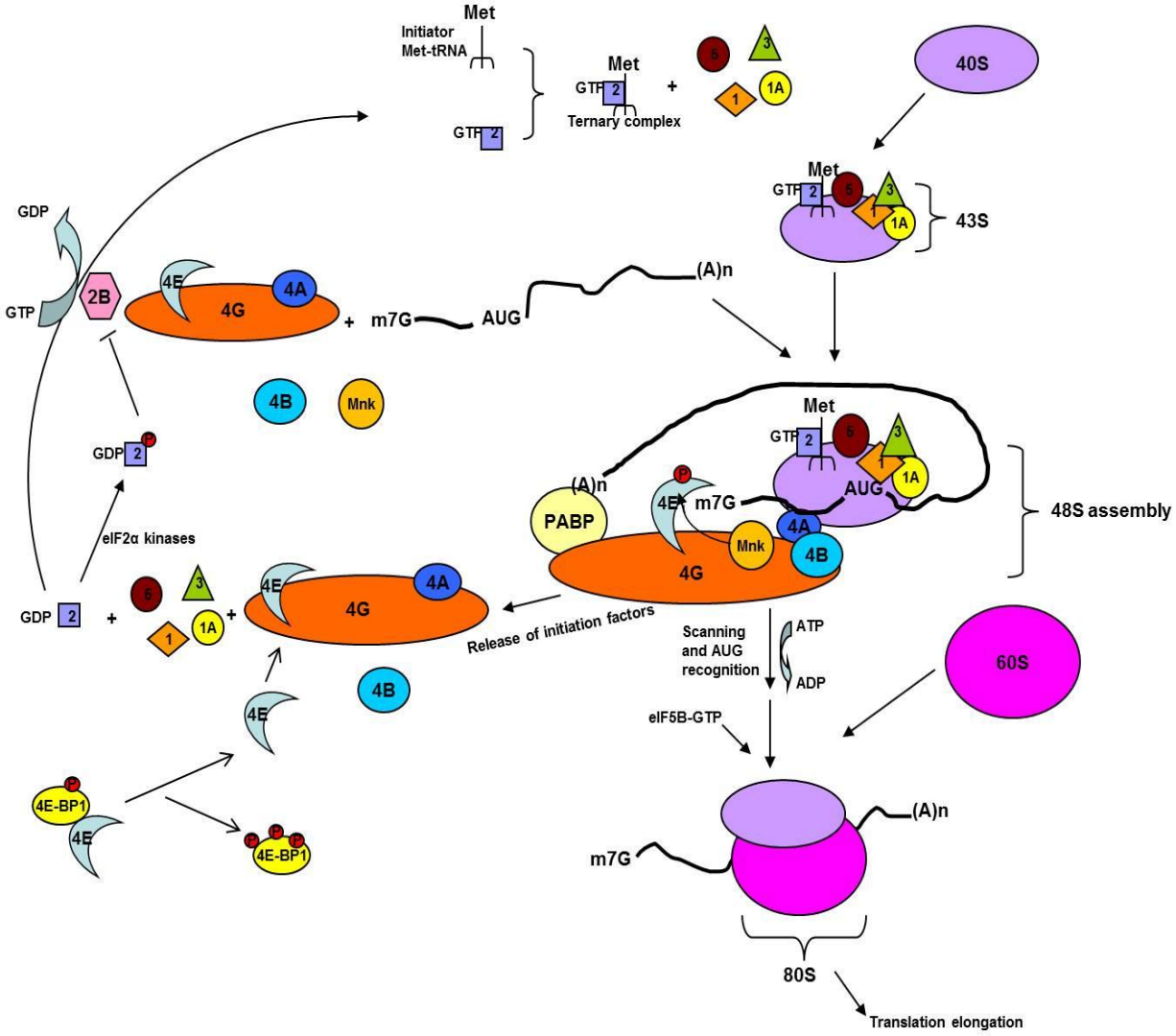


Figure 1.7. Translation initiation.

eIF 1, 1A, and 3, together with eIF5 and the ternary complex (eIF2-GTP-Met-tRNA), bind to the 40S ribosomal subunit and assemble the 43S preinitiation complex (PIC). mRNA is activated by binding of eIF4F (eIF4E, eIF4G and eIF4A) to the m^7GTP cap and PABP to the poly(A) tail, circularizing the mRNA. The 43S PIC, accomplanied by many other protein factors, scans along the mRNA for the start codon (AUG) in an ATP-dependent reaction. When the 43S PIC is bound to the start codon, the 48S PIC is formed. AUG recognition triggers the hydrolysis of GTP in the ternary complex and the dissociation of eIF2-GDP and Pi from 40S. Joining of the 60S subunit and release of other eIFs are catalyzed by eIF5B-GTP. The second GTP hydrolysis triggers release of eIF5B-GDP and eIF1A to form the final 80S initiation complex. The ternary complex formation is inhibited by eIF2α phosphorylation (through inhibition of eIF2B activity), and eIF4F assembly is blocked by 4E-BP1. Phosphorylation of 4E-BP1 by mTOR (not depicted here) induces its release from eIF4E. MAPK signalling promotes phosphorylation of eIF4E by Mnk1/2, which are recruited to eIF4G. 1, 1A, 2, 2B, 3, and 5 represent eIF1, eIF1A, eIF2, eIF2B, eIF3 and eIF5 respectively; 4E, 4G, 4A, 4B, and 5B indicate eIF4E, eIF4G, eIF4A, eIF4B and eIF5B, respectively; PABP: poly (A)-binding protein.

1.2.3 Translation elongation

Peptide chain elongation is the process of polymerizing amino acids into a polypeptide, which is dependent on eukaryotic elongation factors. The amino acid is added to the nascent polypeptide chain in a three-step microcycle: (1) locating the correct aminoacyl-tRNA in the A site of the ribosome, (2) forming the peptide bond, (3) moving the ribosome by one codon forward on the mRNA and translocating the peptidyl-tRNA from the A site to the P site of the ribosome. Three elongation factors are involved in mammals: eEF1A, eEF1B and eEF2.

eEF1A mediates the entry of the aminoacyl-tRNA into a free site (A site) of the ribosome. eEF1A binds to GTP prior to binding the aminoacyl-tRNA. Once activated, a ternary complex (eEF1A-aminoacyl-tRNA-GTP) is formed to deliver the aminoacyl-tRNA into the A site on the ribosome. After the binding of the aminoacyl-tRNA to the A site, the GTP is hydrolysed and inactive eEF1A dissociates from the ribosome. Reactivation of eEF1A requires eEF1B, which serves as the guanine nucleotide exchange factor for eEF1A, catalysing the release of GDP from eEF1A. A peptide bond is formed between the aminoacyl group and the peptidyl group in the P site (catalysis of peptide bond formation is performed by the peptidyl-transferase centre of the 60S subunit). Following the formation of the peptide bond, the polypeptide chain from the tRNA in the P site is released and thus a longer peptidyl-tRNA is formed in the A site (reviewed in Browne and Proud, 2002).

eEF2 catalyses the translocation of the ribosome along the mRNA during the end of each round of polypeptide elongation. Translocation leads to the movement of new peptidyl-tRNA from the A site to the P site and the uncharged tRNA from the P site to the E-site, resulting in an empty A site for the entry of the next aminoacyl-tRNA (reviwed in Browne and Proud, 2002).

1.2.4 Translation termination

Termination of elongation is dependent on eukaryotic release factors. The process is similar to that of prokaryotic termination. This process is initiated when the ribosome encounters a stop codon (UGA, UAG, or UAA). The release factor, eRF1, is involved in the recognition of these codons. Another release factor, eRF3, has been implicated in enhancing this process by acting as a GTPase for eRF1. The ribosome complex is disassembled after the release of the peptide chain.

1.3 mTOR and TOP mRNAs

1.3.1 What are TOP mRNAs?

An important fraction of vertebrate transcripts contain a characteristic 5'UTR, which starts with a uninterrupted sequence of 6-12 pyrimidines (Meyuhas, 2000). Consequently, these mRNAs are referred to as TOP (terminal oligopyrimidine) mRNAs and the genes encoding them are called TOP genes. Since the first discovery in vertebrate genes encoding ribosomal proteins (RPs) in the 1970s by Schibler's group (1977), TOP mRNA expression has acquired increasing attention due to its unique structure and mode of regulation.

There are two essential features for identifying a TOP mRNA: (1) the presence of a 5'-TOP tract and (2) a growth-rate associated regulation at the translational level. According to this definition, it appears that all messengers that encode proteins which are involved in ribosome biogenesis, most notably the RPs (ribosomal proteins), are TOP mRNAs. Among the non-RP TOP mRNAs, the encoded proteins are typically involved in translation, such as some eukaryotic initiation and elongation factors and PABP. Iadevaia *et al.* (2008) reported a systematic analysis of human genes for TOP motifs with a focus on the translation factors. By selecting possible TOP candidates on the basis of the presence of a pyrimidine stretch at their 5' end, they tested these genes by a polysome assay to verify their regulation of translation. In a few cases, they analysed the transcription start site by primer extension. All the translation elongation factors, together with a few translation initiation factors, were found to be encoded by TOP mRNAs. The following table shows the sequence near the transcription start site (TSS) of some known TOP mRNAs in human (Table 1.1).

Table 1.1. A summary of known 5' TOP sequences in human TOP mRNAs. From (Meyuhas, 2000, Iadevaia *et al.*, 2010)

Protein		5' TOP sequence with preferred tss
RPs	rpS4Y	CUCUUCC
	rpS4X	CCUCUUUCCUU
	rpS6	CCUCUUUUCC
	rpS8	CUCUUUCC
	rpS11	CUUUUUUUC
	rpS14	CUCUUUCC
	rpS17	CCUCUUUU
	rpS24	CUUUUCCUCCUU
	rpL7a	CUCUCUCCUCCC
	rpL13a	CUUUUCC
Initiation factors	eIF3e	CUUUUCUUU
	eIF3f	CUUCUUUCU
	eIF3h	CUCUUUCUUCCU
	PABP	CCCCUUCUCCCC
Elongation factors	eEF1A	CUUUUUC
	eEF1B	CUUUUUCCUCUCUUC
	eEF1D	CCCUUUC
	eEF1G	CCUUUCUUU
	eEF2	CUCUUCC
	B23	CUUUCCCU
	P21	CUUUUCC
	hnRNP A1	CUCCUUUCU

*Ribosomal proteins (RPs)***:** After more than 20 years since the initial studies on RP genes, it is evident that all vertebrate RP mRNAs analysed so far are encoded by TOP genes, although it has not been experimentally proven in all cases;

*Initiation factors***:** Among the initiation factors analyzed by Iadevaia *et al.* (2008), only eIF3e, eIF3f, eIF3h exhibit the features of TOP genes;

Elongation factors: eEF1A has a typical 5'-TOP (Uetsuki *et al.*, 1989). The synthesis rate of eEF1A is increased by serum stimulation in Swiss mouse 3T3 cells without being affected by actinomycin D (which is commonly used to inhibit transcription)

(Thomas and Luther, 1981). Subsequently, it was demonstrated that the amount eEF-1A mRNA remained constant while the transcripts shifted between mRNA protein particles and polysomes (Jefferies and Thomas, 1994). eEF2 is also encoded by a TOP mRNA (Terada *et al.*, 1994, Avni *et al.*, 1997). Iadevaia *et al.* (2008) reported that all five translation elongation factors are encoded by typical TOP mRNAs.

Poly (A)-binding protein (PABP): PABP plays a key role in translation initiation. In addition, it has been implicated in mRNA stability (Coller *et al.*, 1998, Amrani *et al.*, 1997, Minvielle-Sebastia *et al.*, 1997), and regulation of poly (A) tail length. Various studies examining its expression have demonstrated that PABP exhibits "TOP" characteristics (Hornstein *et al.*, 1999).

B23 (also called nucleophosmin): B23 is a major phosphoprotein in the interphasic nucleolus and is involved in the pre-ribosome assembly (Zatsepina *et al.*, 1999). As shown in Table 1.1, B23 contains a TOP sequence in its 5' end. Moreover, the translation of this mRNA is repressed in serum-starved cells (Zong *et al.*, 1999). However, during mitosis when cap-dependent protein synthesis is compromised, the synthesis of B23 can be maintained since its 5' UTR contains an IRES element which can recruit ribosomes, and bypass the cap structure (Qin and Sarnow, 2004). This suggests that some TOP mRNAs may be subject to more than one mode of translational regulation.

P21 (also known as TCTP): The growth-related P21 has been shown to possess properties of a tubulin binding protein and contains a typical 5'-TOP (Table 1.1) (Gachet *et al.*, 1999).

*hnRNP A1 (Heterogeneous nuclear ribonucleoprotein A1)***:** hnRNP A1 belongs to the A/B subfamily of ubiquitously expressed heterogeneous nuclear ribonucleoproteins (hnRNPs). The hnRNP A1 transcript was shown to start with a pyrimidine tract (Table 1.1) similar to TOP mRNAs. Moreover, the hnRNPA1 mRNA is more efficiently loaded onto polysomes in growing than in resting cells (Camacho-Vanegas *et al.*, 1997). Thus, hnRNP A1 mRNA is included in the class of TOP genes.

1.3.2 Regulation of TOP mRNA translation

Translational repression of TOP mRNAs occurs when proliferation of mammalian cells is blocked, as reflected by their shift from polysomes into subpolysomal fractions. It appears that TOP mRNAs exist in two states: repressed and active states. A vast majority of TOP mRNAs remain in the translationally inactive state in quiescent cells. In growing cells, the translationally active population increases and they can be translated at maximum efficiency (Meyuhas, 2000). This "all-or-none" phenomenon indicates that the repression likely results from a blockage of the translation initiation.

1.3.2.1 The possible roles of S6 phosphorylation and the PI3K pathway in the activation of TOP mRNA translation

Phosphorylation of ribosome protein S6 is one of the earliest events detected following mitogenic stimulation. The concomitant translational activation of TOP mRNAs under these circumstances led many investigators to propose a link between rpS6 phosphorylation and TOP gene expression. Multiple lines of evidence support this hypothesis, one of which is that the treatment of mammalian cells with rapamycin suppressed translation of TOP mRNAs, an effect thought to be mediated by blocking rpS6 phosphorylation and activation (Jefferies *et al.*, 1997). Nevertheless, subsequent studies have disagreed with this model. During differentiation of murine erythroleukemia (MEL) cells, translation of TOP mRNAs is repressed despite the absence of changes in rpS6 phosphorylation (Barth-Baus *et al.*, 2002). Stolovich and colleague (2002) found that when the phosphorylation of S6 was completely abolished by rapamycin, there was little effect on rpL32 translation in PC12 cells. Furthermore, the mutation of all five phosphorylatable serine residues of rpS6 into alanine does not affect the translational control of TOP mRNAs (Ruvinsky *et al.*, 2005). Also, a mutant possessing a high basal S6K1 activity failed to relieve the translational repression of eEF1A mRNA in serum-starved HEK293 cells (Jefferies *et al.*, 1997). More convincingly, despite the impairment of S6 phosphorylation in cells derived from S6K1 and S6K2 null mice, the translation of 5'-TOP mRNAs were still modulated by mitogens in a rapamycin-dependent manner (Pende *et al.*, 2004). Collectively, S6 and its phosphorylation are unexpectedly dispensable for translational control of mRNAs with a 5'-TOP tract.

A number of studies have demonstrated a role of PI3K in the translational activation of TOP mRNAs. For example, the inhibition of PI3K by LY294002 (a PI3K inhibitor) or overexpression of a dominant-negative mutant of PI3K can block the translation activation of TOP mRNAs. Furthermore, the translational repression of TOP mRNAs in quiescent cells can be relieved by overexpression of constitutively active PI3K (Stolovich *et al.*, 2002).

1.3.2.2 Trans-acting factors

It has been hypothesised that the oligopyrimidine tract in TOP mRNAs can be recognized by specific translational *trans-acting* factors which may be involved in their translational regulation. Indeed, the *Xenopus* homologue of the human La autoantigen has been demonstrated to interact with the 5'-TOP motifs of human and *Xenopus laevis* TOP mRNAs *in vitro* (Pellizzoni *et al.*, 1996, Crosio *et al.*, 2000). However, whether the specific binding promotes or represses the translation of TOP mRNAs remains to be investigated. The results from *Xenopus* cell lines support the idea that La protein positively regulates the translation of TOP mRNAs (Crosio *et al.*, 2000). In contrast, phosphorylation of human La at Ser366 limits 5'-TOP mRNA binding, and increasing non-phosphorylated La leads to greater association with potentially negative effects on translation of TOP mRNA (Schwartz *et al.*, 2004).

Several other *trans-acting* effectors, which may interact with 5'-TOP mRNAs and thus mediate its translation, have also been reported including ZNF9, AUF1 and miRNA-10a (Pellizzoni *et al.*, 1997, Pellizzoni *et al.*, 1998, Kakegawa *et al.*, 2007). miRNA-10a was shown to bind immediately downstream of the 5'-TOP motif and enhance TOP mRNA translation (Orom *et al.*, 2008). More recently, the stress granule (SG)-associated TIA-1 and TIAR proteins have been identified as key regulators of 5'-TOP mRNA translation initiation upon amino acid starvation (Damgaard and Lykke-Andersen, 2011). Therefore, these *trans-acting* factors represent an important group of regulators which are implicated in the control of TOP mRNA translation.

1.4 mTOR and human diseases

mTOR is located in a key position of the crossroads of various signalling pathways that modulate mRNA, ribosome and protein synthesis, which may modulate tumour

proliferation and growth. It has been known that mTOR signalling pathway is frequently activated in many human cancers. Dysregulation in both upstream and downstream effectors of mTOR can lead to activation of mTOR signalling. In fact, abnormal activation of the PI3K-Akt pathway has been widely observed in many cancers. Overexpression and activation of IGF-1R (insulin-like growth factor receptor) frequently occurs in many types of human sarcomas (Baserga, 2005). Amplification of genes which encode subunits of PI3K and Akt occurs in many cases of human cancers, including ovarian, breast, and colon cancer (Vivanco and Sawyers, 2002). As an important upstream regulator of mTOR, PTEN inactivation leads to constitutive activation of PI3K/Akt and thus mTOR. PTEN has been identified as a tumour suppressor, since many human cancers have been found to be accompanied by genetic mutation or loss of PTEN function (Steelman *et al.*, 2004). Furthermore, dysregulation of other upstream effectors of mTOR have also been implicated in cancer genesis. For example, Jones *et al.* (1999) observed that somatic loss of TSC1/2 or intragenic mutation of the corresponding wild-type allele is seen in hamartomas following a comprehensive mutation analysis of TSC1 and TSC2 in a cohort of 150 unrelated TSC patients and their families, and Im *et al.* (2002) found that Rheb is highly activated in several cancer cell lines. Additionally, a high proportion of breast tumours exhibit constitutive activation of mTOR (Perez-Tenorio and Stal, 2002). Various mTOR downstream targets are known to be activated in many human cancers, such as S6K1, which has been linked to the genesis of breast and ovarian cancers (Mamane *et al.*, 2006). Hence, the mTOR signalling pathway has received considerable attention as an important target for cancer therapy and for anticancer drug development.

In addition to its well characterized inhibitory effects on mTORC1, rapamycin inhibits translation of several critical mRNA involved in cell cycle progression, resulting in cell cycle arrest (Hidalgo and Rowinsky, 2000). Hence, rapamycin and its analogues (rapalogs; rapalogs inhibit mTORC1 by forming a complex with FKBP12 and are synthesised with more favourable pharmaceutical characteristics) were expected to exert outstanding effects on many types of cancer. Consistent with this, the rapalog temsirolimus has been approved as a first-line treatment of renal cell cancer (RCC) (Yuan *et al.*, 2009, Wan and Helman, 2007). However, due to their allosteric mode of binding with mTOR, rapamycin and rapalogs only partially inhibit mTORC1 activity and leave mTORC2 unaffected. What is more, the incomplete inhibition of mTORC1 by rapamycin leads to a negative feedback through IRS1, causing increased

Akt signalling and cell survival, which may help explain the resistance of some cancers to rapamycin treatment (O'Reilly *et al.*, 2006). The limited success of rapamycin led to the development of mTOR-KIs which more effectively inhibit mTOR kinase activity (see below).

Besides cancer, mTORC1 is also implicated in many other human diseases, such as cardiac hypertrophy, type II diabetes and obesity (Inoki *et al.*, 2005). Cardiac hypertrophy is one of the main risk factors for heart failure. Strong evidence has shown that hyperactivation of the PI3K-Akt-mTOR pathway is correlated with cardiac hypertrophy (Shioi *et al.*, 2002, Crackower *et al.*, 2002). In type II diabetes, activation of mTORC1 negatively regulates the activity of IRS-1 via the S6K1-dependent feedback loop, resulting in insulin resistance. Activation of mTORC1 is also implicated in obesity. Um *et al.* (2004) have shown that S6K1 null mice exhibit a reduction in adipose tissue mass. Thus, dysregulated mTOR signalling is implicated in many important human diseases, making it an exciting therapeutic target.

1.5 Recent advances in the discovery of small molecule ATP-competitive mTOR-KIs

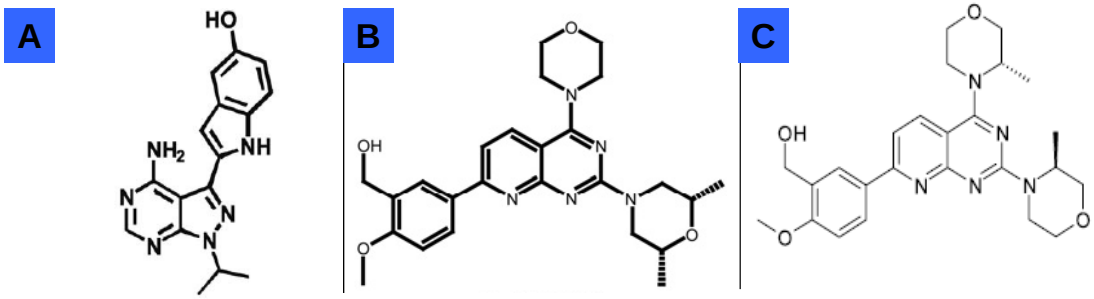
Compared with rapalogs, mTOR-KIs may have some advantages for cancer therapies. In the past few years, this field has made several breakthroughs with the first sets of compounds now in early clinical development [e.g. AZD8055 (developed by AstraZeneca), OSI-027, and INK128 (Chresta *et al.*, 2010, Bhagwat and Crew, 2010, Zask *et al.*, 2011)]. Other compounds including PP242, Ku-0063794 and Torin-1 are currently used in preclinical research (Feldman *et al.*, 2009, Garcia-Martinez *et al.*, 2009, Thoreen *et al.*, 2009). As PP242 and AZD8055 are the compounds mainly used in this study, and Ku-0063794 is structurally similar to AZD8055, they will be discussed below (Fig. 1.8).

PP242 was first developed and reported as an active-site inhibitor of mTOR by Feldman *et al.* (2009). It inhibited mTOR *in vitro* with an IC_{50} (50% inhibitory concentration) value of 8 nM. It strongly inhibited Akt phosphorylation but only modestly inhibited many Akt substrates (e.g. GSK3, TSC2 and FOXO). They also tested the effect of PP242 on the proliferation of primary MEFs and found that PP242 inhibited the proliferation without affecting actins stress fibres, suggesting that the pharmacological inhibition of mTORC2 caused by PP242 did not affect the

morphology or the abundance of these fibres. PP242 was also found to inhibit cap-dependent translation.

Ku-0063794 inhibits both mTORC1 and mTORC2 with an IC_{50} of around 10 nM *in vitro*, but does not suppress the activity of 76 other protein kinases or seven lipid kinases (even at high concentrations). It can also suppress cell growth and induce G1-cell cycle arrest (Garcia-Martinez *et al.*, 2009).

AZD8055 was developed by AstraZeneca and first described by Chresta *et al.* (2010), and is currently in Phase 2 clinical trials. It was identified through the screening of a small library of compounds based around a pyridopyrimidine scaffold. The IC_{50} of AZD8055 against native mTOR complex was approximately 0.8 nM (which is much lower than PP242, suggesting improved potency). AZD8055 binds competitively with ATP and results in significant inhibition of tumour growth in breast, lung and colon xenograft models at a dose of 20mg/kg/day (Chresta *et al.*, 2010).



PP242 Ku-0063794 AZD8055

Figure 1.8. Chemical structures of (A) PP242, (B) Ku-0063794 and (C) AZD8055 (Feldman *et al.*, 2009, Garcia-Martinez *et al.*, 2009, Chresta *et al.*, 2010).

1.6 Aims of this study

The mTOR signalling pathway is an exciting target for the development of pharmaceuticals to treat cancer and other diseases. However, rapalogs, due to their allosteric mode of interaction with mTOR, inhibit some functions of mTORC1, but not all. They also leave mTORC2 unaffected (in the short term). This has led to the development of various new inhibitors which can inhibit both mTORC1 and mTORC2. The recently available inhibitors, PP242 and AZD8055, have been identified as potential mTOR inhibitors with high selectivity. By exploring the distinct effects of rapamycin and these new inhibitors (PP242 and AZD8055), I mainly focused on the role of mTOR signalling pathway in the control of general protein synthesis and particularly the synthesis of specific proteins. I also studied the involvement of eIF4E phosphorylation in its sumoylation, TNFα biosynthesis and the interaction with specific mRNAs using a novel Mnk inhibitor, AZ'9224.

CHAPTER 2
MATERIALS AND METHODS

2.1 Chemicals, biochemicals and other materials

All laboratory reagents were purchased from Sigma-Aldrich and Fisher Scientific, unless specified below.

Agilent Technologies	Prime-It II Random Primer Labeling kit
AstraZeneca, UK	AZD8055 and AZ'9224
Bio-Rad	Protein assay dye (Bradford) Reagents for SDS-PAGE gel PVDF (0.45 μm) and nitrocellulose membranes (0.45 μm) for western blot
Cell Signaling Technology	All the antibodies, unless otherwise stated: 4E-BP1 (raised in rabbit; Wang et al., 1998a), eIF4GII and eIF4A (kind gift from Prof. Simon Morley, University of Sussex), α-tubulin (TU-02) (Santa Cruz Biotechnology), anti-HA high affinity (Roche Applied Science) and anti-SUMO-1 (Sigma-Aldrich)
Calbiochem	Rapamycin
GE Healthcare Life Sciences	m^7GTP Sepharose™ 4B Protein G Sepharose Illustra ProbeQuant™ G-50 Micro Columns
Invitrogen	Tissue culture reagents
Macherey-Nagel	PCR clean up Gel extraction kit Plasmid mini, midi, maxi prep kit
Perkin Elmer	[^{35}S]methionine GreenScreen Plus membrane
Primerdesign	Precision Mastermix with SYBRgreen
Promega	100bp DNA Ladder GoTaq DNA polymerase ImProm-II™ Reverse Transcription System pGEM-T vector RNase inhibitor
Roche Applied Science	Protease inhibitor cocktail
Thermo Scientific	SILAC DMEM Media PageRuler™ Prestained Protein Ladder
Whatman	3 MM filter paper

2.2 Buffers and solutions

Buffers and solutions used in this thesis are listed in alphabetical order below.

Cell freezing medium

40% (v/v) Dulbecco's modified Eagle's medium (DMEM)

50% (v/v) fetal bovine serum (FBS)

10% (v/v) dimethyl sulphoxide (DMSO)

Cell lysis buffer

50 mM Tris-HCl

50 mM β-glycerophosphate (β-GP)

1 mM Ethylenediaminetetraacetic acid (EGTA)

1 mM Ethylene glycol tetraacetic acid (EDTA)

1% (v/v) Triton X-100

β-mercaptoethanol (β-ME), Na_3VO_4 and protease inhibitor cocktail were added to the lysis buffer just before use.

Formaldehyde-agarose gel (1%)	100ml
1% (w/v) Ultra agarose	1g
10% (v/v) 10 x MOPS [3-(N-morpholino) propanesulfonic acid] buffer	10ml
6% (v/v) formaldehyde	16.4ml of 36.5% formaldehyde

MOPS buffer (10x)

200 mM MOPS

25 mM NaAc

10 mM EDTA

adjust pH to 7.0

Northern mix buffer

18% (v/v) formaldehyde

30% (v/v) 20x saline-sodium citrate **(**SSC) buffer

1% (w/v) SDS

0.1 mg/ml salmon sperm DNA

Phosphate-buffered saline (PBS) (10x)

1.7 M NaCl

40 mM KCl

100 mM Na_2HPO_4

22 mM KH_2PO_4

Phosphate buffer (20x)

127 mM Na_2HPO_4

428 mM NaH_2PO_4

Polysome gradient buffer

30 mM Tris-HCl pH 7.5

100 mM NaCl

10 mM $MgCl_2$

RNP immunoprecipitation assay (RIPA) buffer 1 for RIP assay

50 mM Tris-HCl, pH7.5

1% (w/v) NP-40

0.5% (w/v) sodium deoxycholate

0.05% (w/v) SDS

1 mM EDTA

150 mM NaCl

Protease inhibitor cocktail tablet (Roche) is added before use

RIPA buffer 2 (high-stringency) for RIP assay

50 mM Tris–HCl, pH7.5

1% (w/v) NP-40

1% (v/v) sodium deoxycholate

0.1% (v/v) SDS

1 mM EDTA

1 M NaCl

3 M urea,

0.2 mM phenylmethylsulfonyl fluoride (PMSF)

RIPA buffer 3 for RIP assay

50 mM Tris–HCl, pH7.0

5 mM EDTA

10 mM dithiothreitol (DTT)

1% (w/v) SDS

SDS-PAGE sample buffer

62.5 mM Tris-HCl

7% (w/v) SDS

20% (w/v) sucrose

0.01% (w/v) bromophenol blue

SDS-PAGE running buffer (1x)

(Diluted from 10x Tris/Glycine/SDS buffer from Bio-Rad)

25 mM Tris-HCl, pH 8.3

192 mM glycine

0.1% SDS

SSC buffer (20x)

3 M NaCl

0.3 M Sodium Citrate Dihydrate

TAE buffer (50x)

2 M Tris-base

1 M Acetic acid

50 mM EDTA

adjust pH to 8.4

TNM lysis buffer

10 mM Tris-HCl pH7.5

10 mM NaCl

10 mM $MgCl_2$

Western blot transfer buffer (1x)

(Diluted from 10x Tris/Glycine buffer from Bio-Rad)

25 mM Tris-HCl, pH 8.3

192 mM glycine

Methanol was added at a final concentration at 15% (v/v).

SDS was added at a final concentration at 0.2% (w/v).

2.3 Cell Biology and Protein biochemistry

2.3.1 Cell lines

All the cell lines used in this thesis were from the stock in Prof. Chris Proud's lab, otherwise stated: PC3 (human prostate cancer cell line) and ZR75 (human breast carcinoma cell line cell) cell lines were kind gift from Dr. Jeremy Blaydes, School of Medicine, University of Southampton; RAW cell line was kind gift from Dr. Malcolm East, Centre for Biological Sciences, University of Southampton.

2.3.2 Culture and treatment of adherent cell line

Cells were grown in DMEM (high glucose, GlutaMAX^{TM}, HEPES) supplemented with 10% (v/v) FBS and 1% penicillin/streptomycin, and cultured in 5% CO_2 incubator at 37°C. When cells reached approximately 80% confluence, they were passaged. To do this, the culture medium was aspirated and the cells were rinsed with sterile PBS. One ml trypsin was added into the flask/dish and the cells were incubated at 37°C for 3 min. Then the detached cells were resuspended in complete medium and seeded in new sterile vessels to the desired density. For long-term storage, the cells were digested

with trypsin, resuspended in cell freezing medium and aliquoted into storage vials. After freezing at -80°C overnight, the cells were moved to liquid nitrogen for long-term storage.

2.3.3 Transient transfection of cells

Lipofectamine™ 2000 (Invitrogen) was used for plasmid DNA transfection according to the manufacturer's protocol. Typically, one day before transfection, cells were plated in DMEM without antibiotics to achieve a 70%-80% confluence at the time of transfection. On the day of transfection, plasmid DNA and Lipofectamine™ 2000 were each diluted with DMEM. After 5 min incubation at room temperature, diluted plasmid DNA was gently mixed with diluted Lipofectamine™ 2000 and incubated for 20 min at room temperature. The mixture was then added to the cells. The cells were incubated at 37°C for 4-6 h before the medium was changed. Transgene expression can be tested after 48-98 h incubation.

Amounts of reagents used for the transfection:

Culture vessel	**Vol. of dilution medium[2]**	**DNA**	**Lipofectamine™ 2000**
6-well	2 x 100 µl	1 µg	4 µl
6-cm	2 x 250 µl	2 µg	8 µl

2.3.4 Gene silencing

The gene expression of 4E-BP1 was transiently knocked down with Stealth RNAi in HeLa cells. Stealth RNAi (Invitrogen) directed against 4E-BP1 (sequence 5′-UCUAUGACCGGAAAUUCCUGAUGGA) was transfected into the cells using Lipofectamine™ 2000 as the methods described in section 2.3.3. A Stealth RNAi with the same nucleotide composition was used as negative control (sequence 5′-UCUCCAGAAGGCUUAAGUCUUAGGA) (Averous *et al.*, 2008).

2.3.5 Protein extraction

After various treatments, the cells were moved out from the incubator and placed on ice. The culture medium was aspirated and the cells were washed once with ice-cold PBS. The cells were lysed with cell lysis buffer by thorough scraping. The lysates were transferred to microfuge tubes and cleared by centrifugation at 20,000g for 10 min at

4°C. Potein concentration was determined using the Bio-Rad Bradford reagent. Each serial dilution of standard (BSA) and unknown protein sample was pipetted into separate disposable cuvettes in triplicate. 1 x Bradford dye reagent was added to each cuvette and mix. The solution was incubated at room temperature for at least 5 min. The spectrophotometer was set to 595 nm and zeroed with the blank sample. The absorbance of the standards and unknown samples was measured. Protein concentration of each unknown sample was determined using the standard curve derived from the absorbance of the standards.

2.3.6 Pull-down assays

For anti-HA immunoprecipitations, 10 µl of protein G beads were added to cell lysis buffer and washed once. 1 µg of anti-HA high affinity was then added and mixed with the beads for 1 h at 4°C. Subsequently, total cell lysates were added and mixed with the beads for 1-2 h at 4°C. The beads were then washed twice with cell lysis buffer and absorbed proteins were eluted in SDS-PAGE sample buffer at 95°C for 5 min. To pull down eIF4E and associated proteins, cell extracts were incubated with m^7GTP-Sepharose 4B beads which were diluted with two volumes of Sepharose CL-4B for 1.5 h at 4°C under constant shaking. Beads were washed twice with cell lysis buffer and absorbed proteins were eluted in SDS-PAGE sample buffer at 95°C for 5 min.

2.3.7 Evaluation of in vivo eIF4E sumoylation

Sumoylation of total cellular eIF4E was evaluated by m^7GTP pull down assay and subsequent immunoblotting with anti-SUMO1 or anti-eIF4E. 20 mM SUMO isopeptidase inhibitor NEM (N-ethylmaleimide; Sigma-Aldrich) was added to the lysis buffer.

2.3.8 SDS-PAGE electrophoresis and electrotransfer

Cell lysates or immunoprecipitated samples were heated at 95°C for 5-7 min in SDS-PAGE sample buffer. Typically, protein samples are subjected to 12.5% (w/v) acrylamide gel and run using Bio-Rad electrophoresis system. Proteins of high molecular weight (more than 150 kDa) were analysed by resolving samples on a 10% acrylamide gel. Proteins of low molecular weight (less than 20 kDa; such as 4E-BP1)

were studied on a 13.5% (w/v) acrylamide gel with high concentration of 2% bisacrylamide [0.36% (v/v)]. Gels were run in SDS-PAGE running buffer at constant voltage (180 V) until the bromophenol blue dye was approximately 1cm from the bottom of the gel. Samples separated by SDS-PAGE were transferred to nitrocellulose/PVDF membranes using Bio-Rad Electrotransfer system for 1 h at 100V. PVDF membranes were activated with methanol for 15 sec to reduce its hydrophobicity. To prevent the non-specific binding, membranes were then blocked in PBS-0.05% (v/v) Tween 20 containing 5% (w/v) skimmed milk for 1 h at room temperature. The membranes were probed with the indicated primary antibody diluted in 2% BSA overnight at 4°C. Excess antibody was removed by washing the membrane 3 times, 5 min each, in PBS-0.05 % (v/v) Tween 20. After incubation with fluorescently-tagged secondary antibody, the signals were scanned and quantified using a Licor Odyssey imaging system (LI-COR Bioscience).

Composition of separation gel and stacking gel (0.75 mm)

Separation gel	**12.5%**	**10%**	**13.5%**
H_2O (ml)	1.3	2.12	0.76
1.5 M Tris-HCl pH8.8 (ml)	0.88	1.25	0.88
40% acrylamide (ml)	1.11	1.26	1.18
2% bisacrylamide (ml)	0.18	0.2	0.63
10% (w/v) SDS (µl)	35	50	35
10% (w/v) APS (µl)	17.5	25	17.5
TEMED (µl)	1.75	2.5	1.75
Total volume (ml)	3.5	4.9	3.5

Stacking gel	
H_2O (ml)	1
1.0 M Tris-HCl pH6.8 (ml)	0.19
40% acrylamide (ml)	0.19
2% bisacrylamide (ml)	0.1
10% (w/v) SDS (µl)	15
10% (w/v) APS (µl)	7.5
TEMED (µl)	1.5

2.3.9 Measurements of protein synthesis rates

Cells were incubated in the presence of inhibitors for the indicated time at 37°C. For the final 60 min, [^{35}S]methionine was added to a final concentration of 10 μCi/ml. After incubation, the medium was removed completely; the cells were washed with ice-cold PBS and then lysed. The protein concentrations in the extracts were then quantified using the Bradford method as described in section 2.3.5. Lysates were applied to 3 MM filter papers and allowed to dry at room temperature. After two brief washes with 5% (w/v) trichloracetic acid (once at 100°C) and one in acetone, filters were then dried. The incorporated radioactivity was measured by scintillation counting.

2.3.10 Pulsed SILAC, SILAC labelling and mass spectrometry

For pulsed SILAC experiments, HeLa cells growing in SILAC medium (and treated as described in the legends) were incubated with the medium containing 154 mg/L L-[^{13}C]$_6$,[^{15}N]$_2$-Lys and 89 mg/L L-[^{13}C]$_6$,[^{15}N]$_4$-Arg for 2-6 h at 37°C. Where serum was used, only serum dialysed against PBS was used to decrease the levels of free Arg and Lys. After incubation, the cells were washed twice and then harvested in PBS. After a short centrifugation at 600g, the pellets were resuspended in buffer containing 50 mM ammonium bicarbonate, 1% sodium deoxycholate and immediately heated to 95˚C.

For degradation analysis, two sets of HeLa cells were grown in the medium either containing 77 mg/L L-[^{13}C]$_6$,[^{15}N]$_2$-Lys and 44.5 mg/L L-[^{13}C]$_6$,[^{15}N]$_4$-Arg (heavy-labelled) or 75 mg/L L-[^{2}H]$_4$-Lys and 43.5 mg/L L-[^{13}C]$_6$-Arg (medium-labelled). Cells were passaged five times in the above medium. Then the heavy-labelled cells were moved into DMEM containing normal Lys/Arg and treated with the indicated inhibitors. After incubation, the cells were lysed as the methods described for pSILAC. Equal amounts of proteins from heavy-labelled and untreated medium-labelled cells were mixed prior to trypsin digestion. The medium-labelled samples act as an internal control for the heavy-labelled one.

After cooling, the protein concentration of the lysates was measured using the bicinchoninic acid (BCA) assay kit according to the manufacturer's instructions (Thermo Scientific). The kit came with two reagents, reagent A and reagent B, which needed to be mixed at 50:1 ratio to prepare the working reagent. The unknown sample replicate and a serial dilution of standard protein (BSA) are incubated with the working

reagent at 37°C for 30 min. After cooling to room temperature, the average 562 nm absorbance measurement of the blank replicates was subtracted from that of all other individual standard and unknown sample replicates. The protein concentration of each unknown sample was determined with a standard curve by plotting the average blank-corrected 562 nm measurement for each BSA standard.

25 µg of total protein was aliquoted, reduced, alkylated and digested as described by Rogers and Foster (2007). After digestion, the resulting deoxycholate precipitate was pelleted by centrifugation. The supernatant containing the peptides was then desalted, concentrated, filtered on C_{18} STop and Go Extraction tips (Rappsilber et al., 2003), and eluted into a 96-well plate. Peptide mixtures were analyzed on a linear trapping quadrupole-Orbitrap (ThermoFisher Scientific) tandem mass spectrometer as described (Chan *et al.*, 2006).

2.4 Molecular Biology

2.4.1 Vectors

Vectors for rat 4E-BP1 and HA-tagged eIF4E were taken from the stock in Prof. Chris Proud's lab and have been described (Waskiewicz et al., 1999, Tee and Proud, 2002). The vector for HA-eIF4E-Ser209Ala was generated using Quick-Change site-directed mutagenesis kit.

The mutagenic primer for generate HA-eIF4E-Ser209Ala (sense primer 5'-> 3')	CAGCTACTAAGAGCGGCGCCACCACTAAAAATAGG

2.4.2 Reverse Transcription (for real-time PCR)

Reverse Transcription was performed using ImProm-II™ Reverse Transcription System (Promega) according to the manufacturer's protocol. Briefly, the experiments were performed in 20 µl reactions comprised of components of the ImProm-II™ Reverse Transcription System. Experimental RNA was combined with the cDNA primer in RNAse/DNAse free water for a final volume of 5 µl reaction. The RNA/primer mix was thermally denatured at 70°C for 5 min and chilled on ice for at least 5 min. A reverse transcription reaction mix was prepared containing nuclease-free

water, reaction buffer, reverse transcriptase, magnesium chloride, dNTPs and ribonuclease inhibitor on ice. As a final step, the RNA/primer combination was added to the reaction mix on ice. Following an initial annealing at 25°C for 5 min, the reaction was incubated at 42°C for 1 h. Finally, the reverse transcriptase was inactivated by incubation of 15 min at 70 °C.

2.4.3 Real-time RT-PCR

A. Bench-side protocol

1. Tubes containing lyophilised primer mix were pulse-spun before opening, and the lyophilised primer mix was then resuspended in RNAse/DNAse free water.

2. When using PrimerDesign 2 x Precision™ Mastermix with SYBR green, a mix containing all real-time RT-PCR reagents was made up according to the protocol below.

Component	**Each reaction**
Resuspended primer mix	1 µl
PrimerDesign 2X Precision™ Mastermix*	10 µl
RNAse/DNAse free water	4 µl
Final volume	15 µl

*working concentration of primers = 300 nM in a 20 µl reaction

4. 15 µl of this mix was added into each well according to the experimental plate set up.

5. cDNA was prepared using ImProm-II™ Reverse Transcription System (Promega) as described above, and diluted in RNAse/DNAse free water at 1:10.

6. 5 µl of diluted cDNA was added into each well, according to the experimental plate set up. The final volume in each well was 20 µl.

7. Wells where the cDNA was replaced with RNAse/DNAse free water was included. Any amplification in this sample was indicative of cDNA cross contamination between wells, or contamination of one or more reagents.

B. Amplification protocol

Amplification was performed on Applied Biosystems StepOnePlus™ Real-Time RT-PCR instrument using the following conditions.

Stage	Time	Temperature	Cycle
Enzyme activation	10 min	95ºC	1x
Denaturation	15 sec	95ºC	40-50x
Data collection	60 sec	60ºC	
Melt Curve Stage			1x

2.4.4 Microarray Analysis

A. Sucrose gradient density centrifugation and RNA detection

Briefly, HeLa cells were lysed and post-nuclear lysates were applied to sucrose gradients (10% to 60%) which were then centrifuged at 16, 0000 g for 2 h at 4 °C. Gradients were fractionated with continuous monitoring the absorbance at 254 nm. Eleven 1 ml fractions were collected into guanidine-HCl, and followed by precipitation with ethanol. The RNA was then resuspended in water and further purified by sodium acetate/ethanol precipitation.

B. Microarray hybridisation

The human cDNA microarrays were kindly obtained from Dr Paddy McTighe at the University of Nottingham Genomics Facility. RNA from sucrose density gradient fractionation was pooled into subpolysomal or polysomal fractions based on the polysome profile, precipitated first with 2.5 M LiCl and then with sodium acetate and ethanol. Fluorescently labelled DNA probes were generated using equal proportions of RNAs of pooled subpolysomal fractions (Cy3) and pooled polysomal fractions (Cy5) and hybridised to the microarrays as described previously (Bushell et al., 2006). Microarray slides were scanned using a GenePix 4200B microarray scanner and GenePix Pro 6.0 software (Axon Instruments).

C. Analysis of Microarray Data

Fluorescence intensities for individual spots on the microarray were quantified using GenePix Pro 6.0. All statistical analysis was performed by Dr. Lindsay Wilson,

University of Leicester.

2.4.5 Polysome analysis and Northern blot analyses

A. To prepare cell lysates

HeLa cells that had been washed three times with ice-cold PBS buffer were lysed directly on the plate with 300 μL of lysis buffer (268 μL TNM lysis buffer, 1% Triton X-100, 10 mM DTT, 40 U RNase inhibitor) and transferred into a microcentrifuge tube. The lysates were centrifuged for 5 min at 17,000 g at 4°C. The supernatant was transferred to a new tube in the presence of 3 μL heparin (10 μg/μL) and stored at -80°C after freezing in liquid nitrogen.

B. To prepare the density gradients

The polyallomer centrifuge tube was placed in dry ice, and 1.5 ml of 50% sucrose solution was carefully added in the bottom of the tube. After the first layer was frozen, the next lighter sucrose solution was added, and so on until all six layers were frozen well. The gradients were stored at -80°C until required. The day before the sample centrifugation, the gradients were taken out into 4°C overnight to allow them to thaw slowly.

C. Sample centrifugation and fraction collection

The lysates were carefully applied onto the top of the gradient and the gradient was inserted into the pre-cold bucket. The metal cup was screwed onto the bucket and placed in the rotor. The samples were centrifuged at 160000 g for 110 min at 4°C. Immediately after the centrifugation, the fractions were collected into 9 microcentrifuge tubes using the fraction collectors (Gilson FC 203B/FC 204) with continuous monitoring the absorbance at 254 nm.

D. RNA extraction and Northern analysis

RNA from each fraction was extracted using the Proteinase K method and precipitated by isopropanol. For Northern blot analysis, RNA was separated on 1% formaldehyde-agarose gels and transferred to GreenScreen Plus membrane (Perkin Elmer) as recommended by the manufacturer. Typically, samples were loaded on 1% agarose-formaldehyde gel and run in 1x MOPS buffer at 150 V for 20 min. After running, the gel was placed in an RNase-free dish and rinsed twice with sufficient deionized water. The transfer was performed in 20x phosphate buffer overnight at 4°C. After transfer, the membrane was washed in pre-warmed Northern mix buffer at 42°C for at least 20

min. The purified radioactive probe was added and incubated with the membrane overnight at 42°C. The radioactive probes were prepared by the random primer technique (Prime-It II Random Primer Labeling kit, Agilent Technologies) using DNA fragments isolated from plasmids containing PCR-amplified cDNA sequences.

Primers for cloning DNA fragments used in generating the radioactive probes (sense primer 5'-> 3')	
HNRNPA3	tgagaaatgg ggcacactcacaga
NAP1L1	gacttgatggtctggtagaaacacc
PRDX6	ctggtgctgt gagccagaggat

(For RPL11, RPS19 and β-actin, plasmids containing these PCR-amplified cDNA sequences were kind gifts from Professor Fabrizio Loreni's lab, University of Rome, Italy)

First, DNA fragments and random primers were added into a microcentrifuge tube and heated in a boiling water bath for 5 min. 5x dATP buffer, labelled nucleotide (α-^{32}P –dATP; Perkin Elmer) and Exo(-) Klenow enzyme were then added to the reaction tube and incubated at 37°C for 10 min. Finally, the reaction was stopped by adding stop mix buffer following the incubation period. Before applied to the hybridisation, the probes were purified using illustra ProbeQuant™ G-50 Micro Columns (GE healthcare). Primers for amplification were designed according to sequences present in the GenBank/EBI Data Bank. After hybridisation, the membrane was washed in 2x SSC plus 1% SDS buffer twice (1st wash at room temprature for 10 min, and 2nd wash at 65°C for 20-30 min). Northern blots were then visualised and quantified using a phosphorimager and the Image-Quant software (Amersham Biosciences).

2.4.6 RNA-protein interaction assay (Niranjanakumari et al., 2002)

A. *In vivo* formaldehyde fixation of HeLa cells.

HeLa cells transfected with HA-eIF4E, HA-eIF4E-S209A or the corresponding empty vector were collected with trypsin digestion. After washed once with DMEM, twice with ice-cold PBS, the cells were resuspended in 10 ml of ice-cold PBS. Formaldehyde (36.5% HCHO/10% methanol) was added to a final concentration of 1% (v/v) and incubated with slow shaking for 10 min at room temperature, followed by further

incubation with glycine (pH7.0, 0.25 M) for 5 min at room temperature (to quench the crosslinking reaction). The cells were harvested by centrifugation at 200 g for 4 min followed by two washes with ice-cold PBS. The cell pellets were frozen in dry ice and stored at -80°C for subsequent analysis.

B.Solubilization of crosslinked complexes by sonication.

Fixed cell pellets were resuspended in 2 ml of RIPA buffer 1 and lysed by three rounds of sonication, 20 s each. Between each cycle, the samples were kept in ice-water bath for 2 min. Insoluble material was removed by centrifugation at 16,000 g for 10 min at 4°C.

C. Preclearing lysates

An aliquot of the above lysate (500 µl) was mixed with 30 µl (packed volume) protein G-Sepharose beads in the presence of nonspecific competitor tRNA (final concentration of 100 µg/ml). The mixture was rotated for 1 h at 4°C. The precleared supernatant was collected by centrifugation.

D. Immumoprecipitation of crossslinked RNP (Ribonucleoprotrein) complexes.

Protein G-Sepharose beads (30 µl, packed volume) were coated with anti-HA (1.5 µg) for 2 h at 4°C followed by extensive washing with RIPA buffer 1. Before immunoprecipitation, the beads were incubated for 10 min with 0.5 µl of RNasin (40 U/µl, Promega). The precleared lysate (500 µl) was mixed with the anti-HA–coated beads, and incubated with rotation at 4°C for 90 min. The beads were collected using a minicentrifuge at 6000 rpm for 45 s and the supernatant was saved for RNA extraction. The beads were then washed six times with 1 ml of RIPA buffer 2 by 10 min rotation at room temperature. The beads containing the immunoprecipitated samples were collected and resuspended in 100 µl of RIPA buffer 3.

E. Reversal of crosslinks and RNA purification

Samples (resuspended beads) were incubated at 70°C for 45 min to reverse the crosslinks. The RNA was extracted from these samples using Trizol (Invitrogen) according to the manufacturer's protocol. RNA precipitates were collected by microcentrifugation at 16,000 g for 10 min, washed with 70% ethanol, air-dried, and resuspended in 10 µl of RNase-free water.

F. Analysis of immunoprecipitated RNA by RT-PCR

The RNA (3/10th of the total sample) purified from the previous step was used as a template to synthesize cDNA using the method as described before. PCR was performed in a 50-µl volume using GoTaq DNA polymerase (Promega). After an

initial incubation at 95°C for 2 min, the reaction mixtures were subjected to 25–35 cycles of amplification using the following sequence: 95°C for 30 s, 46-51°C for 30 s, and 72°C for 30 s, followed by a final extension step at 72°C for 5 min. 25 µl of each reaction mixture was run on a 2% agarose gel for analysis.

G. Details of primers used in the RIP assay [primers were designed by PrimerDesign; they used a modified version of Beacon Designer and the industries finest DNA analysis software (visual OMP) to model in silico the thermodynamic properties of each real-time PCR probe they designed].

Gene symbol	**Sense Primer 5'-> 3'**	**Product length (bp)**
BIRC2	GTGGGTCGCAATGATGATGT	115
CCL7	TGTAAAAACTGTGGGATGCTC	114
MMP3	ATGATGAACAATGGACAAAGGATAC	101
VEGFC	CCCCAAACCAGTAACAATCAGT	96
RBMX	GTGGAGGAAGCCGATCTGA	92
MCL1	CTGATTGTTCTGCTGCCTCTAC	104

CHAPTER 3
THE DISTINCT EFFECTS OF RAPAMYCIN AND mTOR KINASE INHIBITORS ON mTOR DOWNSTREAM SIGNALLING AND PROTEIN SYNTHESIS

3.1 Introduction

As already discussed, mTOR forms two types of multiprotein complex with distinct components and functions, mTORC1 and mTORC2. mTORC1 controls many essential functions in cells, such as mRNA translation, cell growth and cell proliferation, while mTORC2 is thought to promote cell survival (reviewed in Corradetti and Guan, 2006). mTORC1 is sensitive to rapamycin inhibition, whereas mTORC2 is considered rapamycin-insensitive in the short-term treatment (Rosner and Hengstschlager, 2008). Rapamycin does not directly inhibit mTOR kinase activity; rather, it works through an unusual allosteric mechanism which requires interaction with the immunophilin FKBP12 and binding to a domain far from the kinase's active site. Since rapamycin does not inhibit mTORC2, it is less well studied than mTORC1. Recently, many new mTOR-KIs have been developed as possible anti-cancer agents. These new compounds are reported to be highly-selective and ATP-competitive, and some can induce significant inhibition of tumour cell line growth both *in vitro* and *in vivo* (reviewed in Zask et al., 2011). Because these novel mTOR-KIs affect both mTORC1 and mTORC2, they would be very useful to identify many mTOR functions that are resistant to inhibition by rapamycin. In this chapter, I have studied the effects of rapamycin and two mTOR-KIs (PP242, AZD8055) on a number of aspects of mTOR signalling and protein synthesis, in order to identify differences in their effects.

3.2 Results

3.2.1 The mTOR-KI AZD8055 strongly inhibits insulin-induced phosphorylation of targets of mTORC1 and mTORC2.

Insulin and insulin-like growth factors (IGFs) initiate the PI3K-Akt-mTOR signalling pathway at the plasma membrane, resulting in the phosphorylation of a number of targets, such as S6K1 and 4E-BP1 (reviewed in Hay and Sonenberg, 2004). In order to examine the effects of mTOR-KIs on the ability of insulin to induce the phosphorylation of mTOR signalling downstream effectors, HeLa cells were starved of serum for at least 16 h and then treated with insulin. In the presence or absence of rapamycin or AZD8055, the phosphorylation of different targets of mTORC1 and mTORC2 was studied using phosphospecific antibodies.

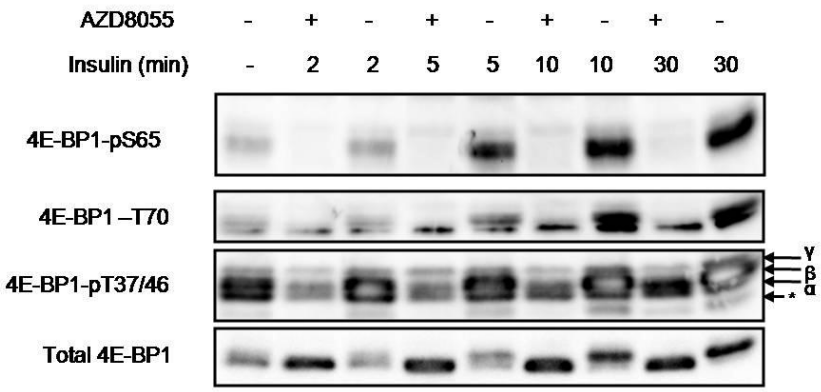


Figure 3.1. AZD8055 inhibits insulin-induced phosphorylation of 4E-BP1 at different sites in serum-starved HeLa cells.

HeLa cells were starved of serum (16 h) and then treated with AZD8055 (100 nM) for 1 h prior to stimulation with insulin. Lysates were analyzed by western blot using the indicated antibodies. The arrows indicate different species of 4E-BP1 α, β and γ.

* indicates nonspecific bands.

As an important downstream target of mTORC1, phosphorylation of 4E-BP1 is often used as readout of mTORC1 activity. Although rapamycin inhibits mTORC1, it does so incompletely. For example, phosphorylation at Thr37/46 and Thr70 of 4E-BP1 is not blocked by rapamycin in various cell lines (Thoreen *et al.*, 2009). As expected, insulin increased the phosphorylation of 4E-BP1 at all the sites, even though 4E-BP1 was already substantially phosphorylated at Thr37/46 (Fig. 3.1). This is consistent with the previous findings, suggesting that the phosphorylation at the N-terminal sites (Thr37/46) of 4E-BP1 is not sensitive to serum deprivation but is maintained by the amino acids in the medium (Wang *et al.*, 2005, Thoreen *et al.*, 2009). Moreover, the phosphorylation of Ser65 and Thr70 gradually increased along the time course of insulin treatment. The increase in Ser65 phosphorylation was probably more rapid than that in Thr70, as it was already dramatically enhanced after 5 min incubation with insulin. AZD8055 had a strikingly strong effect on the phosphorylation of 4E-BP1. It completely blocked Ser65 phosphorylation and substantially decreased Thr70 phosphorylation induced by insulin (Fig. 3.1). The basal level of Thr37/46 phosphorylation was already considerably high, resulting in saturated signal when stimulated by insulin. However, AZD8055 still reduced insulin-induced phosphorylation at these sites.

Ribosomal protein S6K is another well-characterized downstream target of mTORC1. S6Ks have many substrates, including rpS6 which has several phosphorylation sites (Ferrari *et al.*, 1991). Since Ser240/244 of rpS6 is not phosphorylated by p90 RSK (it phosphorylates Ser235/236; Pende et al., 2004) and is, therefore, more specific to mTOR regulation, it was chosen to test the effect of the inhibitors. The basal level of phosphorylation of rpS6 at Ser240/244 was very low (in serum-starved HeLa cells), demonstrating that S6K was not activated (Fig. 3.2). Insulin induced rapid phosphorylation of rpS6 at Ser240/244. The phosphorylation level was stronger after 10 min incubation with insulin. Rapamycin decreased the phosphorylation induced by insulin to the basal level, consistent with this site being regulated by mTORC1 and S6Ks. AZD8055 also showed a strong inhibition at this site and completely blocked the insulin-induced phosphorylation of rpS6 at these sites.

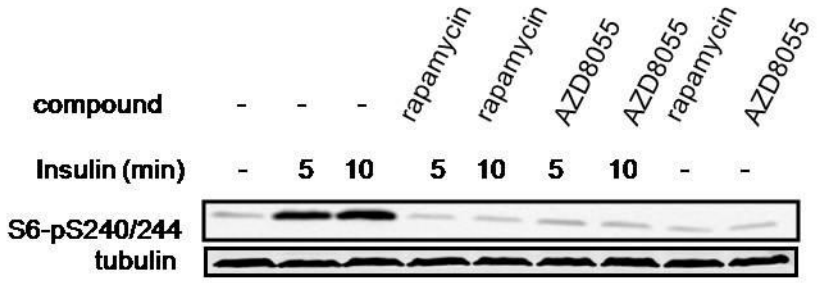


Figure 3.2. Rapamycin and AZD8055 strongly inhibit the phosphorylation of rpS6 at Ser240/244 in response to insulin in HeLa cells.

HeLa cells were starved of serum (16 h) and then treated with rapamycin (100 nM) or AZD8055 (100 nM) for 1 h prior to stimulation with insulin. Lysates were analyzed by western blot using a S6-pSer240/244 antibody. The same blot was reprobed with a tubulin antibody as a loading control.

The phosphorylation of Akt at Thr308 and Ser473 was also examined in serum-depleted HeLa cells (Fig, 3.3). Of the two sites, Thr308 is located in the T loop and phosphorylated by PDK1 (Alessi et al., 1997b, Stokoe et al., 1997), whereas Ser473 is in the hydrophobic motif and is considered as mTORC2 site (Sarbassov et al., 2005). The interplay between these two sites is ambiguous, but it has been reported both of them are required for Akt to achieve its optimal activity (Andjelkovic *et al.*, 1997, Alessi *et al.*, 1996).

Insulin rapidly increased the phosphorylation of Akt at Thr308 and Ser473 and its substrate, FOXO after 2 min (Fig. 3.3). However, the phosphorylation of Akt at Ser473 (and its substrate FOXO) was reduced after 30 min of stimulation by insulin. AZD8055 abolished the phosphorylation of Akt at Ser473 and also remarkably reduced its phosphorylation at Thr308. The phosphorylation of FOXO was partially inhibited by AZD8055, suggesting that phosphorylation of this site is only modestly affected by loss of phosphorylation of Akt at Ser473. The mechanism by which AZD8055 inhibits phosphorylation of Akt at Thr308 is unclear. Feldman *et al.* (2009) found that another mTOR kinase inhibitor PP242 also inhibited Akt phosphorylation at both Ser473 and Thr308 in WT MEFs. By contrast, when phosphorylation of Akt at Ser473 was abolished by genetic approaches (either by loss of SIN1 or transfecting cells with a Ser473Ala mutant of Akt), Thr308 remained phosphorylated even in the presence of PP242. Their findings support the idea that the inhibition of phosphorylation of Thr308 by PP242 is dependent on its inhibition of Ser473 (Feldman *et al.*, 2009). In a very recent report on AZD8055 by Chresta *et al.* (2010), the authors found that the inhibition of phosphorylation of Akt at Thr308 by AZD8055 is not as potent as Ser473 and generally requires higher doses of this compound. They hypothesised that the inhibition of Thr308 by mTOR-KIs is probably caused by a reduction in the ability of PDK1 to phosphorylate Akt on Thr308 when Ser473 phosphorylation is transiently but fully inhibited, rather than the ability of mTOR-KIs to directly inhibit PDK1.

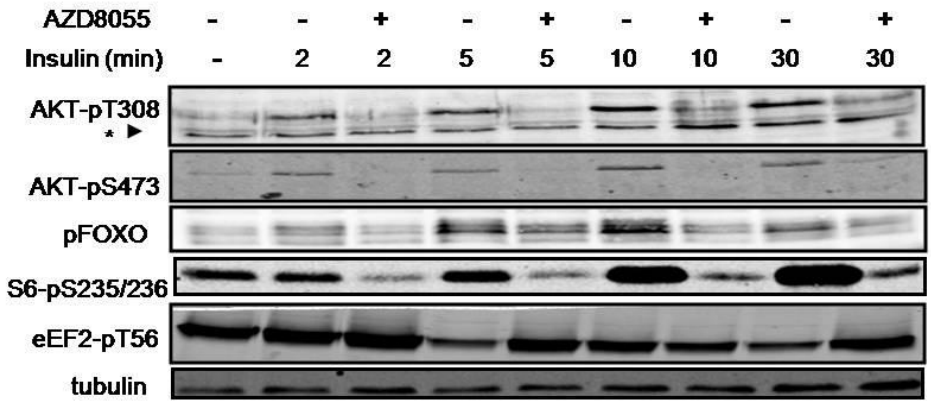


Figure 3.3. Effects of AZD8055 on the phosphorylation of Akt, Akt substrates, eEF2 and rpS6 in response to insulin.

HeLa cells were starved of serum (16 h) and then treated with AZD8055 (100 nM) for 1 h prior to stimulation with insulin. Lysates were analyzed by western blot using the indicated antibodies. The multiple bands of FOXO are due to the antibody detecting the phosphorylation of different isoforms of FOXO: FOXO1, FOXO3a and FOXO4.

* indicates nonspecific bands.

The effect of AZD8055 on phosphorylation of rpS6 at Ser235/236 and eEF2 at Thr56 was also examined (Fig. 3.3). Similarly to Ser240/244 (Fig. 3.2), insulin induced the phosphorylation of Ser235/236 very quickly. AZD8055 substantially decreased the phosphorylation of this site. The remaining signal might be due to a small contribution from p90 RSK. It has been previously shown that phosphorylation of eEF2 at Thr56 by eEF2 kinase inhibits its activity, and eEF2 kinase is phosphorylated by p70 S6K at Ser366 (Ryazanov and Davydova, 1989, Redpath *et al.*, 1993, Wang *et al.*, 2001). Insulin caused the dephosphorylation of eEF2 after 5 min, resulting in its activation, whereas AZD8055 increased the phosphorylation of eEF2, leading to its inactivation (Fig 3.3).

To confirm that the above effects of AZD8055 on Akt and its substrate were not confined to HeLa cells, two other cell lines were chosen: the breast cancer cell line MCF7 and the human embryonic kidney cells HEK293. As seen in Fig. 3.4, AZD8055 inhibited both basal and insulin-induced Akt phosphorylation at Thr308 and Ser473 in the two cell lines. The phosphorylation of GSK3 was also reduced by treatment with AZD8055. In agreement with the effects observed in HeLa cells (Fig. 3.3), AZD8055 only slightly decreased the phosphorylation of FOXO. However, it also blocked its basal phosphorylation in MCF7 cells (Fig. 3.4).

Akt can directly phosphorylate TSC2 at two sites, Ser939 and Thr1462 on the full-length human protein (Dan *et al.*, 2002). Phosphorylation of TSC2 inhibits its activity as a GTPase-activating protein for Rheb, thereby allowing Rheb-GTP to activate mTORC1 signalling (reviewed in Manning and Cantley, 2003). As shown in Fig. 3.4, AZD8055 strongly inhibited insulin-induced phosphorylation of TSC2 at Thr1462.

Collectively, AZD8055 suppressed the insulin-induced activation of various targets of mTORC1 and mTORC2, showing its strong effects on both complexes.

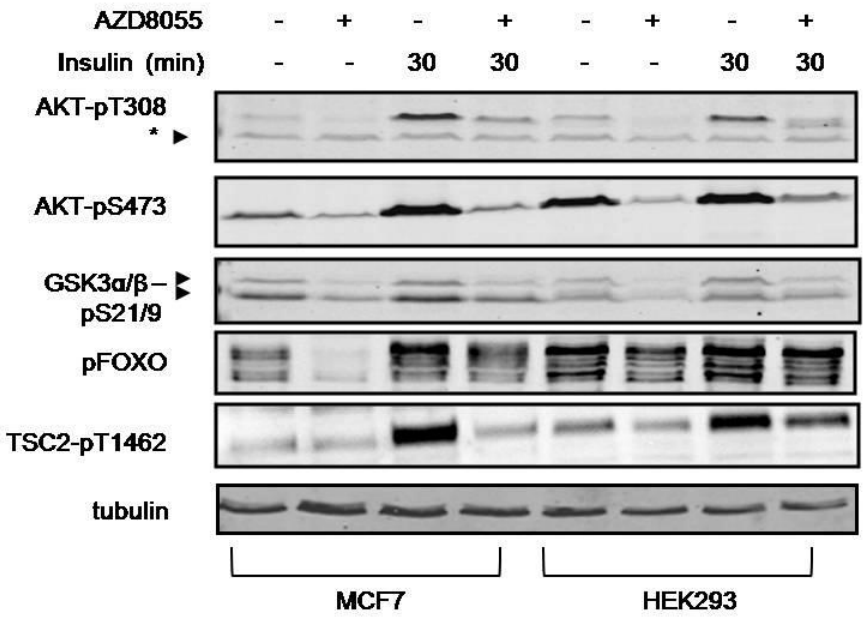


Figure 3.4. Effects of AZD8055 on the phosphorylation of Akt and its substrates in MCF7 and HEK293 cells.

Cells were starved of serum (16 h) and then treated with AZD8055 (100 nM) for 1 h prior to stimulation with insulin. Lysates were analyzed by western blot using the indicated antibodies. * indicates unspecific bands.

3.2.2 The mTOR-KIs PP242 and AZD8055 inhibit the phosphorylation of targets of mTORC1 and mTORC2 in serum-fed cells.

The above data demonstrated the effects of AZD8055 on mTOR signalling in response to insulin. These data are obviously very useful and valuable for understanding the effects of mTOR-KIs. However, for cancer therapy, examining these compounds in serum-replete culture cells may provide more useful insights.

To compare the different effects between rapamycin and AZD8055 in serum-maintained cells, HeLa cells growing in serum-replete medium were treated with rapamycin and AZD8055 over a range of times up to 60 min. The phosphorylation of 4E-BP1 at different sites was tested. As seen in Fig. 3.5, in the presence of serum, the basal level of phosphorylation of 4E-BP1 was already high. Rapamycin did not lead to a significant change in the phosphorylation of these sites. In contrast, AZD8055 substantially suppressed phosphorylation of Thr37/46 and Ser65. For Thr37/46, the phosphorylation of all three isoforms (α, β, γ) was inhibited by AZD8055 even as early as 10 min of treatment. For Ser65 and Thr70, clear band shifts were observed following treatment with AZD8055. The insensitivity of phosphorylation of Thr37/46 and Thr70 to rapamycin in HeLa cells has been reported before (Thoreen et al., 2009), but the lack of effect of rapamycin on Ser65 appears surprising. This might be due to the cross-reaction of the antibody with another site in human 4E-BP1. More details will be discussed below.

The effect of rapamycin and AZD8055 on the phosphorylation of translation factors, eEF2 and eIF2Bε, was also studied (Fig. 3.5). Both rapamycin and AZD8055 inactivated eEF2 through increasing its phosphorylation at Thr56. The phosphorylation of GSK3 leads to its inactivation and loss of function in phosphorylating eIF2B, thus facilitating mRNA translation (Rayasam *et al.*, 2009). Rapamycin slightly increased the phosphorylation of GSK3, whereas AZD8055 decreased it (Fig. 3.5). The distinct effects of rapamycin and AZD8055 on GSK3 were further reflected by the behaviour of eIF2Bε-Ser535 caused by these compounds (Fig. 3.5). Rapamycin also caused the enhancement of Akt-pThr308 and a minimal change of PRAS40-pThr246 whereas AZD8055 inhibited both of them. The finding that rapamycin increased the phosphorylation of Akt and its substrates probably provides new insights into its limited success towards some tumour types.

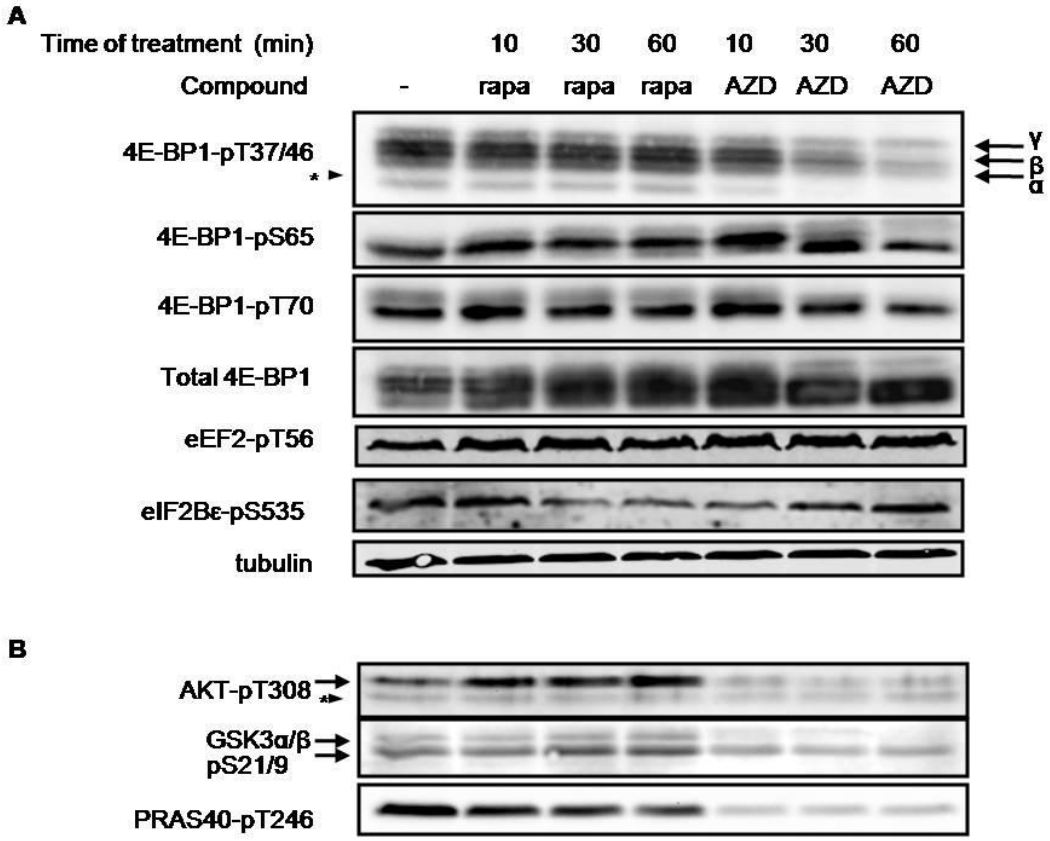


Figure 3.5. Effects of differing periods of treatment with rapamycin or AZD8055 on selected proteins in serum-fed HeLa cells.

(A, B) HeLa cells growing in serum were treated with rapamycin (rapa; 100 nM) or AZD8055 (AZD; 100nM) for the indicated times, and then lysed. Lysates were analyzed by western blot using the indicated antibodies. The signal for tubulin in A also serves as a loading control for B. Three isoforms (α, β, γ) of 4E-BP1 are indicated; * denotes nonspecific bands.

To have a better understanding of the distinct effects of rapamycin and mTOR-KIs, another mTOR kinase inhibitor PP242 was included for analysis (Fig. 3.6). PP242 was developed by Feldman *et al.* (2009) and showed profound effects on both mTORC1 and mTORC2 in L6 myotubes and HEK293 cells. Therefore, I examined its effects in HeLa cells, the main cell type used in this study. HeLa cells growing in serum-fed medium were treated with PP242 at different concentration, rapamycin at 100nM or AZD8055 at 100nM for 60 min. As shown in Fig. 3.6, rapamycin completely inhibited the phosphorylation of rpS6 at Ser235/236 and decreased the signal seen with an antibody that recognises the Ser240/244, specific sites for S6Ks (Ferrari *et al.*, 1991, Pende *et al.*, 2004). The residual signal of pSer240/244 may be due to an ability of this batch of antibody to detect non-phosphorylated rpS6. Similar effects on rpS6 phosphorylation were seen with PP242 and AZD8055 (even the lowest concentration used here had a substantial inhibition). PP242 also increased the level of phosphorylation of eEF2 at Thr56 (Fig. 3.6 A).

In addition, PP242 also exhibited similar effects on 4E-BP1 to AZD8055. No obvious effects of rapamycin were observed on these sites. As mentioned before, the finding that rapamycin failed to inhibit Ser65 may reflect the recognition by some batches of this antibody of another site in human 4E-BP1. Indeed, the cross-reaction of the antibody with Ser101 may account for the remaining signal for Ser65 in cells treated with rapamycin. Since the corresponding residue in rat or mouse 4E-BP1 lies in a different sequence context (Wang *et al.*, 2003), HeLa cells were transfected with a vector encoding rat 4E-BP1, where the corresponding site is S64, not 65. The data in Fig. 3.6C clearly showed that rapamycin partially inhibited and PP242 completely blocked the signal seen with this antibody for rat 4E-BP1. Phosphorylation of Akt at Ser473 was diminished by PP242, as seen in Fig. 3.6D, while that of Akt-Thr308, GSK3 and PRAS40 was not completely inhibited by PP242. Rapamycin again slightly increased the phosphorylation of all these sites (Fig. 3.6D).

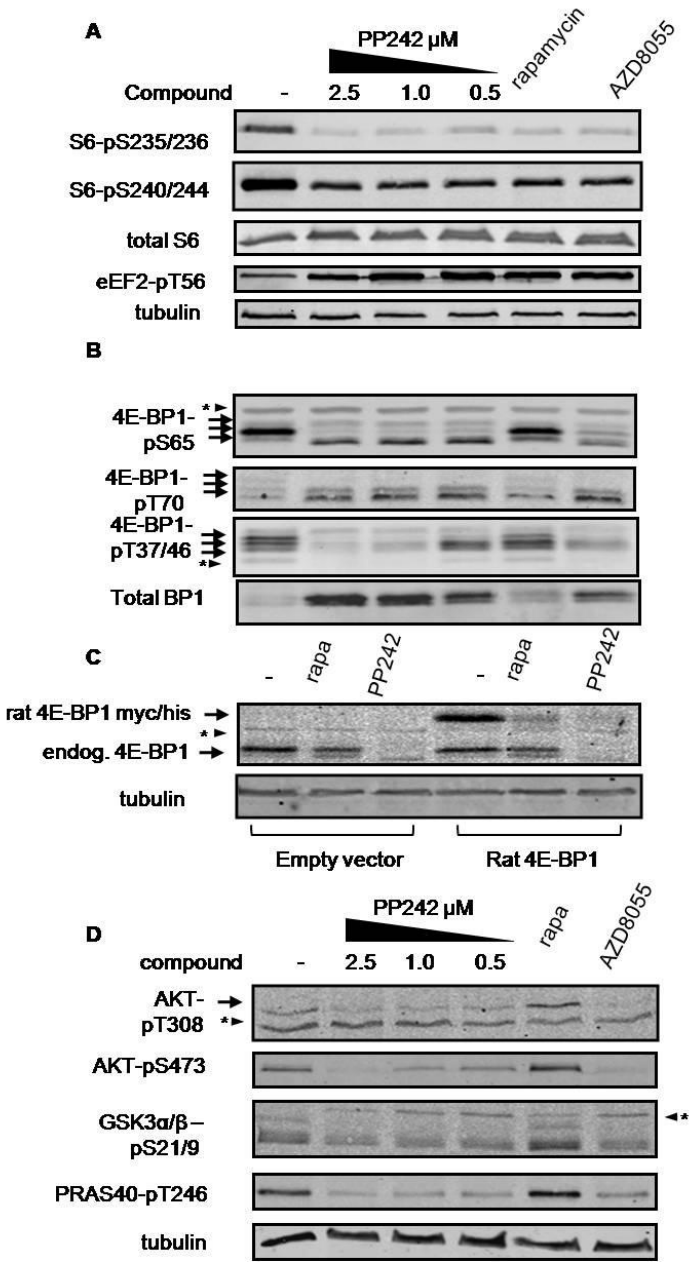


Figure 3.6. Effects of rapamycin, PP242 and AZD8055 on downstream signalling targets of mTORC1 and mTORC2 in serum-fed HeLa cells.

(A), (B) and (D): serum-fed HeLa cells were treated with the PP242 at the indicated concentrations, rapamycin (rapa; 100 nM) or AZD8055 (100 nM) for 60 min. Cell lysates were analyzed by western blot using the indicated antibodies. (C) HeLa cells were transfected with an empty vector (pcDNA 3.1) or a vector encoding rat 4E-BP1 (containing C-terminal Myc and His tags). Where indicated, cells were treated with rapamycin or PP242 for 60 min. Lysates were analysed by western blot using the indicated antibodies. * denotes nonspecific bands; arrows indicate the species under study.

In order to have a complete understanding of the distinct effects of rapamycin and mTOR-KIs on mTOR signalling, a summary of all the targets tested is given in Table 3.1. Although rapamycin induced an inhibition of rpS6 phosphorylation at both Ser235/236 and Ser240/244, it had limited effect on 4E-BP1 phosphorylation, suggesting its incomplete inhibition of mTORC1 activity. In contrast, mTOR-KIs (PP242 and AZD8055) significantly inhibited the phosphorylation of these rapamycin-insensitive sites. Both rapamycin and mTOR-KIs caused inactivation of eEF2 by increasing its phosphorylation at Thr56. Rapamycin treatment increased phosphorylation of Akt at Ser473 as well as its substrates GSK3 and PRAS40, whereas mTOR-KIs inhibited the phosphorylation of those targets, revealing a lack of function of rapamycin on mTORC2. Rapamcyin also increased phosphorylation of Akt at Thr308, which might be mediated by the feedback loop via S6Ks (Harrington *et al.*, 2004).

Table 3.1. Summary of effects of rapamycin and mTOR-KIs (PP242 and AZD8055) on the phosphorylation of mTORC1 and mTORC2 targets in serum-fed cells.

	Compound / Target	Rapamycin	mTOR kinase inhibitors	Functions
Serum-fed Cells	4E-BP1-Thr37/46	/	↓	mTORC1 sites
	4E-BP1-Ser65	↓	↓	
	4E-BP1-Thr70	/	↓	
	rpS6-Ser235/236	↓	↓	
	rpS6-Ser240/244	↓	↓	
	eEF2-Thr56	↑	↑	S6K substrate
	Akt-Thr308	↑	↓	PDK1 site
	Akt-Ser473	↑	↓	mTORC2 site
	GSK3α/β-Ser21/9	↑	↓	Akt Substrates
	PRAS40-Thr246	↑	↓	

↑: Increase;

↓: Decrease;

/: No effect;

3.2.3 mTOR-KIs, but not rapamycin, decrease the binding of eIF4G to eIF4E in HeLa cells.

Activation of mTORC1 stimulates cap-dependent translation via its downstream substrates. It is well understood that the hypophosphorylated 4E-BP1 interacts strongly with the cap-binding factor eIF4E, inhibiting the formation of the translation initiation complex eIF4F and, therefore acting as a negative regulator of cap-dependent translation (Pause *et al.*, 1994). Identification of the strong effects of PP242 and AZD8055 on mTOR signalling, especially on the phosphorylation of 4E-BP1, suggested it would be very interesting to assess the effects of mTOR-KIs on the formation of eIF4F.

To assess the binding between eIF4E and 4E-BPs/eIF4G (i.e. the formation of eIF4F), m^7GTP Sepharose beads, which mimic the mRNA 5'cap to precipitate cap-interacting proteins, were used to perform a pull down assay. Firstly, effect of AZD8055 on the formation of eIF4F in response to insulin was examined in serum-starved HeLa cells (Fig 3.7).

Insulin rapidly promoted the binding of eIF4GI to eIF4E after 2 min. Surprisingly, increasing the length of insulin treatment did not further enhance the interaction between eIF4GI and eIF4E. AZD8055 substantially inhibited the amount of eIF4GI bound to eIF4E when it caused a large increase of bound 4E-BP1 (Fig. 3.7).

PMA (phorbol myristate acetate) can also activate mTOR independently of Akt through inhibition of TSC2 (Roux *et al.*, 2004). Therefore effect of AZD8055 on PMA-induced eIF4F formation was studied as well (Fig. 3.8). PMA induced eIF4GI binding to eIF4E in serum-starved HeLa cells. This effect was blocked by adding AZD8055 in a dose-dependent manner. Rapamycin however did not inhibit the binding of eIF4GI to eIF4E. The data in Fig. 3.7 and Fig. 3.8 demonstrate that AZD8055 blocks both insulin and PMA-induced eIF4GI binding to eIF4E in HeLa cells.

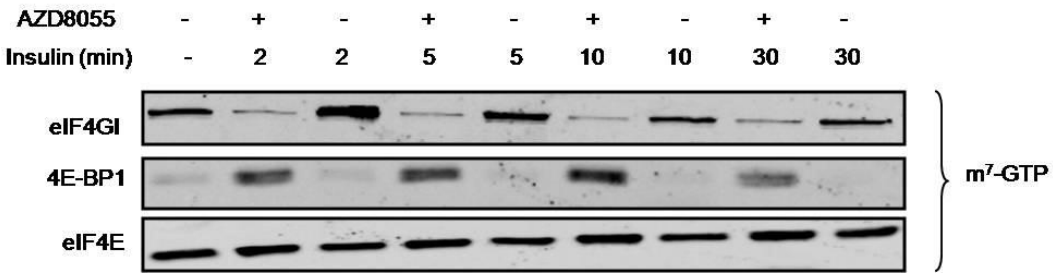


Figure 3.7. Effects of AZD8055 on the insulin-induced formation of eIF4F in HeLa cells.

HeLa cells were serum-starved for 16 h, then treated with AZD8055 at 100 nM for 60 min, and then incubated with insulin for the indicated time. Equal amounts of lysates were subjected to affinity chromatography on m^7GTP-Sepharose. Bound proteins were analysed by western blot using the indicated antibodies. Note: samples were also applied to CL4B sepharose in order to test any nonspecific binding (one typical data will be shown later).

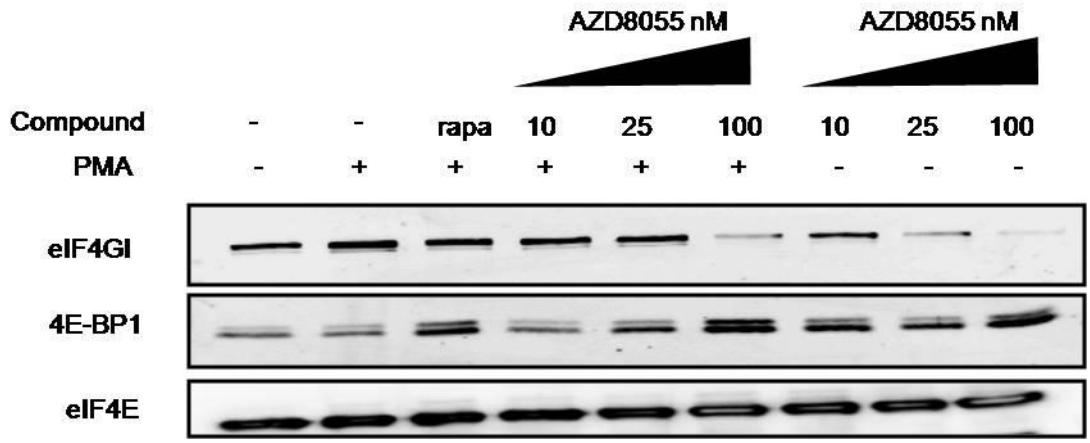


Figure 3.8. AZD8055 inhibits PMA-induced eIF4F formation in HeLa cells. HeLa cells were serum-starved for 16 h, treated with rapamycin at 100 nM or AZD8055 at the indicated concentrations for 60 min, and then incubated with PMA for 30 min. Equal amounts of lysates were subjected to affinity chromatography on m^7GTP-Sepharose. Bound proteins were analysed by western blot using the indicated antibodies.

Due to the promising results of AZD8055 on the formation of eIF4F, it would be interesting to compare its effects with rapamycin in serum-fed cells. Differing times of treatment with rapamycin and AZD8055 was carried out to compare their effects (Fig. 3.9). Rapamycin and AZD8055 had significantly different effects on the association of the translation initiation factors in serum-replete HeLa cells. Treatment with AZD8055 resulted in a large increase of bound 4E-BP1, raising the level of 4E-BP1 on the beads above that seen in untreated cells. Because the binding of 4E-BP1 and eIF4G to eIF4E are mutually exclusive (Haghighat *et al.*, 1995), it appears logical that the increase of bound 4E-BP1 was accompanied by a significant loss of eIF4GI (as observed for AZD8055 treatment). Treatment with rapamycin only caused a slight increase in the amount of 4E-BP1 bound to eIF4E, consistent with its limited inhibition of 4E-BP1 phosphorylation. Surprisingly, but reproducibly, rapamycin enhanced the scaffold protein eIF4GI bound to eIF4E, rather than reducing it, which is very difficult to explain based on the current knowledge.

To further clarify this finding, prolonged treatment with the compounds was carried out and another important component of eIF4F, eIF4A was included in the analysis (Fig. 3.10).

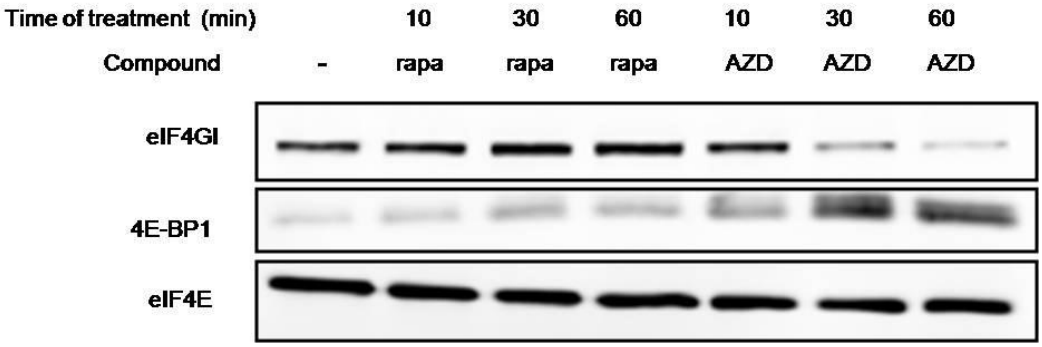


Figure 3.9. Effect of short time treatment with rapamycin or AZD8055 on eIF4F formation in serum-fed HeLa cells.

HeLa cells growing in serum-maintained medium were treated with rapamycin (rapa; 100 nM) or AZD8055 (AZD; 100 nM) for the indicated times (up to 60 min). Equal amounts of lysates were subjected to affinity chromatography on m^7GTP-Sepharose. Bound proteins were analysed by western blot using the indicated antibodies.

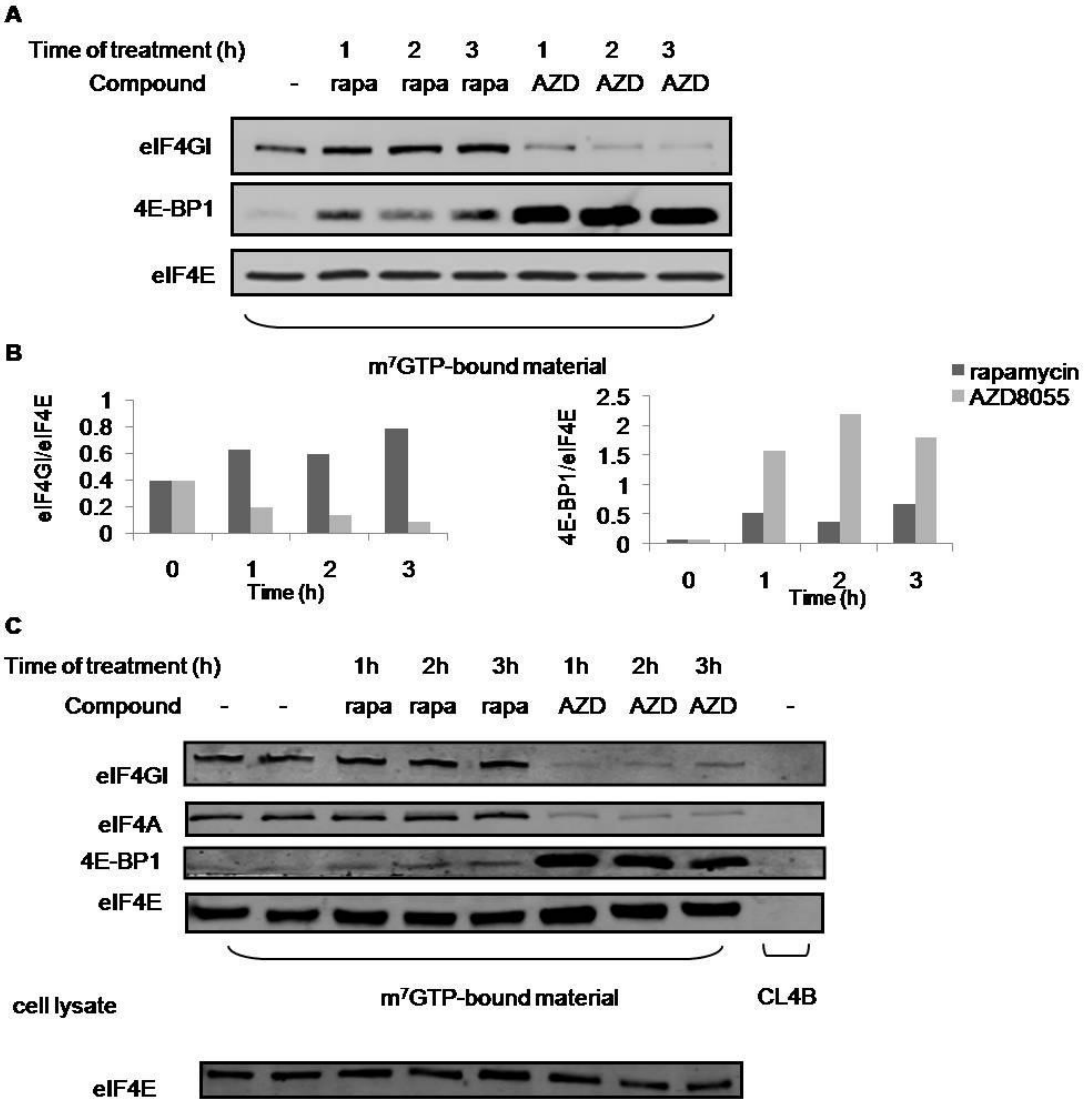


Figure 3.10. Effect of prolonged treatment with rapamycin or AZD8055 on eIF4F formation in serum-fed HeLa cells.

HeLa cells growing in serum-maintained medium were treated with rapamycin (rapa; 100 nM) or AZD8055 (AZD; 100 nM) for the indicated times (up to 3 h). Equal amounts of lysates were subjected to affinity chromatography on (A) and (C) m^7GTP-Sepharose or (C) CL4B Sepharose. Bound proteins were analysed by western blot using the indicated antibodies. (B) Quantification of (A) for eIF4GI/eIF4E and 4E-BP1/eIF4E binding. The bottom blot of eIF4E from whole cell lysate in (C) indicates input control.

Increasing the time (3 h) of AZD8055 treatment inhibited the association of eIF4GI with eIF4E, and strongly increased the binding of 4E-BP1 to eIF4E (Fig. 3.10A). Additionally, eIF4A, which unwinds the secondary structure of the mRNA 5' UTR to render it more conducive to ribosomal binding and subsequent translation (Rogers et al., 1999), also dissociated from eIF4E upon AZD8055 treatment up to 3 h (Fig. 3.10C). The presence of all the components of eIF4F (eIF4A, eIF4G and 4E-BP1) in the m^7-GTP bound material shows the integrity of this complex. Thus, treatment with AZD8055 causes the dissociation of both eIF4GI and eIF4A from eIF4E, showing its strong inhibitory effect on the assembly of eIF4F. The enhanced binding of eIF4GI to eIF4E also occurred following the prolonged treatment with rapamycin (Fig. 3.10A and C), which clearly shows the amount of bound eIF4GI was further increased by long time treatment with rapamycin. Similar results were observed for eIF4A following rapamycin treatment (Fig. 3.10C). As seen in the right lane of Fig. 3.10C, there was no nonspecific binding to the CL4B beads.

eIF4G and 4E-BP have been reported to have other paralogs (Imataka *et al.*, 1998, Poulin *et al.*, 1998, Lin *et al.*, 1994, Pause *et al.*, 1994), therefore, I examined whether rapamycin and mTOR-KIs have differing effects on those isoforms.

Treatment with AZD8055 caused a substantial decrease in the amount of eIF4GII bound to eIF4E, as seen for eIF4GI (Fig. 3.11A). Rapamycin induced a small increase of the amount of bound eIF4GI and apparently had little effect on eIF4GII (it was difficult to determine change of eIF4GII exactly, due to the weak detection by the antibody for eIF4GII). Both PP242 and AZD8055 elicited a large retention of 4E-BP1 and 4E-BP2, accompanied by a loss of eIF4GI (Fig. 3.11B). The increase in binding of 4E-BP1 and 4E-BP2 induced by rapamycin was very slight, as observed in Fig. 3.11B. The finding that rapamycin had similar effects on 4E-BP1 and 4E-BP2 excluded the possibility that 4E-BP2 was the second factor which dissociated with eIF4E upon rapamycin treatment. To summarise, rapamycin and mTOR-KIs have differing effects on the assembly of eIF4F.

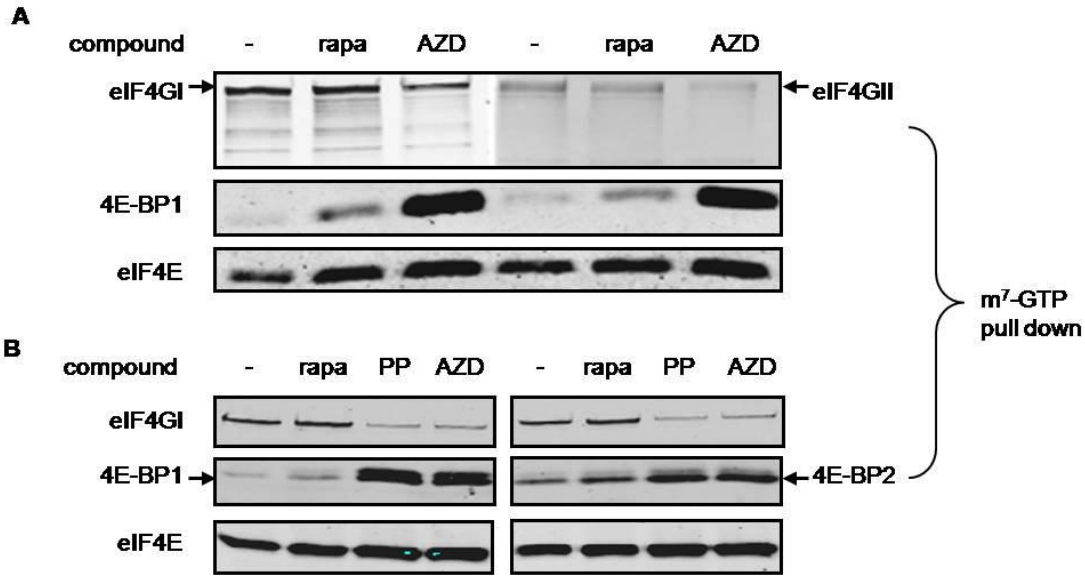


Figure 3.11. Effects of rapamycin and mTOR-KIs (PP242 and AZD8055) on the binding of different paralogs of eIF4G and 4E-BPs.

(A) and (B) HeLa cells growing in serum-maintained medium were treated with rapamycin (rapa; 100 nM) , PP242 (PP; 1 μM) or AZD8055 (AZD; 100 nM) for 60 min. Equal amounts of lysates were subjected to affinity chromatography on m^7GTP-Sepharose. Bound proteins were analysed by western blot using the indicated antibodies.

3.2.4 mTOR-KIs inhibit protein synthesis more strongly than rapamycin.

Given the contrasting effects of rapamycin and the mTOR-KIs, their effects on the rate of protein synthesis were examined. HeLa cells maintained in serum were treated with rapamycin or mTOR-KIs for 2 h. Short times were first chosen to minimise the influence of the effects of the compounds on ribosome biosynthesis (Mayer and Grummt, 2006). The rate of protein synthesis was assessed by measuring the incorporation of [^{35}S]methionine into TCA-insoluble material. This method is able to estimate the amount of protein synthesized in a population of cells.

The data in Fig. 3.12 show that rapamycin only had a small inhibitory effect on the protein synthesis whereas AZD8055 had a much stronger one by around 30%. Therefore, what accounts for the greater effect of mTOR-KIs on total protein synthesis? Thoreen *et al.* (2009) found similar effects in MEFs when treated with Torin 1 (another ATP-competitive mTOR inhibitor) and that this pattern was maintained in Rictor null MEFs. This indicates that the greater effect of mTOR-KIs on protein synthesis is not due to effects mediated through mTORC2, but rather through rapamycin-insensitive effects of mTORC1, for example, 4E-BP1 phosphorylation.

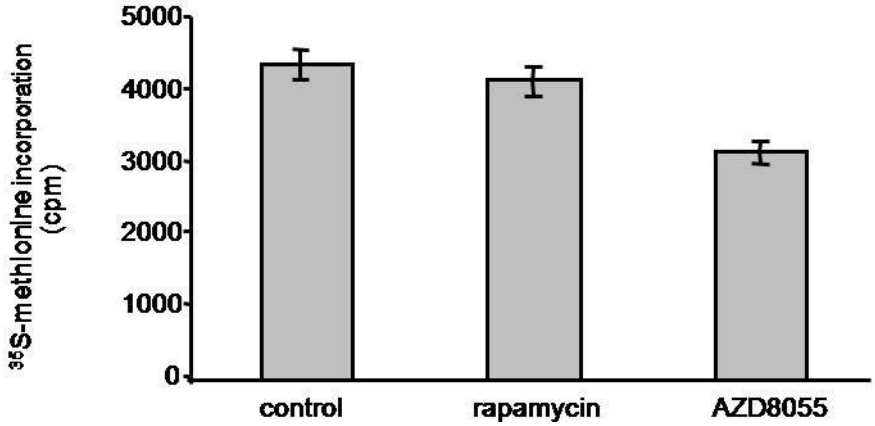


Figure 3.12. Effects of rapamycin and AZD8055 on protein synthesis rates in HeLa cells growing in serum.

Serum-fed HeLa cells were treated with rapamycin (100 nM) or AZD8055 (100 nM) for 1 h and then incubated with [^{35}S]methionine (10 μCi/ml) for 1 h. 30 μg of Lysates were added to 3 MM filter paper and the incorporated radioactivity was measured as described in *Materials and Methods section 2.3.9*. Data are given ± SEM, n = 3.

3.2.5 Role of 4E-BP1 in the effects of rapamycin and mTOR-KIs on protein synthesis.

To assess whether the stronger inhibition of protein synthesis by mTOR-KIs is due to their effects on 4E-BP1, I first attempted to eliminate mTOR inhibition of eIF4G/eIF4E binding by knocking down expression of 4E-BP1 using siRNA (Averous *et al.*, 2008). To achieve a good knockdown efficiency, various incubation times (from 48 h to 96 h) after transfection with siRNA were tested. Expression of 4E-BP1 in the samples transfected with 4E-BP1 siRNA was greatly diminished after 96 h incubation compared to the samples transfected with control siRNA (Fig. 3.13A). Therefore, 96 h was chosen for the subsequent experiments. As shown in Fig 3.13B, compared with control siRNA, transfection with 4E-BP1 siRNA caused a recovery of eIF4GI bound to eIF4E in cells treated with AZD8055. However, AZD8055 can still induce an increase of the amount of 4E-BP1 bound to eIF4E in 4E-BP1 siRNA-transfected cells, revealing an incomplete knockdown of 4E-BP1 expression. More importantly, AZD8055 still decreased eIF4GI binding (Fig. 3.13B). In total, since knocking down 4E-BP1 could not eliminate the inhibitory effects of AZD8055 on eIF4GI/eIF4E binding, this approach is not suitable for studying the contribution of 4E-BP1 to overall protein synthesis.

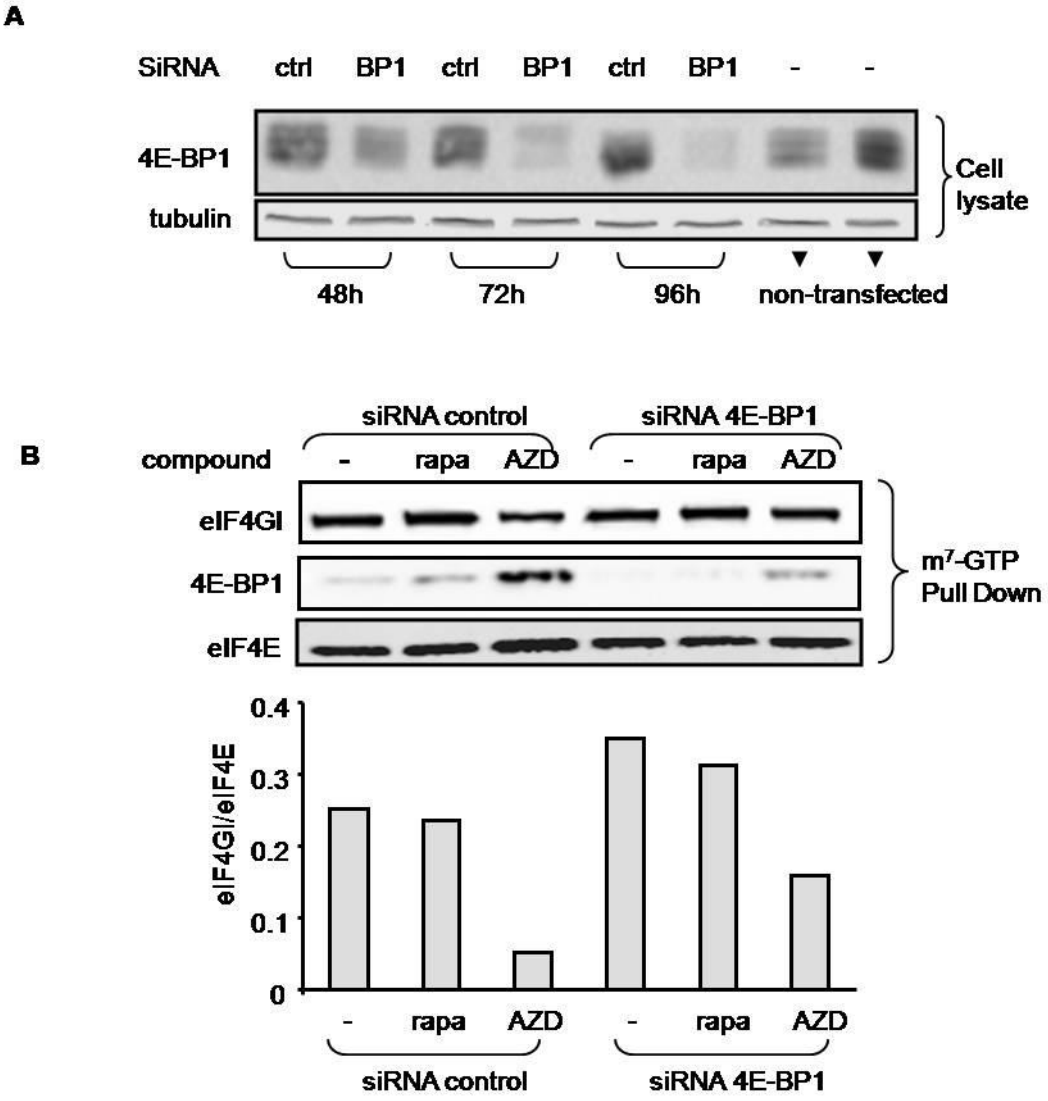


Figure 3.13. Knocking down 4E-BP1 diminishes the inhibition of eIF4GI/eIF4E binding by AZD8055.

(A) and (B) Serum-fed HeLa cells were transfected with siRNA targeted to the 4E-BP1 mRNA (BP1) or, as a negative control, scrambled siRNA (ctrl). (A) After indicated times (up to 96 h), cell lysates were analysed by western blotting using the indicated antibodies. (B) After 96 h incubation, cells were treated with mTOR inhibitors where indicated. Cell lysates were then subjected to affinity chromatography on m^7GTP Sepharose and the bound material was analyzed by western blot using the indicated antibodies. The graphic below represents the ratio of bound eIF4GI/eIF4E under each condition.

Therefore extra eIF4E was introduced into the cells, arguing that these eIF4E could bind to increased amounts of non-phosphorylated, active 4E-BPs induced by mTOR inhibitors. First, HeLa cells were transfected with untagged eIF4E vectors (Fig. 3.14). Treatment with AZD8055 caused a dissociation of eIF4GI with eIF4E when cells were transfected with empty vector, eIF4E mutant vector (4E-W73A) or without transfection. This dissociation was recovered by overexpression of wild type eIF4E, accompanied by the retention of 4E-BP1, suggesting more free eIF4E bound to eIF4GI as well as to dephosphorylated 4E-BP1. Interestingly, expression of 4E mutant form (4E-W73A) failed to recover the loss of eIF4GI caused by AZD8055, which agrees with earlier finding that when Tryptophan at position 73 in eIF4E was mutated into alanine, eIF4E was incapable of interacting with eIF4G *in vivo* (Marcotrigiano *et al.*, 1997). However, no significant increase in levels of eIF4E was observed in total cell lysates (Fig. 3.14). Since this untagged eIF4E comigrates with endogenous eIF4E, it was not easy to assess its overexpression level.

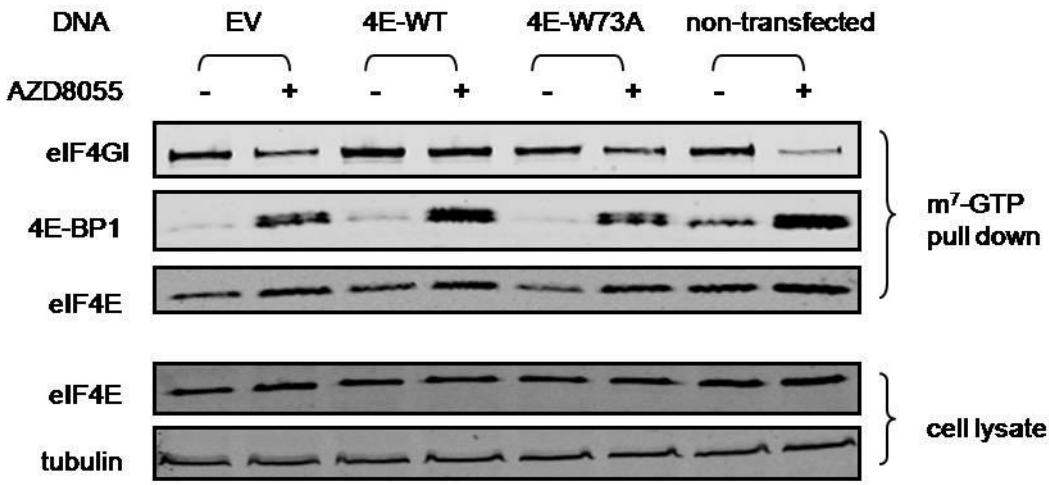


Figure 3.14. Overexpression of untagged eIF4E wild type (4E-WT) and eIF4E mutant form (4E-W73A) in serum-fed HeLa cells.

HeLa cells growing in serum were transfected with a vector encoding wild type eIF4E or a vector encoding mutant eIF4E (or the corresponding empty vector, EV). The cells were then treated with AZD8055 (100 nM) for 1 h. The lysates were directly analyzed by western blot (the bottom two blots) or were first subjected to affinity chromatography on m^7GTP-Sepharose and the bound material was analyzed by western blot.

Therefore an N-terminal HA-tagged eIF4E was used. A number of different conditions were tested to achieve a good expression level (data not shown). To verify that the HA-tag did not interfere with the binding of eIF4E to its partners, this HA-tagged eIF4E was immunoprecipitated and the immunoprecipitates were analysed for eIF4GI, eIF4GII and 4E-BP1 (Fig. 3.15). The data in Fig. 3.15 show that HA-eIF4E bound to each of these three partners. Moreover, treatment with PP242 and AZD8055 did not cause a reduction of the amount of eIF4GI and eIF4GII bound to eIF4E, suggesting that sufficient amount of eIF4E was introduced to moderate the effects of the dephosphorylated, and active 4E-BPs.

Given that HA-eIF4E is able to bind these partners in a similar manner to endogenous eIF4E, HeLa cells were transfected with this vector as well as a corresponding empty vector as the control. After 48 h, rapamycin and mTOR-KIs (PP242 and AZD8055) were added to the cells for 60 min prior to lysis. The lysates were then applied to m^7-GTP sepharose to analyse the effect of these compounds on cap-bound proteins following eIF4E overexpression. In contrast to control cells (which received the empty vector), cells expressing HA-eIF4E showed similar levels of bound eIF4GI compared to untreated cells even when exposed to mTOR-KIs (Fig. 3.16). Thus, overexpressing HA-eIF4E is able to remove the inhibitory effect of mTOR-KIs on binding of eIF4GI to eIF4E.

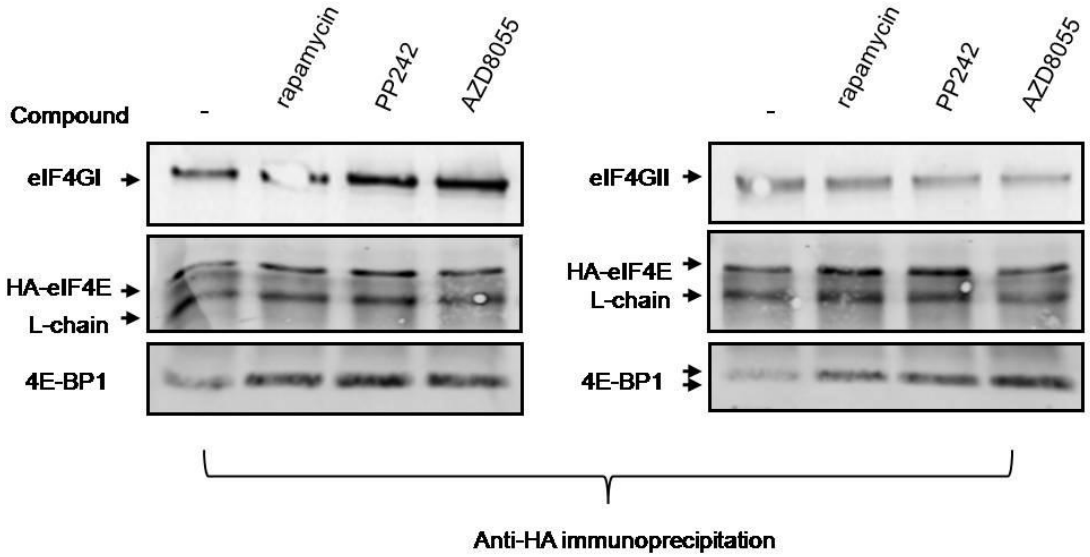


Figure 3.15. The interaction of HA-eIF4E with eIF4Gs and 4E-BP1.

HeLa cells were transfected with a vector encoding HA-tagged eIF4E. After 48 h, the cells were treated with rapamycin (100 nM), PP242 (1 μM) and AZD8055 (100 nM) for 60 min and then lysed. The lysates were subjected to immunoprecipitation using HA antibodies; the immunoprecipitates were analyzed by western blot using the indicated antibodies. The positions of the HA-tagged eIF4E and the light chain of the HA antibody are shown.

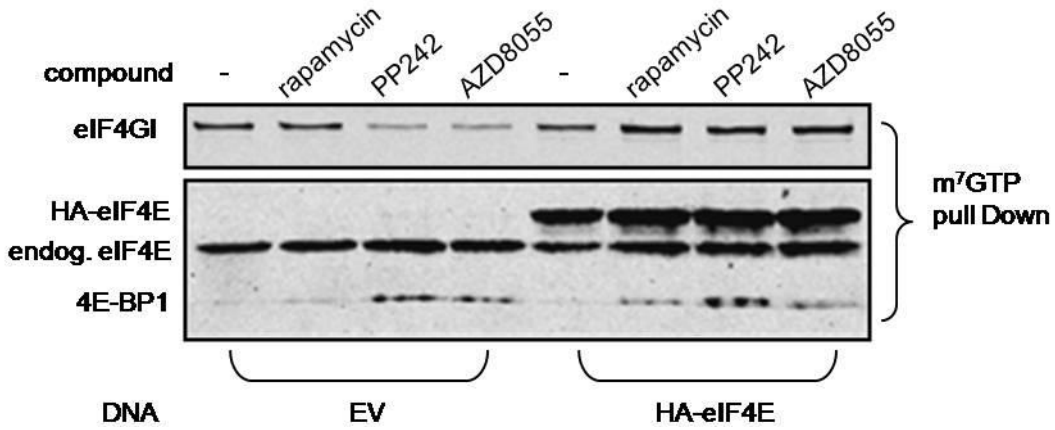


Figure 3.16. The effects of overexpressing HA-eIF4E on eIF4GI/eIF4E binding. Serum-fed HeLa cells were transfected with HA-tagged eIF4E (or the corresponding empty vector, EV). After 48 h, the cells were treated with rapamycin (100 nM), PP242 (1 μM) and AZD8055 (100 nM) for 60 min. The lysates were subjected to m^7GTP-Sepharose affinity assay and the bound material was analyzed by western blot using the indicated antibodies.

Since the inhibition of eIF4F formation by mTOR-KIs was eliminated by introducing exogenous eIF4E, it was logical to compare the effects of rapamycin and mTOR-KIs on protein synthesis under this condition, i.e. whether eliminating the inhibitory effects of mTOR-KIs could rescue their inhibition to protein synthesis. [^{35}S]methionine incorporation experiments performed in HeLa cells transfected with HA-eIF4E or a corresponding empty vector showed that overexpressing HA-eIF4E significantly attenuated the small inhibitory effect of rapamycin on protein synthesis in control HeLa cells (-eIF4E; Fig. 3.17). In contrast, overexpressing HA-eIF4E did not significantly reduce the inhibition of protein synthesis caused by PP242 or AZD8055 (Fig. 3.17). This suggests that the level of eIF4G/eIF4E binding in HeLa cells does not primarily account for the inhibition of protein synthesis caused by these inhibitors. Since rapamycin and mTOR-KIs completely block S6 phosphorylation (Fig. 3.6), the distinct effects of rapamycin and mTOR-KIs on protein synthesis are not due to this event. As discussed above, neither is the difference due to mTORC2 because the effects of another mTOR kinase inhibitor, Torin 1, were nearly equivalent in wild-type and Rictor-null MEFs (Thoreen *et al.*, 2009).

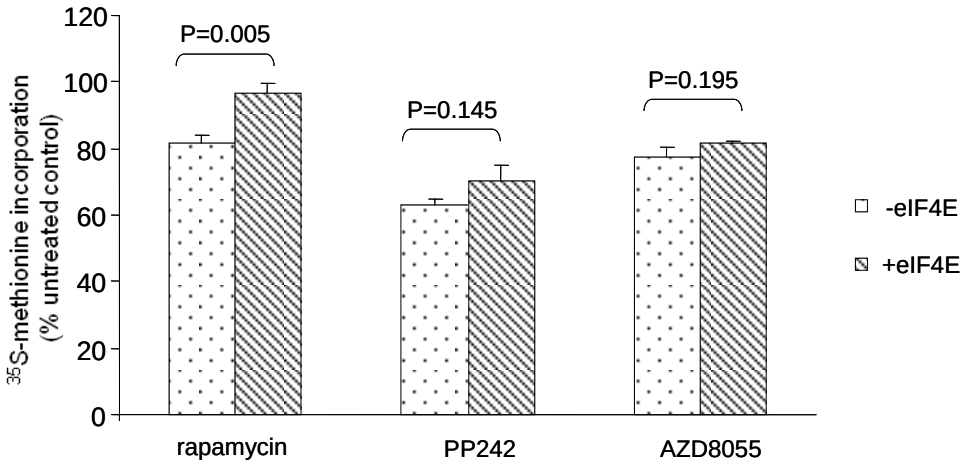


Figure 3.17. Effects of eIF4E overexpression on the inhibition of protein synthesis by mTOR inhibitors.

HeLa cells transfected with a vector encoding HA-eIF4E (or the corresponding empty vector) were treated with rapamycin (100 nM), PP242 (1 μM) and AZD8055 (100 nM) for 60 min. [^{35}S]methionine was then added into the medium for labelling (60 min) and the incorporation of [^{35}S]methionine was determined as described in *Materials and Methods section 2.3.9.* The data are derived from at least three independent experiments in each case; bars indicate the standard deviation. P values between the two groups (-/+ eIF4E) were calculated using individual t test. Data are given ± SEM, n>= 4.

3.3 Discussion

In this chapter, I have carried out a detailed study comparing the effects of rapamycin and the recently developed mTOR-KIs (AZD8055 and PP242) on mTOR signalling pathways. Consistent with earlier reports (Garcia-Martinez *et al.*, 2009, Feldman *et al.*, 2009, Thoreen *et al.*, 2009), mTOR-KIs exhibit a much stronger effect than rapamycin on both mTOR complexes. More importantly, they inhibit protein synthesis to a substantial extent where rapamycin has almost no effect. Therefore, what is the main mechanism accounting for the distinct effects of rapamycin and mTOR-KIs on protein synthesis? Given that mTOR-KIs inhibit the rapamycin-insensitive mTORC2 (shown by effect on Akt phosphorylation at Ser473), the difference might be a reflection of mTORC2 function. However, the studies of Thoreen *et al.* using another ATP-competitive mTOR inhibitor Torin 1 excluded this possibility, since they found that the effects of Torin 1 on protein synthesis were similar in wild type and Rictor null MEFs which lack functions of mTORC2 (Thoreen *et al.*, 2009). In addition, both rapamycin and mTOR-KIs blocked the activation of S6Ks, indicating the stronger effects of mTOR-KIs on protein synthesis were not due to S6Ks either.

My results clearly show that while mTOR-KIs were effective at blocking the phosphorylation of 4E-BP1 on multiple sites, rapamycin did not block 4EBP1 phosphorylation as completely as mTOR-KIs. Indeed, only the phosphorylation of rat-4E-BP1 on Ser64 was shown to be suppressed by rapamycin (Fig. 3.6), which is consistent with earlier findings (Feldman *et al.*, 2009, Garcia-Martinez *et al.*, 2009, Wang *et al.*, 2005). With respect to the activation of Akt, my data show that rapamycin always stimulated the phosphorylation of Akt on both Ser473 and Thr308. This reflects its lack of effect on mTORC2 and may be due to the relief of the feedback loop from S6K to IRS1, a key signalling molecule that links activation of the insulin receptor to PI3K (Harrington et al., 2004, Um et al., 2004). In contrast to rapamycin, inhibition of both mTORC1 and mTORC2 by mTOR-KIs suppresses rather than facilitates Akt activation.

Consistent with the dramatic impact of mTOR-KIs on 4E-BP1 phosphorylation, these compounds increased the association of eIF4E and 4E-BP1 to a much greater extent than rapamycin. Unexpectedly, while mTOR-KIs always decreased the binding of eIF4G to eIF4E (i.e. the assembly of eIF4F complex), rapamycin consistently

caused a modest increase in eIF4G binding. I attempted to address this by comparing the effects of these inhibitors on other isoforms such as 4E-BP2 or eIF4GII. However, the impact of these inhibitors remained similar to that observed for 4E-BP1 and eIF4GII. Nevertheless, these observations together with the activation of Akt by rapamycin are potentially important for explaining the limited value of rapamycin as an effective anti-cancer drug. Therefore, it would be very important to study the effects of these compounds on the synthesis of specific proteins on a proteome-wide scale. I first attempted to perform the isobaric tags for relative and absolute quantitation (iTRAQ) experiment to address the differences of eIF4E-bound proteins caused by the inhibitors. However, I did not successfully identify any new regulator which might be involved in the control of eIF4F assembly.

PP242 and AZD8055, like other newly-developed mTOR-KIs (Zask *et al.*, 2011, Feldman *et al.*, 2009), exhibit strong inhibition of protein synthesis whereas rapamycin does not. Given the marked differences between rapamycin and mTOR-KIs on the formation of the cap-dependent translation initiation complex eIF4F, I examined whether this difference resulted in distinct effects on protein synthesis. Introducing wild-type eIF4E allows the increased levels of hypophosphorylated 4E-BP1 to bind to extra eIF4E, thus removing the inhibition of the association between eIF4G and eIF4E caused by mTOR-KIs. However, in cells overexpressing eIF4E, mTOR-KIs again greatly reduced the rate of general protein synthesis, indicating decreased levels of eIF4G/eIF4E are not responsible for the impact of these compounds on protein synthesis. Puzzlingly, although rapamycin did not decrease the binding of eIF4G to eIF4E, overexpressing eIF4E recovered the modest inhibition of rapamycin on protein synthesis.

CHAPTER 4
DISTINCT EFFECTS OF RAPAMYCIN AND mTOR KINASE INHIBITORS ON PROTEIN SYNTHESIS REVEALED BY pSILAC METHOD

4.1 Introduction

The experiments described in Chapter 3 addressed the different effects of rapamycin and mTOR-KIs on mTOR signalling pathways and general protein synthesis. Key questions remain unanswered, including whether the greater effects of mTOR-KIs on overall protein synthesis reflect their bigger effect on the synthesis of proteins in general or on the synthesis of specific proteins. Therefore, it would be very important to study the effects of these compounds on the synthesis of specific proteins on a proteome-wide scale.

Since its invention (O'Farrell, 1975), two-dimensional (2D) gel electrophoresis has been widely used for differential expression proteomics. This method separates proteins through two systems, isoelectric focusing and polyacrylamide gel electrophoresis. Coupled with staining techniques, 2D gel electrophoresis can visualize a large number of protein spots. However, 2D gel electrophoresis does have several challenges, including that some of very acidic and basic proteins will fall outside of the pI (isoelectric point) range, or that low abundance proteins are poorly covered. Microarray and other current methods for analysis of system-wide gene expression only detect changes in mRNA abundance (Hoheisel, 2006). Therefore more quantitative and powerful tools on a proteome-wide scale are needed.

Ong *et al.* (2002) reported a simple, inexpensive yet accurate MS-based approach to expression proteomics, which is named stable isotope labelling by amino acids in cell culture (SILAC). SILAC utilizes stable isotope-labelled amino acids in cell medium to investigate cellular proteomes quantitatively. Compared to classical approaches like microarray, SILAC is able to provide new insights into bioanalytical problems quantitively. This approach was originally performed in mammalian cell line NIH 3T3 and C2C12 (Ong *et al.*, 2002), which were grown in medium lacking a standard essential amino acid but supplemented with a non-radioactive, isotopically labelled amino acid. Ong *et al.* (2002) found no difference between growth of these cells and those in normal medium (as proved by doubling time, ability to differentiate and cell morphology). More importantly, the complete incorporation of the isotopically labelled amino acid into the cells can be achieved after 5 rounds of cell divisions (Ong *et al.*, 2002). Using this strategy, Ong *et al.* (2002) studied the process of muscle cell differentiation and found that glyceraldehyde-3-phosphate dehydrogenase, fibronectin, and pyruvate kinase M2 were up-regulated in this process. After that, SILAC has been

diversely used in other systems such as yeast (Jiang and English, 2002), bacteria (Kerner *et al.*, 2005) and plants (Gruhler *et al.*, 2005). A schematic of typical SILAC protocol is presented in Fig. 4.1.

As shown in Fig. 4.1, cells growing in normal DMEM are split into two culture dishes, each containing light (L) or heavy (H) SILAC medium respectively. Cells are subcultured in their respective SILAC medium 5-6 times until full incorporation has been achieved. Treatments are added to one cell population to induce a different response between the control and experimental cell populations. Cells are then lysed and equal amounts of proteins from heavy-labelled and light-labelled cells are mixed. Subsequent fraction procedure is performed to produce peptide mixtures which are suitable for MS analysis.

The SILAC method has also been applied to measure protein turnover (Doherty *et al.*, 2005, Pratt *et al.*, 2002, Milner *et al.*, 2006). However, as we known, the rates of protein turnover is a reflection of both synthesis and degradation. So a high H/L ratio could be either due to the high translation rate of a stable protein or the low translation rate of a rapidly degraded protein. To directly compare translation rates without interference from degradation, Selbach and colleagues developed a new pulsed labelling strategy, pSILAC (Schwanhausser *et al.*, 2009). Instead of labelling cells with several rounds of doubling for a few days, they only cultivated the cells in the SILAC medium with isotopically labelled amino acids for a few hours and then subjected the cell lysates to MS analysis. Fig. 4.2 describes the workflow of their pSILAC approach. Based on this pulsed-labelling method, the differences in protein stability could be ignored since pre-existing proteins remain labelled with the “L” form of amino acids. It is likely that newly synthesized proteins could also be affected by degradation. However, this can be ignored because this degradation will occur equally in both the “M” and “H” labelled proteins (Fig. 4.2). Therefore H/M ratios in this method can provide accurate information about the synthesis rate of a specific protein.

In summary, SILAC provides relative quantification in proteomics while pSILAC directly detects changes at the translation level without interference from protein degradation. Given the increasing interest in the regulation of translation, SILAC and pSILAC can be diversely applied to a wide range of biological questions.

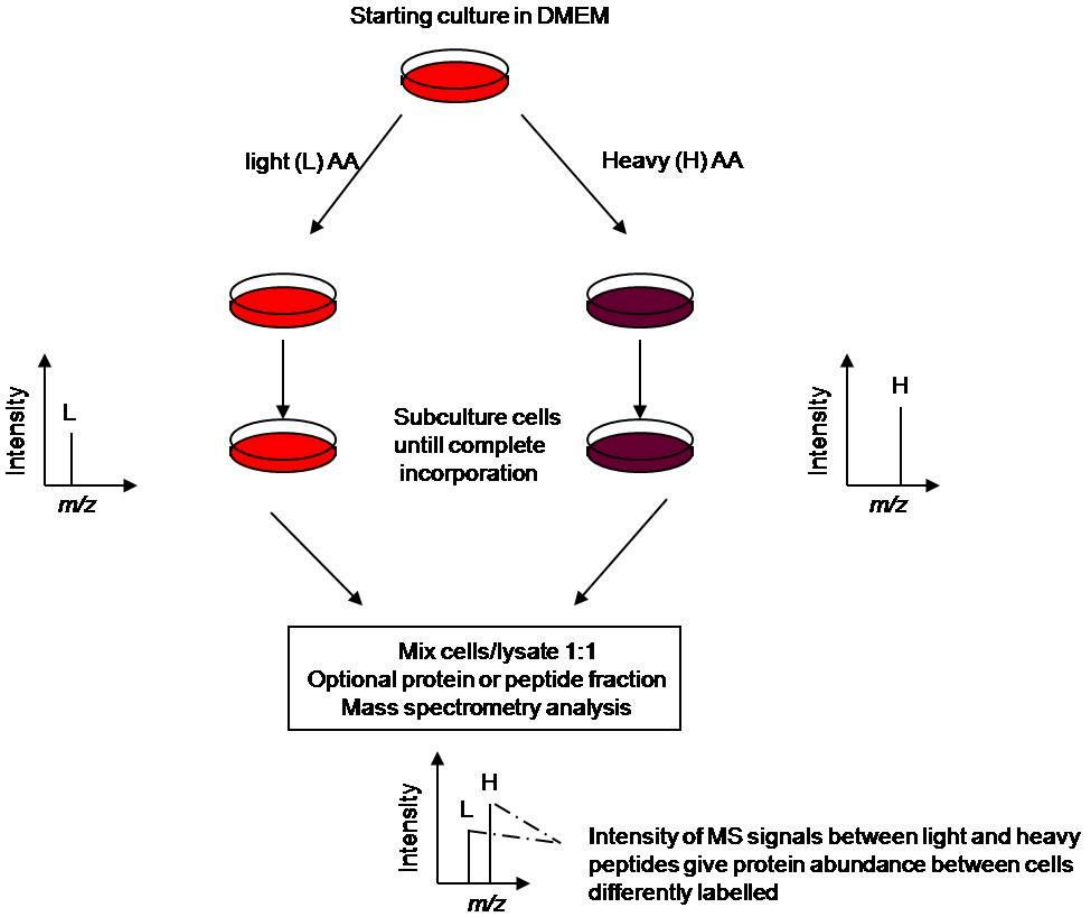


Figure 4.1. An overview of a typical SILAC protocol.

Cells are first grown in normal DMEM and then transferred into light (L) or heavy (H) SILAC medium until cells have fully incorporated the isotoplabelled amino acids. After the adaptation, the two cell populations are differentially treated and mixed. A subproteome can be digested to peptides with trypsin and then analyzed by MS for protein identification and quantification. *m/z*: the mass to charge ratio.

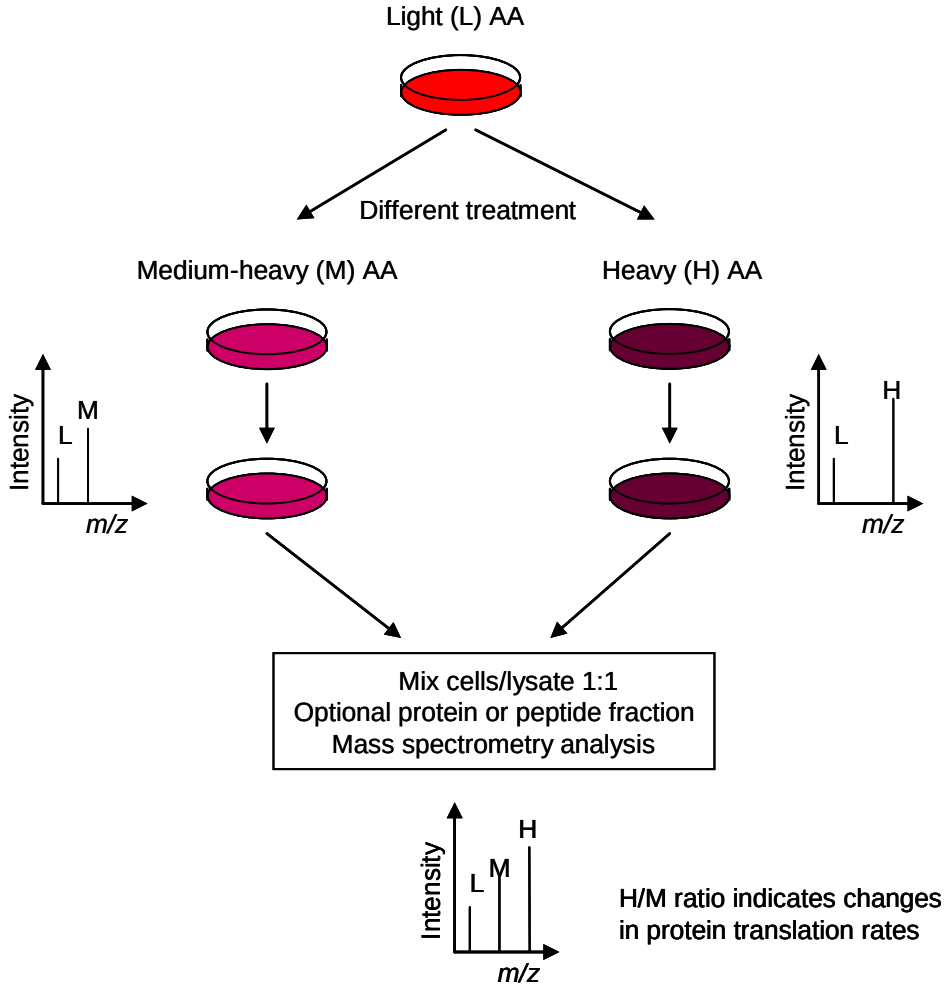


Figure 4.2. The pSILAC approach (Schwanhausser *et al.*, 2009).

Cells growing in "light" (L) medium are either transferred into "heavy" (H) or "medium heavy" (M) SILAC medium. From this time point on, cells are pulse-labelled because all newly synthesized proteins incorporate either the "H" or the "M" amino acids, and pre-existing proteins remain labelled in the "L" amino acids. After this short labelling phase (a few hours), the two cell populations are equally mixed and analyzed by MS. The ratio of peak intensities of H versus M peptides is a direct reflection of differences in synthesis rates of the corresponding protein. *m/z*: the mass to charge ratio

4.2 Results

4.2.1 Polysome-microarray analyses

DNA microarray is the most classical and useful way to measure the level of mRNAs and investigate the translation regulation. Therefore, I first applied this approach to study the distinct effects of mTOR inhibitors. HeLa cells were treated with rapamycin, PP242 and AZD8055 for 2 h (as used in [^{35}S] methionine incorporation experiments) and were subjected to sucrose gradients and polysome profile analyses in School of Pharmacy, University of Nottingham (with the kind help from Dr. Lindsay Wilson, Dr. Martin Bushell and Professor Anne Willis, University of Leicester, UK). The polysome profiles are presented in Fig. 4.3. In these profiles, a substantial proportion of the total mRNA was located in the polysomes in HeLa cells growing in serum (Fig. 4.3, control). Rapamycin only caused a slight shift of the polysomes into subpolysomes compared to the control (Fig. 4.3, rapamycin). In contrast, PP242 and AZD8055 strongly decreased the size of the peaks of polysomes (Fig. 4.3, PP242, AZD8055), implying that mTOR-KIs have much greater inhibitory effects on translation than rapamycin. This finding is consistent with the general protein synthesis data (Fig. 3.12), which is that rapamycin only decreased protein synthesis by around 10% after 2 h treatment, while mTOR-KIs have a much stronger effect (around 30%).

RNA was then extracted from sucrose gradients and pooled into subpolysomal or polysomal fractions. Fluorescently labelled DNA probes were generated and hybridised to the human cDNA microarrays (with the help of Dr. Lindsay Wilson, University of Leicester, UK). Surprisingly, after the initial data analysis, a number of mRNAs encoding ribosomal proteins such as rpLP1, rpLS2L1, rpL39L1, rpL17 and rpL2L3 whose translation are known to be suppressed by rapamycin (Jefferies *et al.*, 1994, Jefferies *et al.*, 1997, Terada *et al.*, 1994, Grolleau *et al.*, 2002), were found to be more loaded onto polysomes after 2 h treatment by rapamycin. In order to confirm this and also obtain more information, further analysis of the microarray data is currently being performed by Dr. Lindsay Wilson. However, for the studies performed in this thesis, another approach was required to further study the effects of mTOR inhibition on the synthesis of specific proteins, especially ribosomal proteins on a proteome-wide scale.

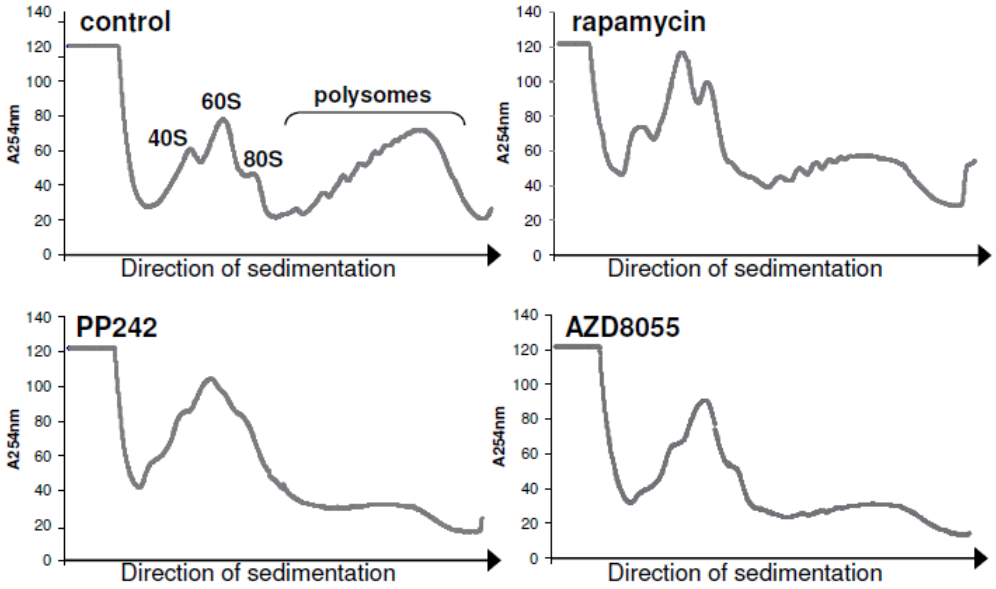


Figure 4.3. Polysome profiles.

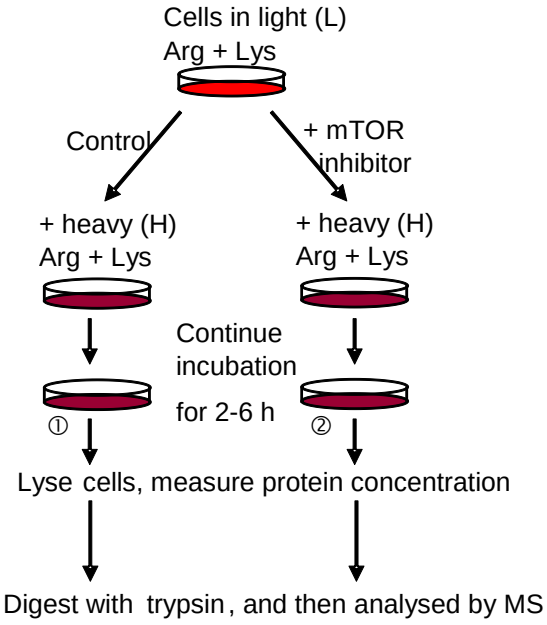
HeLa cells growing in serum were treated by rapamycin (100 nM), PP242 (1 μM) and AZD8055 (100 nM) for 2 h before harvesting. Cell lysates were then centrifuged through sucrose gradients. Eleven fractions were collected from each gradient while recording the absorbance profile. More details and subsequent microarray analysis are described in *Materials and Methods section 2.4.4.*

4.2.2 pSILAC analysis reveals qualitative differences in the effects of mTOR-KIs and rapamycin on the synthesis of specific proteins.

As described earlier, SILAC is a simple but powerful approach for the accurate relative quantification by proteomics. It has been recently adapted to measure protein synthesis rates in response to iron (Schwanhausser *et al.*, 2009). In this case, the proteins in experimental samples are pulsed labelled with heavy amino acids with simultaneous addition of irons. Control cells are pulsed labelled with medium amino acids in parallel without addition of iron. We therefore applied this method to tag newly-synthesized proteins and identify their changes in the rates of synthesis which may be affected by mTOR inhibitors. However, this method reported by (Schwanhausser *et al.*, 2009) was not designed for examining the effects of different inhibitors on protein synthesis. As the aim was to investigate the difference between rapamycin and mTOR-KIs, it was necessary to combine treatments of these compounds with different forms of amino acids labelling. Thus, a modified protocol was applied in the experiments (Fig. 4.4).

As shown in this protocol, HeLa cells are first incubated in normal DMEM and then transferred to the SILAC medium containing heavy lysine and arginine isotopologs together with the relevant compounds for short periods of time (a few hours). Control HeLa cells are also transferred to heavy SILAC medium but without the addition of the compounds. This short labelling time allows the tagging of only newly-synthesized proteins by the heavy isotopes of the amino acids, as the pre-existing proteins remain in the light form. After the labelling phase, cells are lysed and proteins are digested with trypsin which cleaves after arginine and lysine. Peptides are analysed by mass spectrometry. As shown in Fig. 4.4B, the proportions of the two variants (heavy/light) provide information about the relative rates of synthesis of the specific protein. Multiple peptides from the same protein are analysed to derive accurate information about their rates of accumulation.

A



B

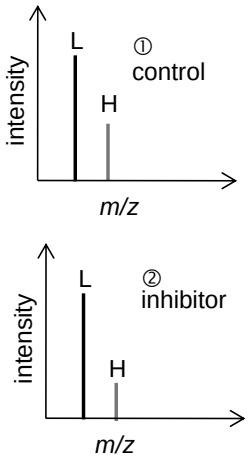


Figure 4.4. Cartoon of the pSILAC method applied in this thesis.

(A) HeLa cells previously maintained in normal medium are placed in medium containing stable isotope-labelled arginine and lysine. After a pulse labelling period of, e.g., 2-6 h, cells are lysed, proteins are subjected to tryptic digestion and then analysed by mass spectrometry. (B) Two species are observed for any given tryptic peptide, one with normal amino acids that are not labelled (L; 'untagged'), and one with heavy arginine and/or lysine (H; 'tagged'). The ratio of tagged to untagged peptides reflects the relative rate of synthesis of the studied protein; data from multiple peptides from the same protein are averaged. Triplicate experiments are performed independently for final data analysis.

Together with Dr Valentina Iadevaia, a number of different labelling times from 2 h to 6 h were tested. 2 h was first chosen based on the [^{35}S]methionine incorporation experiments. The longer time 6 h gave a higher proportion of labelled peptides and ability to provide more accurate and quantifiable data than labelling for 2 h. Importantly, treating cells with rapamcyin or mTOR-KIs for 6 h had similar effects as 2 h treatment on general protein synthesis (Fig. 3.12 and Fig. 4.5). As seen in Fig. 4.5, rapamycin has almost no effect while PP242 and AZD8055 have very strong inhibitory effect on protein synthesis. Therefore, 6 h labelling time was chosen as the one used in the experiments.

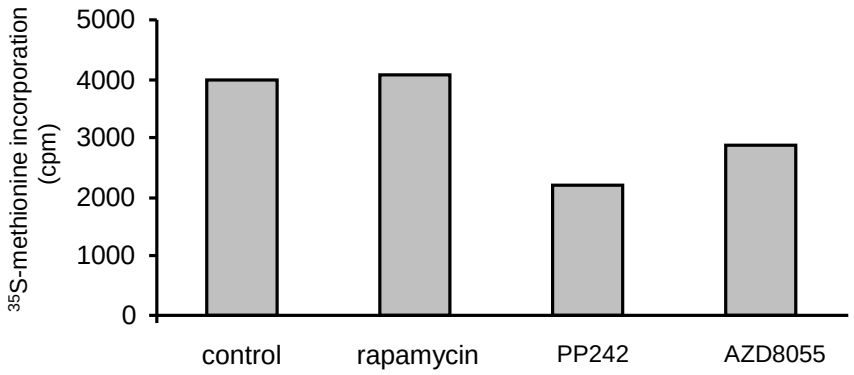


Figure 4.5. Effect of prolonged treatment with rapamycin or mTOR-KIs on [^{35}S] methionine incorporation.

HeLa cells were treated with rapamycin (100 nM), PP242 (1 μM) and AZD8055 (100 nM) for 6 h. [^{35}S]methionine was then added into the medium for the final 1 h. Cells were lysed and 30 μg of lysates were added to 3 MM filter paper. The incorporation of [^{35}S]methionine was determined as described in *Materials and Methods section 2.3.9.*

Together with Dr Valentina Iadevaia, we labelled and treated serum-fed HeLa cells with rapamycin and mTOR-KIs based on the protocol shown in Fig. 4.4. After denatured at 95°C, the lysates were sent to University of British Columbia, Canada and analysed by MS by Dr Leonard J. Foster's group. Generally, our pSILAC data reveal that mTOR-KIs had a particularly striking effect on the synthesis of a number of proteins. Among this set of proteins, those encoded by 5'-TOP mRNAs are prominent. The pie chart data presented in Fig. 4.6 show that PP242 and AZD8055 strongly inhibited the synthesis of proteins encoded by 5'-TOP mRNAs in serum-fed HeLa cells. As seen in this figure, the synthesis of the great majority of this set of proteins was inhibited by more than 50% by PP242 and AZD8055. Similarly, the majority of the most inhibited proteins by mTOR-KIs were those encoded by known 5'-TOP mRNAs, except NAP1L1 which will be discussed later (Table 4.1).

In contrast to the profound effects of mTOR-KIs, rapamycin inhibited their synthesis much more weakly. In fact, inhibition by more than 50% by rapamycin was only observed for a small proportion of the 5'-TOP mRNA-encoded proteins (Fig. 4.6). The inhibitory effects of rapamycin on more than half of the 5'-TOP mRNA-encoded proteins analysed were less than 50%, while the translation of a significant proportion of such proteins only decreased by less than 20%. The most inhibited protein by rapamycin is rpS3A (60%), which is much more inhibited by PP242 (89%). With respect to those proteins that are not encoded by 5'-TOP mRNAs, PP242 and AZD8055 had a much smaller effect in general. As shown in Fig. 4.6, the synthesis of the majority of this type of mRNAs (non-TOPs) was inhibited by less than 50%. Rapamycin also had less effect on this set of proteins. In line with this, the ten least inhibited proteins by each compound were those encoded by non-TOP mRNAs (Table 4.1). Table 4.1 also shows that PP242 always had slightly greater effects than AZD8055 on the synthesis of individual protein, which is consistent with its bigger effects on general protein synthesis. For example, the synthesis of RPS17 was decreased by PP242 by 85%, which was stronger than effect of AZD8055 (77%; Table 4.1). The Transcription Start Site (TSS) of individual protein in Table 4.1 was also annotated according to the searching results from the DataBase of human Transcriptional Start Sites and full-length cDNAs (DBTSS; http://dbtss.hgc.jp) (Suzuki *et al.*, 2002). It can be seen that the transcripts of ribosomal proteins and translation factors in Table 4.1A start with a sequence of polypyrimidines.

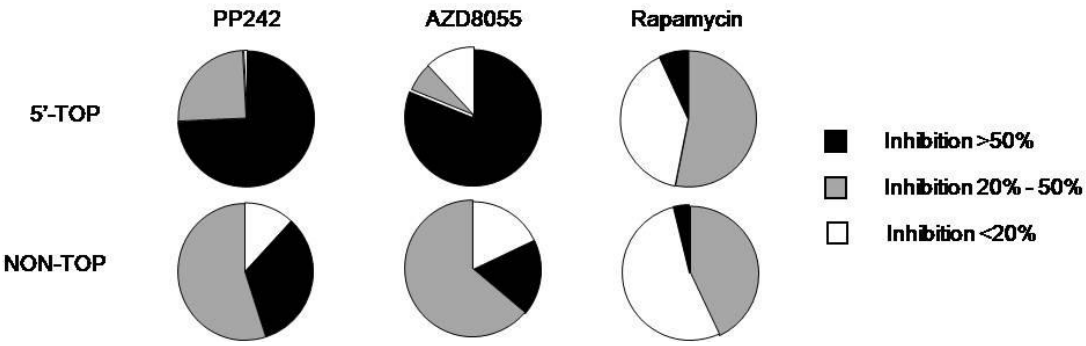


Figure 4.6. pSILAC data.

This figure shows the degree of inhibition by the three different inhibitors used (rapamycin, PP242 and AZD8055). 5'-TOP: proteins encoded by known 5'-TOP mRNAs; non-TOP: proteins not to be encoded by known 5'-TOP mRNAs.

Table 4.1 Proteins whose synthesis is most (A) or least (B) inhibited by each compound (PP242, AZD8055 or rapamcyin) as revealed by the pSILAC method.

A

Proteins whose synthesis is most inhibited by each compound (PP242, AZD8055 or rapamycin) as revealed by the pSILAC method								
PP242			AZD8055			Rapamycin		
Gene symbols	Transcription start site[1] (DBTSS)	Inhibition by PP242	Gene symbols	Transcription start site[1] (DBTSS)	Inhibition by AZD8055	Gene symbol-s	Transcription start site[1] (DBTSS)	Inhibition by rapa
RPS3A	CCCTTTT	89%	EEF1A1	CTTTTTCG	82%	RPS3A	CCCTTTT	60%
RPS17	CTGTTTCCTCTTTT	85%	EEF2	CTCTTCC	82%	RPS5	CTCTTCCT	59%
EEF2	CTCTTCC	83%	RPS17	CTGTTTCCTCTTTT	77%	RPS9	CTCTTTCTC	55%
RPS5	CTCTTCCT	82%	RPS5	CTCTTCCT	76%	EEF1G	CCTTTCTTT	54%
RPS20	CTTTTT	81%	RPS19	CCTTTCCCCT	76%	RPS20	CTTTTT	52%
RPS19	CCTTTCCCCT	80%	RPLP0	CCTTCTCTC	75%	CLIC1	AGAGG	52%
RPLP2	CCTTTTCCTC	78%	RPS16	CCTTTTCC	75%	HNRPDL	AGAAGC	52%
RPLP1	CCCTTTCCTC	78%	RPS9	CTCTTTCTC	75%	EEF2	CTCTTCC	51%
RPS14	CTCTTTCC	78%	RPLP2	CCTTTTCCTC	74%	RPL7A	CTCTCTCCTCCC	51%
RPL6	CTCTTTCCC	77%	NAP1L1	CTTTTT	74%	RPS4X	CCTCTTTCCTT	51%

B

Proteins whose synthesis is least inhibited by each compound (PP242, AZD8055 and rapamycin) as revealed by the pSILAC method								
PP242			AZD8055			Rapamycin		
Gene symbols	Transcription start site[1] (DBTSS)	Inhibition by PP242	Gene symbols	Transcription start site[1] (DBTSS)	Inhibition by AZD8055	Gene symbols	Transcription start site[1] (DBTSS)	Inhibition by rapa
PCNA	ACTGAG	9%	BCLAF1	ATCGGA	0.1%	ARF1	AGAGCC	0.2%
SPTAN1	gccACTACCC	11%	RBBP7	GAGAGA	6%	ALDOA	AAAAGGG	0.8%
MYH9	AAAGTC	19%	RCN1	GACGTG	7%	UBQLN1	AATTTC	0.8%
HIST1H1B	TCTTGA	20%	HMGB2	AAACCA	8%	YWHAZ	TTCCGG	0.8%
VIM	GTCCCC	24%	TCOF1	GGGGCG	11%	HMGB2	AAACCA	1%
HPRT1	GAACCT	26%	PARK7	GCAGTG	12%	CD44	GAATAA	2%
ALDOA	AAAAGG	27%	CPS1	tAAAAT	15%	BCLAF1	ATCGGA	3%
VCP	AGTCTC	29%	HIST1H1B	TCTTGA	15%	VCP	AGTCTC	4%
MARCKS	ACTTGG	31%	FLNB	AGAGCA	17%	BASP1	AGTAGC	4%
CPS1	tAAAAT	31%	HIST1H4K	GTAAAC	17%	PSME1	GCTTT	7%

1. TSSs are searched from DBTSS (http://dbtss.hgc.jp/). Lower case letters indicate a less common reported start site

To clearly show the distinct effects of mTOR inhibitors on the proteins encoded by 5'-TOP or non-TOP mRNAs, data for selected proteins from these two sets are presented in Fig. 4.7. The regulation of several proteins known to be encoded by TOP mRNAs, such as ribosomal proteins (RPs), elongation factors (eEFs) and the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1) are included in this list. As seen in Fig. 4.7A, PP242 or AZD8055 caused very strong inhibition (often more than 70%; red or black bars) whereas rapamycin always had a much smaller effect (often less than 50%; blue bars). For example, the accumulation of 40S ribosomal protein S3 (rpS3) was inhibited by PP242 and AZD8055 by more than 70%. Rapamycin only decreased the synthesis of this protein by less than 20% (Fig. 4.7A). For non-TOP mRNAs, as illustrated in Fig. 4.7B, rapamycin typically inhibited their protein synthesis by around 20%. The reduction caused by PP242 or AZD8055 was often less than 50%. These data (Fig. 4.6 and Fig. 4.7) thus define a clear difference between the behaviour of these two sets of proteins (TOP and non-TOP mRNAs) and between the effects of rapamycin and mTOR-KIs.

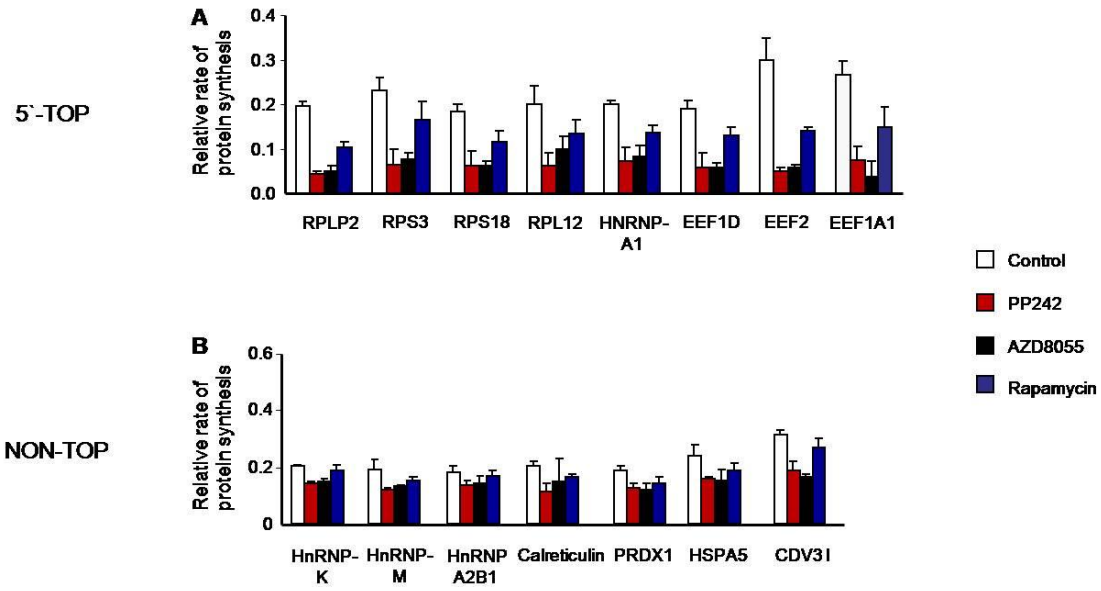


Figure 4.7. The relative rates of synthesis of selected proteins revealed by pSILAC. (A) and (B) show the relative rates of synthesis (H/L ratio) of selected proteins in each category, 5'-TOP and non-TOP; data are derived from three independent experiments, and given as mean $\pm$ SEM (n = 3).

Unexpectedly but interestingly, mTOR-KIs and rapamycin also strongly inhibited the synthesis of several proteins which are not known to be encoded by 5'-TOP mRNAs (Fig. 4.8 and Table 4.1). This group included nucleosome assembly protein 1-like 1 (NAP1L1), nascent polypeptide-associated complex alpha subunit (NACA), peroxiredoxin 6 (PRDX6), transketolase (TKT), heat shock 70 kDa protein 8 (HSPA8), heterogeneous nuclear ribonucleoprotein C (HNRNPC) and L-lactate dehydrogenase B (LDHB). Actually, the behaviour of these proteins appears similar to those of known 5'-TOP mRNA products. It is therefore of interest to examine the transcription start site (TSS) of each gene in this group. Searching the data from the DataBase of human Transcriptional Start Sites and full-length cDNAs (DBTSS; http://dbtss.hgc.jp) (Suzuki *et al.*, 2002) showed that the mRNAs for NAP1L1, NACA, PRDX6 and LDHB start with a sequence of at least 5 pyrimidines (Table 4.2). This structural feature is one of the canonical hallmarks of 5'-TOP mRNAs, suggesting that these mRNAs might belong to this subset of messages. Others in Table 4.2 including HSPA8, HNRNPC and TKT also start with a cytosine but this is not followed by an uninterrupted stretch of several pyrimidines (Table 4.2).

Many of these proteins in Table 4.1 play roles in different aspects of cell physiology. For example, nucleosome assembly proteins (NAPs) are initially identified as chaperones and nucleosome assembly factor while this family has been recently implicated in a large variety of other cellular functions, such as transcription, nuclear import and lysine biosynthesis (Laskey *et al.*, 1978, Zlatanova *et al.*, 2007). Another example, NACA, is known to bind newly synthesised peptides emerging from ribosomes and thus block their inappropriate interaction with the endoplasmic reticulum (ER) (Lauring *et al.*, 1995, Wiedmann *et al.*, 1994). Further studies suggest that NACA also plays a role in cell proliferation and apoptosis (Stilo *et al.*, 2003, Quelo *et al.*, 2004).

Our pSILAC data reveal that mTOR-KIs exhibit different effects on the synthesis of several members of the HnRNP family. They strongly inhibited the synthesis of HnRNP A1 and HnRNP C whereas they only weakly or hardly affected the synthesis of HnRNP A3, K, M, A2/B1 (Fig, 4.7, 4.8 and 4.14). Among this family, only the mRNA for HnRNP A1 is a known 5'-TOP message. However, as mentioned above, HnRNP C, whose synthesis is largely reduced by mTOR inhibitors, does have an unusual 5'-terminal sequence (rich in pyrimidines; Table 4.2).

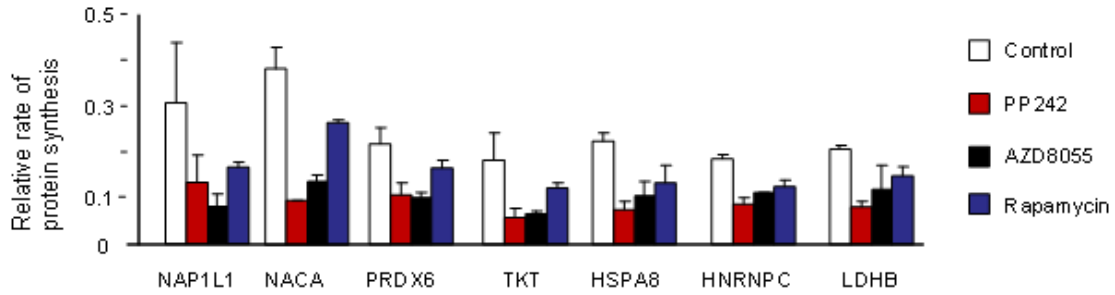


Figure 4.8. Selected proteins whose synthesis is particularly strongly affected by mTOR-KIs.

The effects of rapamycin and mTOR-KIs on the relative rates of synthesis (H/L ratio) of selected proteins are displayed as mean ±SEM. In each case, data are derived from three independent experiments.

Table 4.2. Proteins whose synthesis is significantly inhibited by mTOR-KIs (PP242 and AZD8055) or rapamycin as revealed by the pSILAC method.

Gene Symbols	Transcription start site[1]	Inhibition by PP242	Inhibition by AZD8055	Inhibition by Rapamycin	Functions (provided by NCBI database)
NAP1L1	CTTTTT	57%	74%	46%	The protein encoded by this gene may be involved in modulating chromatin formation and regulating cell proliferation
NACA	tCTTTCTG	75%	64%	32%	NACA prevents nascent ribosome-associated polypeptides from inappropriate interactions with cytosolic proteins.
PRDX6	cTTCTTC	50%	55%	26%	PRDX6 is involved in redox regulation of the cell. It may be also involved in the regulation of phospholipid turnover and in protection against oxidative injury.
TKT	CTCGGC	69%	64%	33%	In mammals, TKT connects the pentose phosphate pathway to glycolysis. It plays a role in the channeling of excess sugar phosphates into the main carbohydrate metabolic pathways
HSPA8 (also known as Hsc70)	tCTCATTG	53%	59%	42%	HSPA8 belongs to the heat-shock cognate subgroup of the heat shock protein 70 family. It binds to nascent polypeptides to facilitate correct protein folding and also functions as an ATPase
HNRNPC	CCATTTTGT	53%	38%	28%	HNRNPC belongs to the subfamily of heterogeneous nuclear ribonucleoproteins (hnRNPs). It binds pre-mRNA and is involved in the assembly of 40S hnRNP particles
LDHB	CTTGC	61%	42%	27%	The enzyme encoded by this gene catalyzes the reversible conversion of lactate and pyruvate, and NAD and NADH, in the glycolytic pathway.

1. Lower case letters indicate a less common reported start site.

4.2.3 Analysis of the behaviour of specific mRNAs by real-time PCR and Northern blot

Subsequently, it is important to examine the behaviour of specific mRNAs, i.e. validation of the microarray and pSILAC data. Real-time RT-PCR (also called qPCR) and Northern blot are the common approaches to perform gene expression analysis.

4.2.3.1 Analysis by real-time PCR

First, the behaviour of specific mRNAs in the microarray samples was examined using real-time RT-PCR. Relative quantification (Comparative CT Method Quantitation) was used for data analysis. When using this method, the expression of a target gene needs to be normalised relative to the expression of an endogenous control gene, which should be expressed at a constant level across all sample groups in a study, and should not be influenced by treatments or conditions. As mentioned in Section 4.2, RNAs from 11 fractions were pooled into subpolysomal (Subs) or polysomal (Polys) fractions, based on the polysome profiles (Fig. 4.3). Several genes were tested in order to find a good endogenous control including 18S rRNA, GAPDH and β-actin. Fig. 4.9 shows the amplification plots for 18S rRNA. As shown in Fig. 4.9, treatment with PP242 and AZD8055 strongly affected the distribution of 18S in the subpolysomal and polysomal samples. Similar results occurred for GAPDH and β-actin, suggesting that it is difficult to find an endogenous control gene whose expression is not affected by mTOR-KIs.

We therefore adopted the standard curve method arguing that the relative expression of a specific gene can be assessed via the standard curve assay. As shown in Fig. 4.10, although β-actin amplified nicely against serial dilutions of cDNAs cloned from total RNA (Fig. 4.10B), the polysomal samples treated with AZD8055 failed to amplify (Fig. 4.10A). Other genes such as GAPDH were also examined using this method, but similar results occurred (data not shown). Although real-time RT-PCR is a highly sensitive technique, the samples from microarray are not suitable for real-time PCR analysis. Hence, alternative approaches (for example, Northern blot) are required.

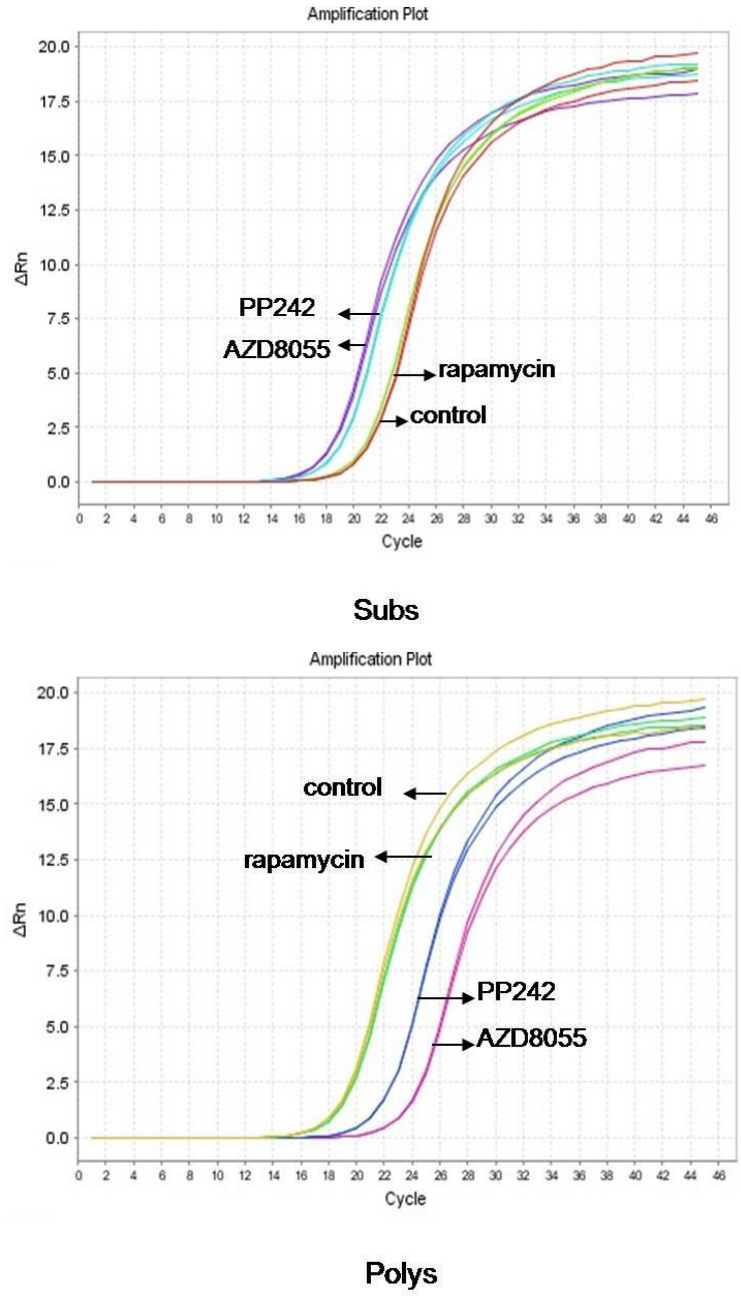


Fig. 4.9 Amplification plots of 18S against cDNAs derived from RNA from the microarray experiments.

cDNAs were synthesized using RNA samples from the microarray experiments described in section 4.2.1. The methods of reverse transcription and real-time RT PCR are described in *Materials and Methods section 2.4.2 and 2.4.3*. Subs: subpolysome fractions; Polys: polysome fractions. Arrows indicate different treatment.

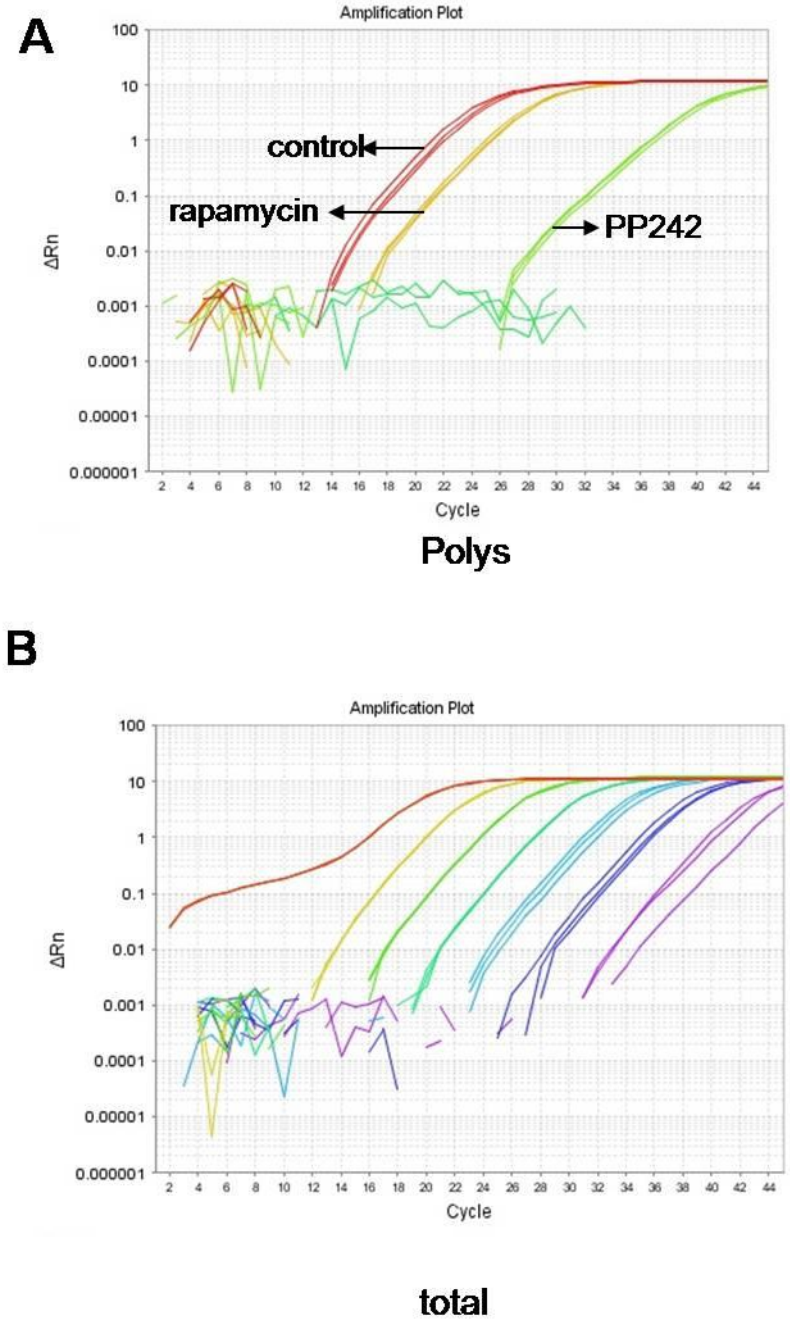


Fig. 4.10. Amplification of β-actin.

Amplification of β-actin against (A) cDNAs derived from the polysome RNA from the microarray experiments described in section 4.2.1 or (B) serial dilutions of cDNAs cloned from total RNA. The methods of reverse transcription and real-time RT PCR are described in *Materials and Methods section 2.4.2 and 2.4.3.* Polys: polysome fractions. Arrows indicate different treatment.

4.2.3.2 Analysis by Northern blot

With the help of Dr. Valentina Iadevaia, Northern blot analysis was then performed to examine the behaviour of specific mRNAs. First, the effects of short treatment (2 h) with mTOR inhibitors on various mRNAs were studied. Fig. 4.11 gives a simplified work flow.

DNA probes were prepared for: (1) known TOP mRNAs (rpL11); (2) non-TOP mRNAs (β-actin); (3) possible "new-TOP" mRNAs (PRDX6). The blots are presented in Fig. 4.12. It can be observed that: (1) treatment with rapamycin and mTOR-KIs caused the association of most of rpL11 mRNA with subpolysomes; (2) β-actin mRNA is mainly associated with polysomes in control and rapamycin-treated cells. AZD8055 and PP242 caused a partial decrease of the amount of this mRNA associated with polysomes; (3) the polysomal distribution of PRDX6 was not greatly affected by rapamycin but shifted strongly to the subpolysomes by treatment with AZD8055 and PP242.

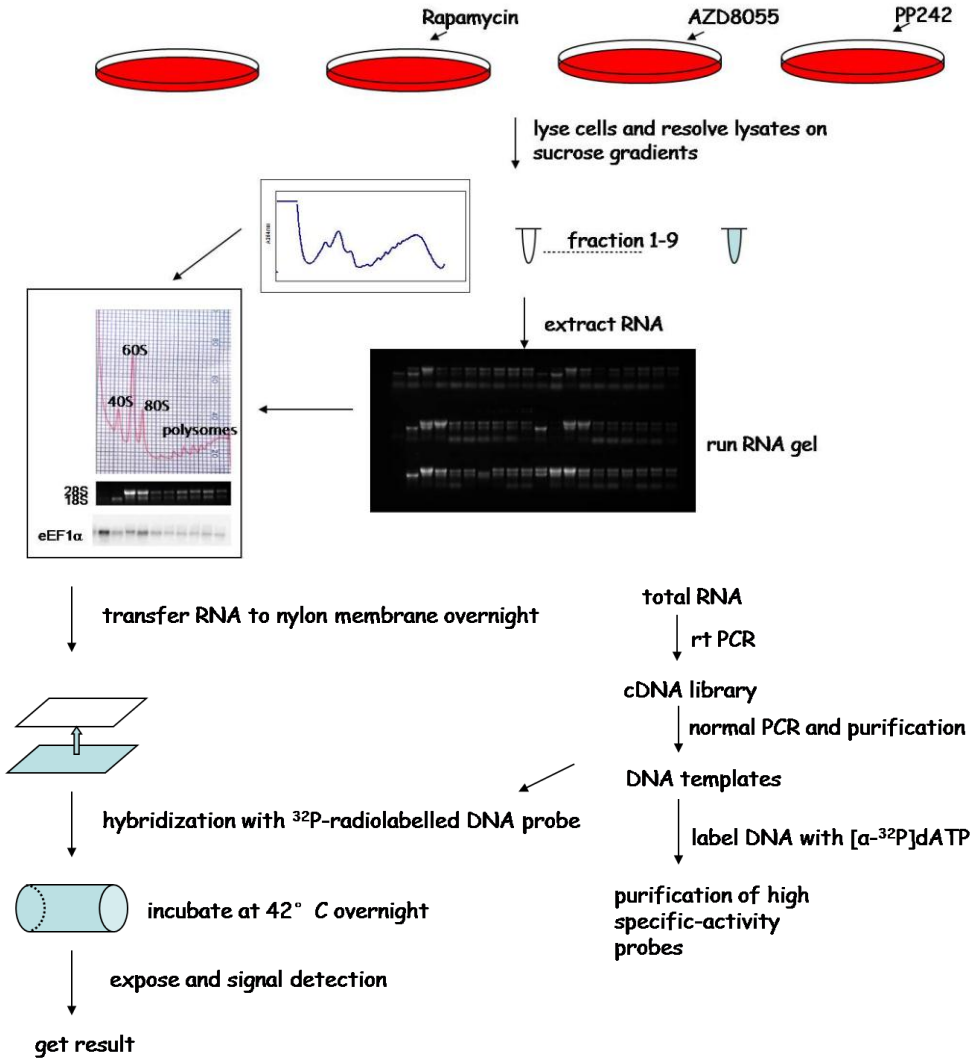


Figure 4.11. Northern blot protocol.

HeLa cells growing in serum were incubated with rapamycin (100 nM), PP242 (1 μM) or AZD8055 (100 nM) for 2 h and then lysed directly on the plate with 300 μL of TNM lysis buffer. The lysates were then centrifuged for 5 min at 16,000 g at 4°C. The supernatant was transferred to a new tube in the presence of 3 μL heparin (10 μg/μL), frozen in liquid nitrogen and stored in -80 ℃ for later analysis. Lysates were resolved into polysomal and non-polysomal fractions by sucrose density gradient centrifugation and the absorbance at 254 nm was recorded. Nine fractions were collected from each gradient. The extracted RNA was then analysed by Northern blot. See *Materials and Methods section 2.4.5* for details.

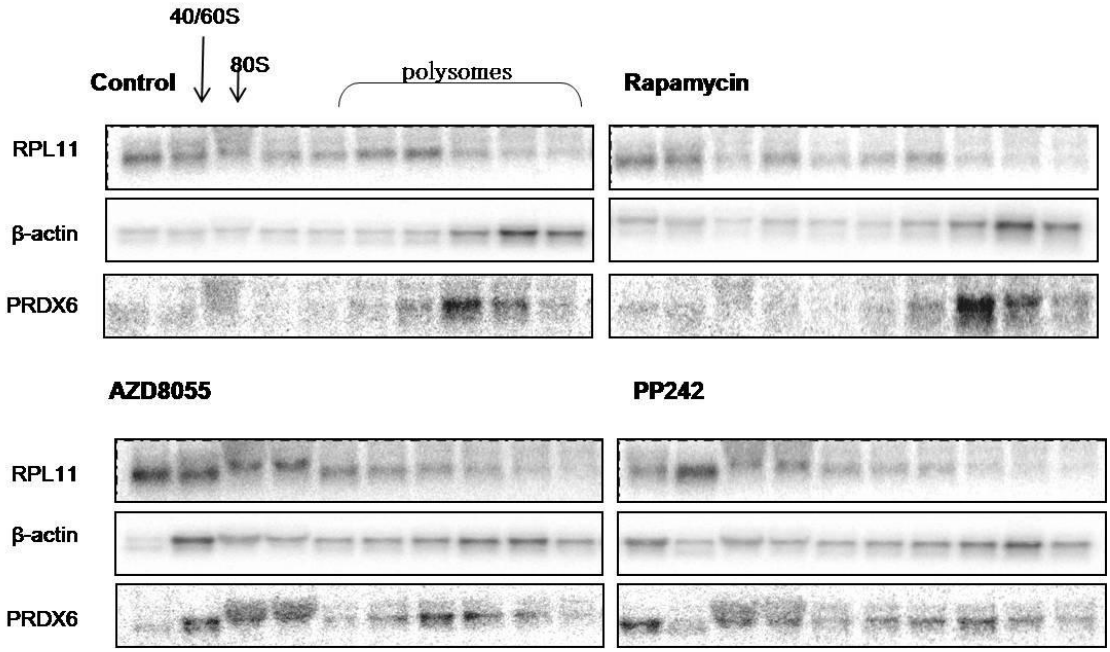


Figure 4.12. Northern blot data (2 h treatment).

Serum-fed HeLa cells were treated with rapamycin (100 nM), AZD8055 (100 nM) and PP242 (1 μM) for 2 h. Northern blots were performed on RNA extracted from fractions separated by sucrose density gradients. The fractions of subpolysomes and polysomes are indicated on the blot.

As mentioned before, a number of different labelling times from 2 h to 6 h were tested in pSILAC experiments. The longer time 6 h was selected since it gave a higher proportion of labelled peptides and ability to provide more accurate and quantifiable data than labelling for 2h. Therefore, in line with the conditions used in pSILAC experiments, prolonged treatment was then performed in Northern blot analysis. HeLa cells were treated with rapamycin, PP242 or AZD8055 for 6 h. Judging from the polysome profile of 6 h (Fig. 4.13), treatment with rapamycin for 6 h had minor effect on the distribution of mRNAs in polysomes. In contrast, both AZD8055 and PP242 caused a huge rise in the peaks of level of 80S monomers (Fig. 4.13), implying a more substantial inhibition of translation initiation.

Based on the pSILAC results, it is of high interest to investigate the behaviour of mRNA from different groups, i.e. known 5'-TOP, non-TOP and possible "new TOP" mRNAs (Table 4.2). Therefore, the polysomal distribution of rpS19 was studied as a typical 5'-TOP mRNA. β-actin and hnRNP A3 were selected as non-TOP mRNAs. More importantly, some of the genes in Table 4.1 whose translation was strongly inhibited by mTOR inhibitors contain a TOP-like stretch in their 5'-end. Therefore, it is important to evaluate the effect of prolonged treatment with mTOR inhibitors on their polysome association using Northern blot. NAP1L1 and PRDX6 were chosen as possible candidates for "TOP" mRNAs.

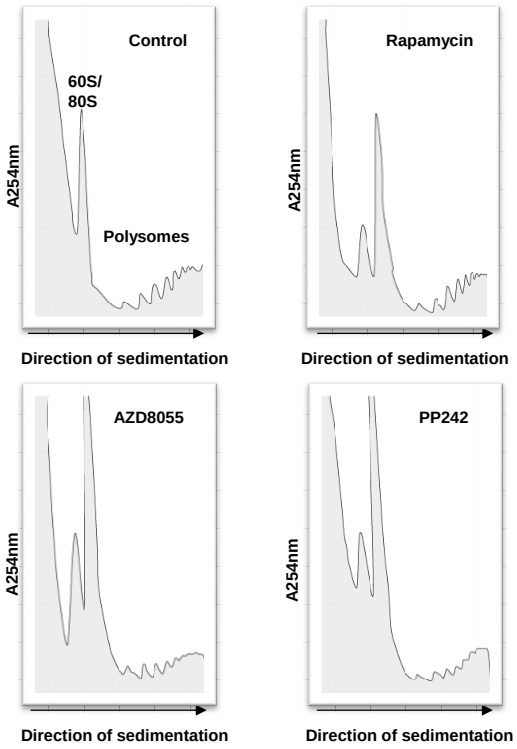
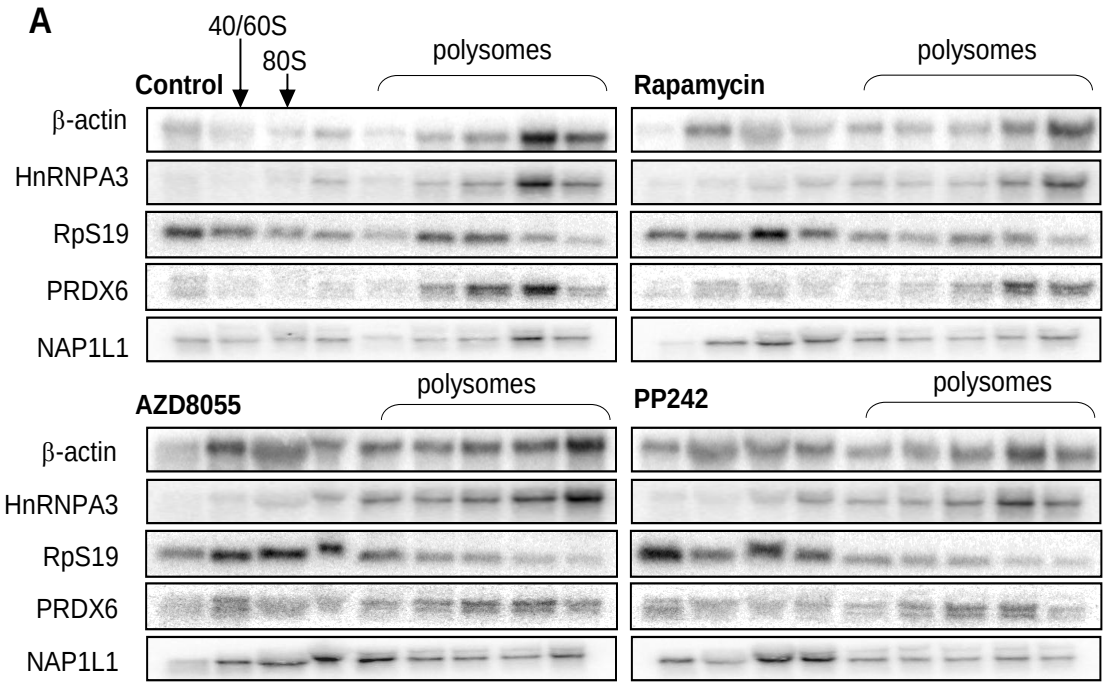


Figure 4.13. Polysome profiles (6 h treatment).

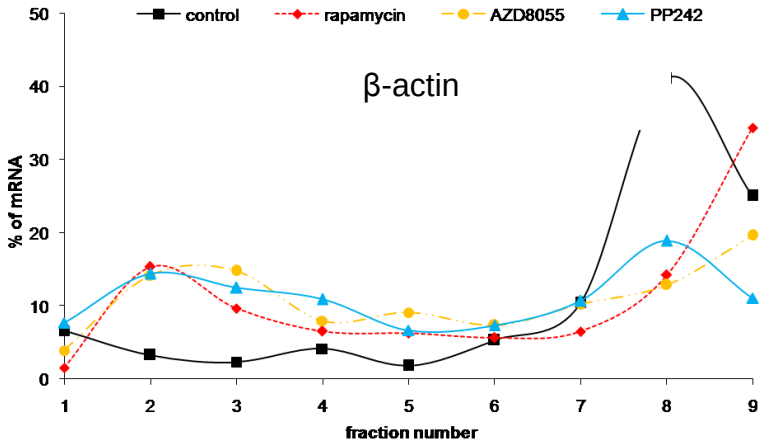
Serum-fed HeLa cells were treated with rapamycin (100 nM), AZD8055 (100 nM), or PP242 (1 μM) for 6 h. Lysates were fractionated on sucrose density gradients. The positions of 80S ribosomal particles and polysomes are labelled; arbitrary units represent absorbance values at 254 nm which are on the same scale for each panel. Detailed procedures are described in *Materials and Methods section 2.4.5*.

As seen in Fig. 4.14A and C, the distribution of the HnRNPA3 mRNA remained unchanged after treatment of cells with various mTOR inhibitors, showing that blocking mTOR activity does not impair the translation of this non-TOP mRNA. mTOR inhibitors did modestly affect the polysomal distribution of the β-actin mRNA (Fig. 4.14A, B). In contrast, the synthesis of the known TOP mRNA rpS19 was strongly blocked by either rapamycin or mTOR-KIs (Fig. 4.14A, D). In fact, AZD8055 and PP242 caused a much more pronounced accumulation of the signals in the non-polysomal fractions than rapamycin. The distinct effect of rapamycin and mTOR-KIs on the polysomal distribution of 5'-TOPs and non-TOPs agrees with the previous finding that they had larger effects on the synthesis of proteins encoded by 5'-TOP mRNAs revealed by pSILAC approach (Fig. 4.6), and more importantly, it suggests that a rapamycin-insensitive component may be involved in the regulation of the translation of this subset of mRNA (5'-TOP) by mTOR signalling.

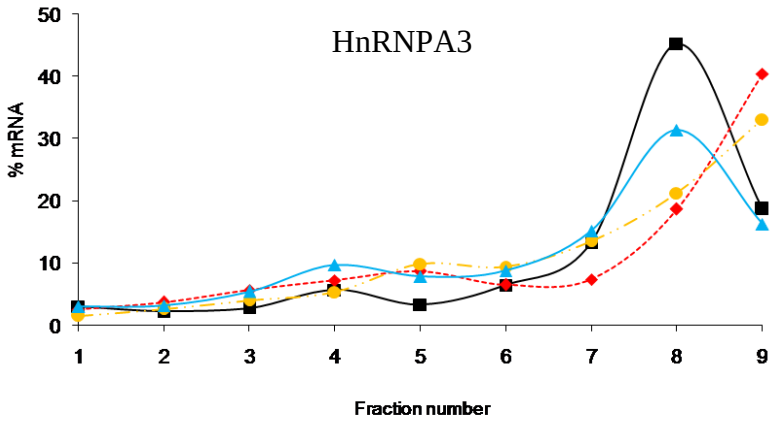
Prompted by the results in Fig. 4.8 and Table 4.1, the distribution of the mRNAs for PRDX6 and NAP1L1 was also investigated. Consistent with the pSILAC data, rapamycin caused a partial shift of the PRDX6 mRNA out of the polysomal fractions but mTOR-KIs had a much greater effect (Fig. 4.8; Fig. 4.14A, E). For NAP1L1 (Fig. 4.8; Fig. 4.14A, F), its behaviour is more similar to that observed for rpS19, again consistent with the possibility that the mRNA for NAP1L is a new 5'-TOP mRNA.


A
40/60S
80S
Control
polysomes
Rapamycin
polysomes
β-actin
HnRNPA3
RpS19
PRDX6
NAP1L1
AZD8055
polysomes
PP242
polysomes
β-actin
HnRNPA3
RpS19
PRDX6
NAP1L1

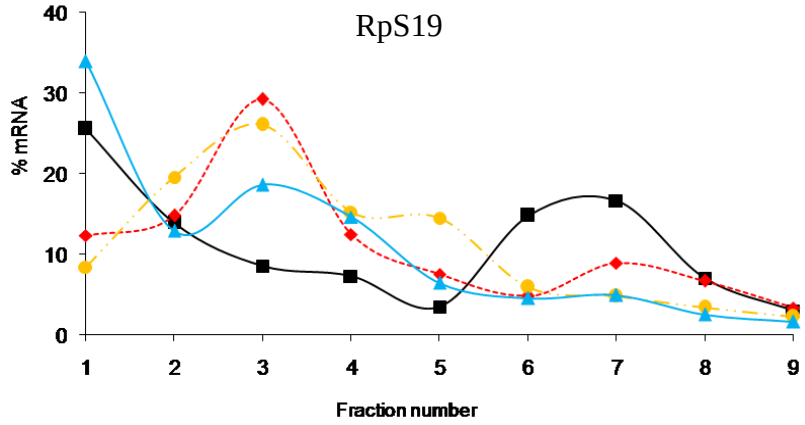
B


control
rapamycin
AZD8055
PP242
β-actin
% of mRNA
fraction number

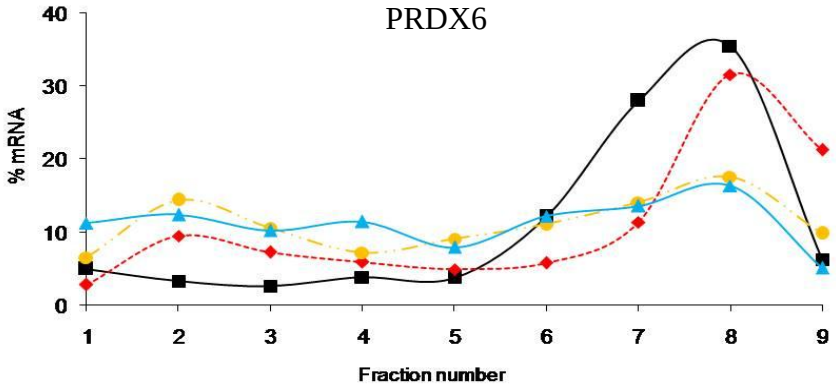
C


HnRNPA3
% mRNA
Fraction number

D



E



F

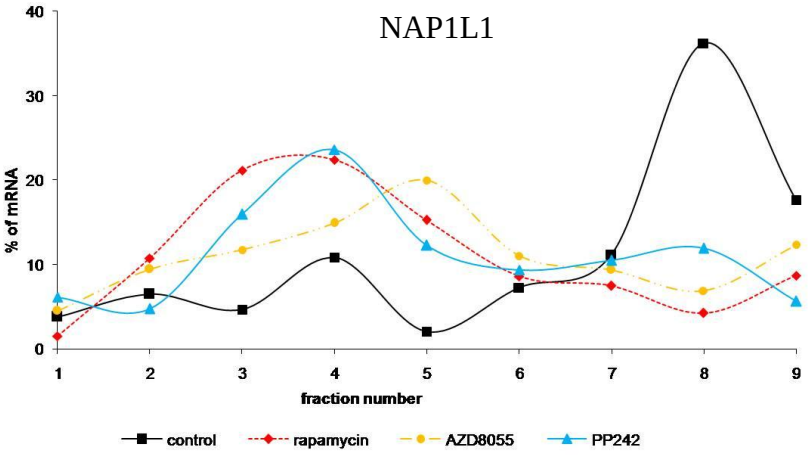


Figure 4.14. Northern blot data (6 h treatment).

Northern blots were performed on RNA extracted from fractions shown in Fig. 4.13. (A) is the Northern blot data for the mRNAs indicated; (B-F) show quantitation of the signals as % of total of each mRNA detected on the blot (in each fraction). In (A), the positions of the ribosomal particles and polysomes are labelled.

4.2.4 Protein breakdown analysis by SILAC approach

As we know, protein turnover is affected by both the rate of synthesis and degradation. Therefore, a modified SILAC approach was used to study the effects of mTOR inhibition by these compounds on the degradation of specific proteins. The protocol of this experiment is given in Fig. 4.15.

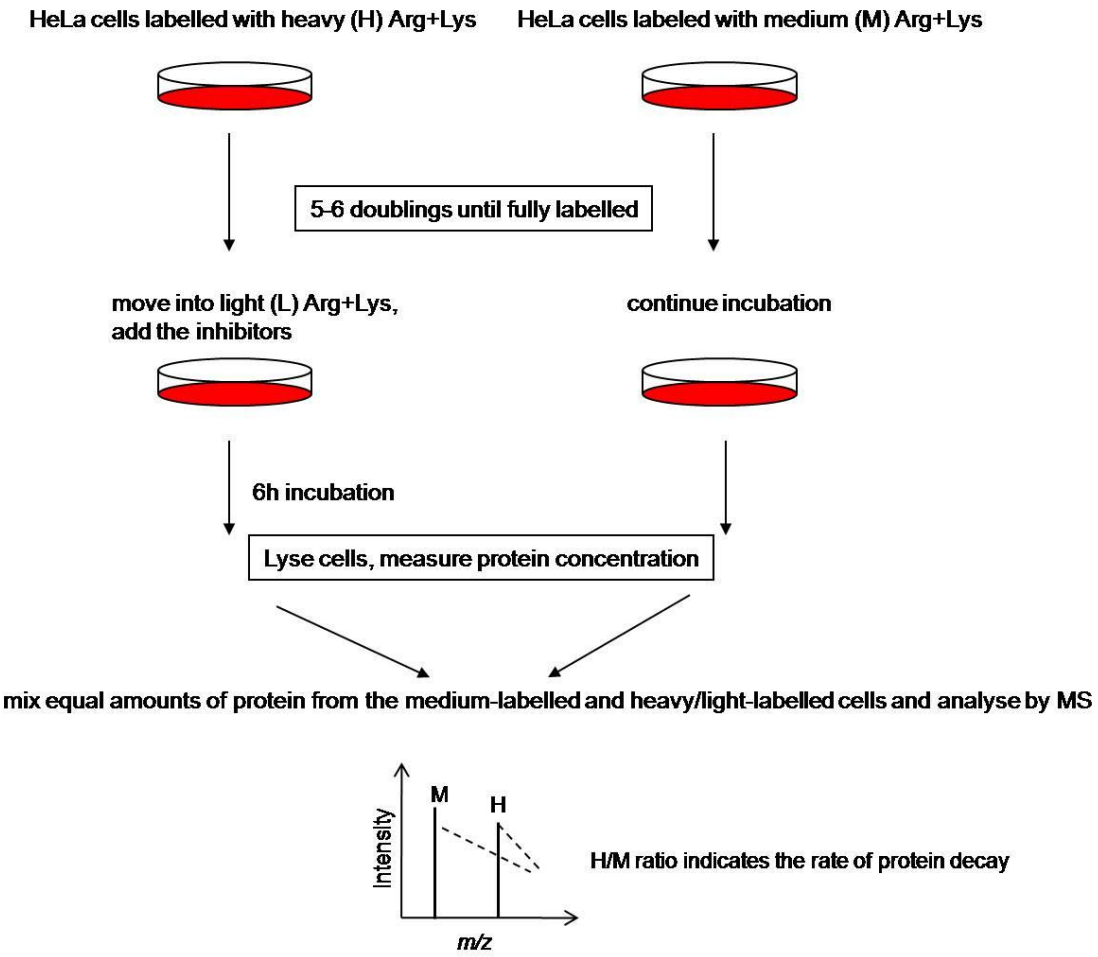


Figure 4.15. A modified SILAC approach for protein breakdown analysis.

Two sets of HeLa cells were grown in the SILAC medium either containing heavy-labelled (H) or medium-labelled (M) Arg and Lys. Cells were passaged five to six times in the above medium. The heavy-labelled cells were then moved into the SILAC medium containing normal Lys/Arg (L) and treated with the indicated inhibitors for 6 h. In this mode, the medium-labelled samples act as internal control for the heavy-labelled ones; the ratio of heavy: medium (H/M) versions of given peptides yields information on the relative rates of decay of the pre-existing 'heavy' version of the specific protein.

The final data for analysis were obtained in triplicate for all four conditions (control, rapamycin, AZD8055 and PP242) from multiple peptides derived from each of more than 300 proteins. Fig. 4.17 shows the H/M ratios for selected proteins which are known to be encoded by 5'-TOP mRNAs, including various ribosomal proteins (RPs) and eukaryotic elongation factors (eEFs).

As seen in Fig. 4.16, it is clear that rapamycin and mTOR-KIs modestly increased the H/M ratios of these proteins, suggesting that inhibition of mTOR activities influences the levels of proteins via decreasing the rates of synthesis rather than accelerating the rates of degradation. From the H/M ratios, the average half-life can be calculated (Table 4.3). The data for AZD8055 are shown, not PP242. Overall, AZD8055 had a stronger effect than rapamycin on half-life of proteins from each category. As seen in Table 4.2, the average protein half-life was about 19 h. AZD8055 extended the average to 22 h while rapamycin slightly increased it (to 19.8 h). In general, proteins not encoded by known 5'-TOP mRNAs have shorter half-lives than those encoded by known 5'-TOP mRNAs (Table 4.3). Interestingly, although rapamycin only had a small effect on overall protein stability, it substantially increased the half-lives of this set of proteins (encoded by known 5'-TOP mRNAs), from 22.7 to 27.2 h. Remarkably, AZD8055 did so to a much greater extent (from 22.7 to 32.4 h; Table 4.3).

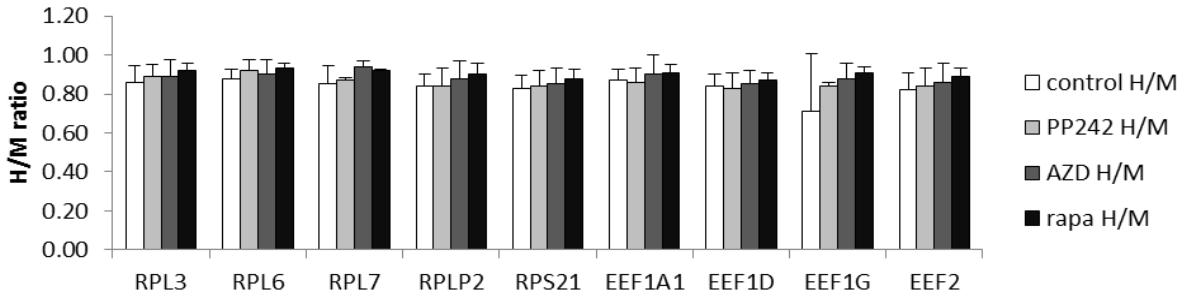


Figure 4.16. The H/M ratio of selected 5'-TOP mRNA encoded proteins

Data are given as mean SEM (n = 3). In each case, data are derived from three independent experiments.

Table 4.3. Effects of mTOR inhibition on protein half-lives which are converted form SILAC data. In each category, proteins with triplicate SILAC data were chosen and analysed. The data are displayed as average ±SEM.

Condition	Average t½ (h) All	Average t½ (h) Proteins encoded by known 5'-TOPs	Average t½ (h) Proteins not encoded by known 5'-TOPs
Control	18.9± 0.33	22.7 ± 1.02	18.5 ± 0.45
Rapamycin	19.8± 0.42	27.2 ± 2.08	18.8 ± 0.36
AZD8055	22.0 ± 0.52	32.4 ± 1.99	21.0 ± 0.51

4.3 Discussion

Multiple approaches were performed to assess the distinct effects of rapamycin and mTOR-KIs on synthesis of specific proteins.

In this chapter, I studied the distinct effects of rapamycin and mTOR-KIs on synthesis of specific proteins using various methods. Microarray was first performed to study the gene expression in cells treated with different inhibitor. However, the initial microarray data analysis showed an unusual pattern of translation of ribosomal proteins caused by rapamycin, which led me to adopt another proteome-wide approach, pSILAC, to further study this. Real-time quantitative RT-PCR and Northern blot were used to validate the data revealed by microarray and pSILAC. The results of real-time PCR showed mTOR-KIs strongly affected the distribution of 18S and β-actin in sub/polysome samples, and also suggest that this method is not suitable as a validation method for my analysis. Northern blot was then carried out to study the behaviour of specific mRNAs revealed by pSILAC. In line with the real-time PCR data, it showed that mTOR-KIs did cause a partial decrease of the amount of β-actin mRNA associated with polysomes.

mTOR-KIs strongly inhibit the synthesis of a wide range of proteins, particularly those encoded by TOP genes.

mTOR-KIs (PP242 and AZD8055) suppressed general protein synthesis to a much stronger extent than rapamycin. The pSILAC data reveal that mTOR-KIs strongly inhibit the synthesis of a subset proteins encoded by 5'-TOP genes, which contain an unusual oligopyrimidine tract (TOP) at their 5'-end. Rapamycin is known to partially suppress the 5'-TOP mRNA translation in serum-stimulated cells (Jefferies *et al.*, 1994, Jefferies *et al.*, 1997, Terada *et al.*, 1994, Grolleau *et al.*, 2002). However, when compared with PP242 and AZD8055, the inhibition caused by rapamycin appears quite mild.

Therefore, what caused the much greater inhibition by mTOR-KIs of the translation of 5'-TOP mRNAs? It has been suggested that rapamycin suppresses 5'-TOP mRNA translation through inhibition of p70 S6K (Jefferies *et al.*, 1997). However, there are multiple lines of evidence supporting the idea that translational activation of TOP mRNAs requires neither S6K1 nor rpS6 phosphorylation (Barth-Baus *et al.*, 2002, Pende *et al.*, 2004, Ruvinsky *et al.*, 2005). Furthermore, both rapamycin and mTOR-

KIs blocked phosphorylation of rpS6, suggesting that this is not the main event resulting in the difference between rapamycin and mTOR-KIs.

Rapamycin and mTOR-KIs also exerted distinct effects on eIF4G/eIF4E binding. My previous data already show that overexpressing eIF4E, which prevents the mTOR kinase inhibitor-caused loss of eIF4G/eIF4E complexes, could not eliminate the mTOR kinase inhibitor-caused inhibition of protein synthesis. More importantly, recent work by Dr. Valentina Iadevaia in our lab has also shown that rapamycin and mTOR-KIs still caused substantial shifts of the rpL11, NAP1L1 and PRDX6 mRNAs out of polysomal fractions, demonstrating that eIF4E (and its partners) is not the main factor resulting in the distinct effects of rapamycin and mTOR-KIs on the translation of 5'-TOP mRNAs.

Nonetheless, mTOR must play a key role in the translational control of 5'-TOP mRNAs and the functions of mTOR involved in the regulation of 5'-TOP mRNA translation may not be strongly affected by rapamycin but more profoundly inhibited by mTOR-KIs. Interestingly, it has been recently suggested that mTOR may exert its effect on the translation of 5'-TOP mRNAs through a novel, unidentified complex rather than the canonical mTOR complexes mTORC1 and mTORC2 (Patursky-Polischuk *et al.*, 2009).

Possible "New" TOP mRNAs revealed by the pSILAC data

So far, all vertebrate genes for the 80 ribosomal proteins and some other genes which are involved in translation are identified as TOP mRNAs (Iadevaia *et al.*, 2008). Recently all five elongation factors were found to be encoded by TOP mRNAs; among the initiation and termination factors, only eIF3e, eIF3f, and eIF3h exhibit the characteristics of TOPs (Iadevaia *et al.*, 2008).

One important finding revealed by the pSILAC data is that the synthesis of certain proteins that are not encoded by known TOP mRNAs is strongly inhibited by mTOR inhibitors. Based on the results from DBTSS database, in several cases, the 5'-UTR of these corresponding mRNAs apparently shows the features of TOP mRNA (Table 4.2), i.e. their transcription start site is a pyrimidine (usually cytosine) followed by an unbroken stretch of pyrimidines. The analysis using Northern blot shows the behaviour of these mRNAs upon treatment with mTOR inhibitors, particularly one of the possible "new" TOPs, NAP1L1 (Table 4.2, Fig. 4.14). Furthermore, analysis by real-time RT-PCR by Dr. Valentina Iadevaia demonstrated that inhibition of mTOR signalling with these inhibitors did not affect the overall levels of these mRNAs

(unpublished work). This promising finding indicates that these mRNAs are possibly new members of 5'-TOP family, although further studies are required to confirm this.

mTOR inhibition affects levels of most proteins via their rates of synthesis rather than degradation

The pSILAC data show the strong inhibitory effects of mTOR-KIs on synthesis of a number of proteins.

It is also interesting to determine whether the levels of specific proteins are affected by changes in their decay rates. Thus, the protein degradation data are very important for several reasons, since they clearly show (1) mTOR inhibitors did not trigger general protein breakdown; instead, they increased the stability of a number of proteins; (2) in general, mTOR kinase inhibitor AZD8055 had bigger effects than rapamycin on stabilising the proteins; (3) interestingly, the stability of proteins encoded by TOP mRNAs was substantially increased by mTOR inhibitors, which agrees with the recent finding that protein half-lives are affected by energetic and dynamic constraints (Schwanhausser *et al.*, 2011). This also makes good physiological sense; the improved stability of existing housekeeping proteins, for example, ribosomal proteins, can help cells to recover from stressful conditions such that caused mTOR signalling inhibition.

CHAPTER 5
THE STUDY OF THE Mnk INHIBITOR: AZ'9224

5.1 Introduction

MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) are protein serine/threonine kinases that are activated by p44/42 MAPK (Erk1/2) or p38 MAPK (Waskiewicz *et al.*, 1997, Fukunaga and Hunter, 1997). The best understood substrate of the Mnks is eIF4E (Mahalingam and Cooper, 2001, Scheper *et al.*, 2001, Ueda *et al.*, 2004).

5.1.1 Discovery and protein structure of Mnks

The Mnks were first discovered simultaneously by two teams during screens for identification of novel Erk2 substrates and Erk1 substrates, respectively (Waskiewicz *et al.*, 1997, Fukunaga and Hunter, 1997). Both Mnk1 and 2 transcripts (gene symbols: *MKNK1* and *MKNK2*) are subject to alternative splicing, giving rise to full length (Mnk1a/2a) as well as truncated versions (Mnk1b/2b) which lack C-terminal MAPK-binding motif (Fig. 5.1). In the N-terminal region, all the Mnk isoforms contain a PBR (polybasic region) which is thought to be involved in the binding of eIF4G and also in nuclear localisation (Pyronnet *et al.*, 1999, Parra-Palau *et al.*, 2003). The catalytic domains of Mnk1a/b and Mnk2a/b share several particular features: two unusual short inserts and a DFD (Asp-Phe-Asp) motif (where most protein kinases have DFG; Jauch et al., 2005, Jauch et al., 2006). The NES (nuclear export sequence) domain in Mnk1a is involved in the export of Mnk1 from nucleus, while its function in Mnk2a is unknown due to the lack of several critical residues (Parra-Palau et al., 2003; Fig. 5.1). Mnk1a contains a MAPK-binding motif in its C terminus, which mediates its interaction with Erk1/2 and p38 MAPK (Waskiewicz *et al.*, 1997, Fukunaga and Hunter, 1997). The corresponding domain in Mnk2a shares high identity with that found in Rsk, leading to its preferred interaction with Erk1/2 (Waskiewicz *et al.*, 1997). Mnk2a also preferably binds to phosphorylated Erk, likely making it constitutively active (Parra *et al.*, 2005). The short versions Mnk1b and 2b lack the C-terminal MAPK-binding motif (Fig. 5.1). No short versions have yet been identified in mice (Parra *et al.*, 2005).

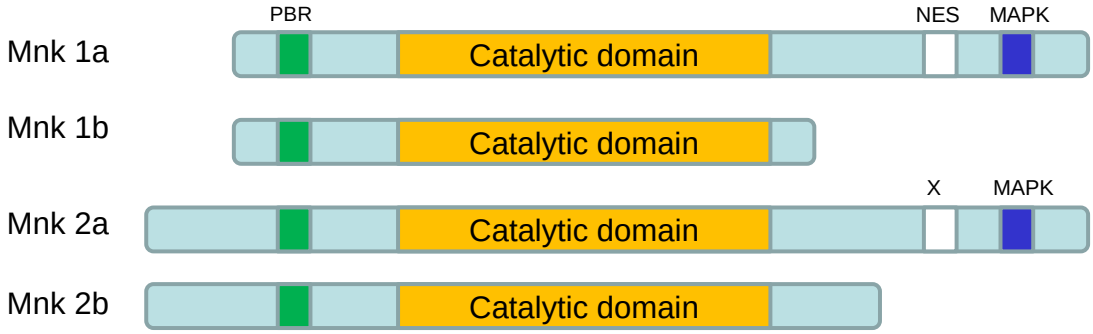


Figure 5.1. The essential structural components of the four Mnk isoforms (Mnk1a, 1b, 2a and 2b) present in human cells. PBR, polybasic region (in green); NES, nuclear-export sequence; X, non-functional NES; MAPK, MAPK-binding motif (in blue).

5.1.2 Activation mechanisms

The activities and regulation of Mnks vary between the isoforms. With respect to Mnk1, the long form Mnk1a is responsive to activation of both Erk1/2 and p38, while the basal activity of Mnk1b is much higher than Mnk1a and is not significantly affected by inhibitors of these modules, consistent with the fact that Mnk1b lacks a MAPK-binding motif (Fukunaga and Hunter, 1997, Wang et al., 1998, Waskiewicz et al., 1997, O'Loghlen et al., 2007). Mnk2a displays high levels of basal activity in cells which can be slightly increased by agonists of Erk1/2 but not P38 MAPK (Scheper et al., 2001). Mnk2b shows much lower activity than Mnk2a and it is not clear which circumstances can promote its activity (Scheper et al., 2003). The various kinases involved in the activation of Mnks are displayed in Fig. 5.2.

The compound CGP57380 has been described as a low-molecular-weight kinase inhibitor of Mnk1 both *in vitro* and *in vivo* (Knauf *et al.*, 2001). This compound was showed to inhibit Mnk1 kinase activity *in vitro* with an IC_{50} of 2.2 μM without inhibiting various other kinases, such as p38 MAPK, JNK1and Erk (Tschopp *et al.*, 2000, Knauf *et al.*, 2001). However, a study against an extended panel of protein kinases indicated that CGP57380 is not a specific inhibitor of Mnks, since it showed similar inhibitory effects against several other protein kinases like MKK1, BRSK2 and CK1δ (Bain *et al.*, 2007). There is therefore a clear requirement to identify novel specific Mnk inhibitors. AZ'9224 is a compound from AstraZeneca and described as a Mnk inhibitor. It is the main aim of my study here to investigate its specificity and its effects.

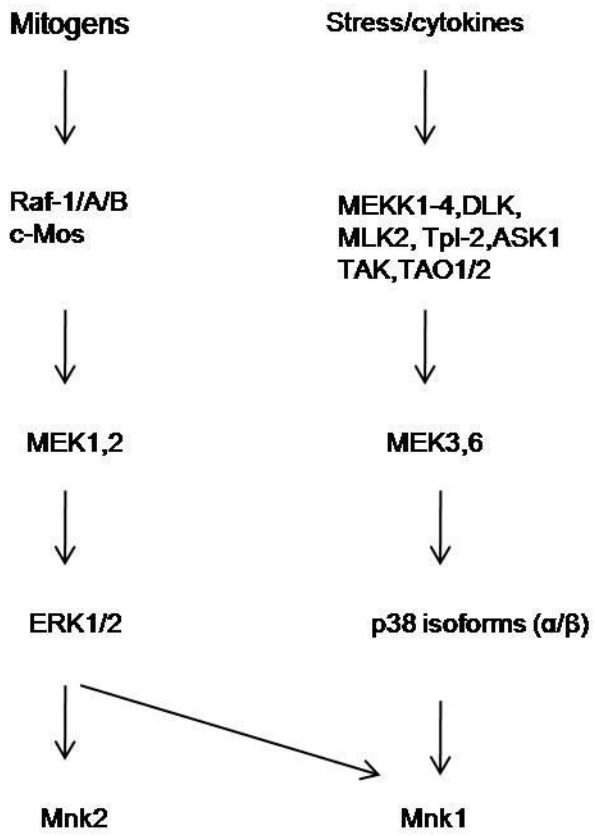


Figure 5.2. MAPK signalling cascades leading to activation of Mnks.

Mitogens, stresses and cytokines promote the activation of different MAPK pathways, which in turn phosphorylate and activate different Mnk isoforms. Mnk1 can be affected by both Erk1/2 and p38 MAPK modules but Mnk2 is only responsive to Erk1/2. It should be made clear that it is the α and β isoforms of p38 MAPK that are responsible for the regulation of Mnks rather than the more-distantly related γ or δ ones.

5.1.3 Substrates

A number of different substrates have been reported to be phosphorylated by Mnks. eIF4E is the best understood one. In eukaryotes, the translation initiation factor eIF4E binds to the mRNA cap structure and interacts with eIF4G, which serves as a scaffold protein for the assembly of eIF4E and eIF4A to form the eIF4F complex (Haghighat *et al.*, 1995, Mader *et al.*, 1995, Marcotrigiano *et al.*, 1999). eIF4E is phosphorylated at Ser209 in response to stress and mitogen stimulation (Flynn and Proud, 1995, Minich *et al.*, 1994). Mnk1 was found to be associated with the eIF4F complex via its interaction with the C-terminal region of eIF4G (Pyronnet *et al.*, 1999). Moreover, Mnk1 and 2 were shown to phosphorylate eIF4E at Ser209 in the presence of mitogen or under cell stress (Waskiewicz et al., 1997, Wang et al., 1998). In embryonic fibroblasts derived from Mnk1 and Mnk2 double knockout mice, the phosphorylation of eIF4E at Ser209 was not detected, even in the presence of the activated Erk and/or p38 MAP kinases (Ueda *et al.*, 2004). Results using cells from single knockout mice further revealed that Mnk1 induced the phosphorylation of eIF4E upon MAPK activation, while Mnk2 was mainly responsible for the basal phosphorylation of eIF4E (Ueda *et al.*, 2004). Taken together, these findings strongly suggest that the Mnk1 and 2 are the only eIF4E kinases, and they mediate inducible and constitutive eIF4E phosphorylation, respectively.

Other identified substrates of Mnks include the heterogeneous nuclear ribonucleoprotein A1 (hnRNP A1; Buxade et al., 2005), the PTB (polypyrimidine tract-binding protein)-associated splicing factor (PSF; Buxade et al., 2008) and sprouty 2 (Bundschu *et al.*, 2006). The Mnks have been found to phosphorylate hnRNP A1 at two residues, Ser192 and Ser310 in response to T cell activation (Buxade *et al.*, 2005). This phosphorylation resulted in decreased interaction of hnRNP A1 with TNF (tumor necrosis factor) α-AREs (AU-rich elements) *in vitro* or TNFα mRNA *in vivo*, suggesting that Mnks play key roles in the regulation of hnRNP A1. Together with its homolog $p54^{nrb}$, PSF forms a multifunctional heterodimer implicated in diverse nuclear processes (reviewed in Shav-Tal and Zipori, 2002). Mnks were found to phosphorylate PSF at two sites *in vitro* during a proteomic approach and the Mnk-mediated phosphorylation appears to increase the binding of PSF to the TNFα mRNA *in vivo* (Buxade *et al.*, 2008). Mnks are also able to phosphorylate cytoplasmic phospholipase A_2 ($cPLA_2$) resulting in enzyme activation and $cPLA_2$-mediated

arachidonate release (Hefner *et al.*, 2000). Furthermore, Mnk1 has been shown to regulate the phosphorylation of human Sprouty 2 (hSpry2) at Ser112 and Ser121 (Bundschu *et al.*, 2006, DaSilva *et al.*, 2006). Enhancement of serine phosphorylation of hSpry leads to the stabilization of hSpry2 and therefore an increased ability of hSpry2 to antagonize RTK induced Ras-Erk activation (DaSilva *et al.*, 2006).

5.2 Results

5.2.1 AZ'9224 inhibits the phosphorylation of eIF4E in various cell lines.

To test whether AZ'9224 inhibits the phosphorylation of eIF4E at Ser209, MCF7 cells were treated with different doses of this compound from 300nM to 1μM for 1 h. As shown in Fig. 5.3, in serum-fed MCF7 cells, the basal level of eIF4E phosphorylation was low but was significantly enhanced by treatment with rapamycin. In contrast, AZ'9224 showed a dose-dependent inhibitory effect on eIF4E phosphorylation. It completely abolished eIF4E phosphorylation at a concentration of 1μM. Concomitant use of AZ'9224 with rapamycin also substantially inhibited eIF4E phosphorylation in MCF7 cells (Fig. 5.3).

In the absence of serum, the basal level of eIF4E phosphorylation is very low as shown in Fig. 5.4. Treatment with PMA can stimulate both Erk1/2 and p38 in a PKC dependent manner (Schultz *et al.*, 1997). AZ'9224 abolished the basal level of eIF4E phosphorylation and PMA-induced eIF4E phosphorylation in MCF7, HEK293 and HeLa cell lines (Fig. 5.4).

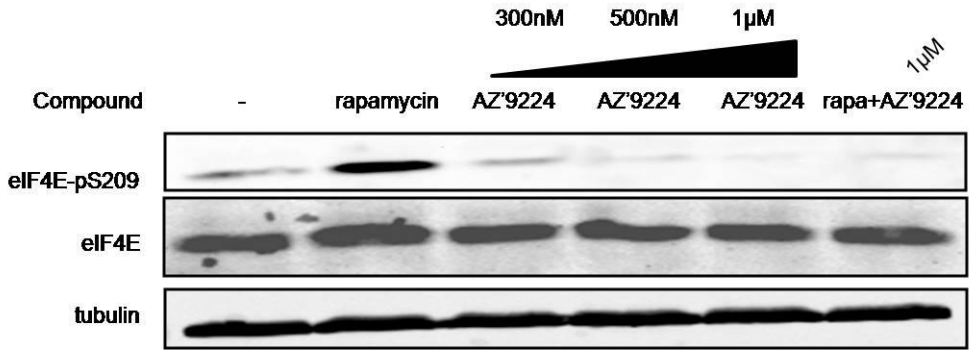


Figure 5.3 Dose dependent effects of AZ'9224 in serum-fed MCF7 cells.

Serum-fed MCF7 cells were treated with AZ'9224 at the indicated concentrations and/or rapamycin (rapa; 100 nM) for 60 min. Cell lysates were analyzed by western blot using the indicated antibodies. Tubulin was examined as a loading control.

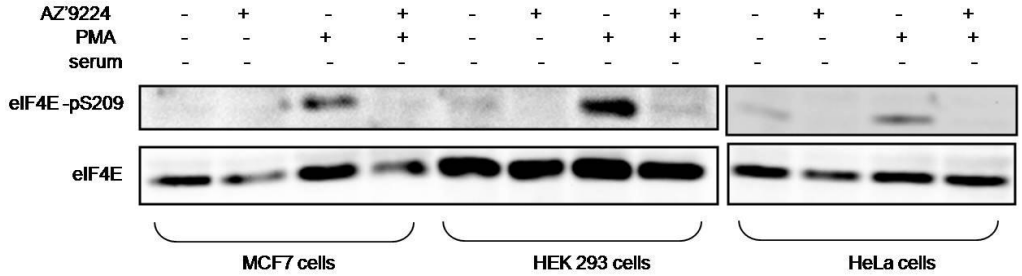


Figure 5.4. Effects of AZ'9224 on eIF4E phosphorylation in response to PMA in various cell types.

MCF7, HEK293 and HeLa cells were starved of serum overnight and then treated with 1 μM AZ'9224 prior to PMA stimulation. Whole cell lysates were subjected to western blot analysis.

Subsequently, a wide variety of cell lines including MCF7, MEFs (mouse embryonic fibroblasts), PC3 (human prostate cancer cell line) and ZR75 (human breast carcinoma cell line cells) were tested. The effects of the mTOR inhibitors rapamycin and AZD8055 on eIF4E phosphorylation were also analysed. AZ'9224 strongly inhibited eIF4E phosphorylation at Ser209 in serum-fed MCF7, PC3 and ZR75 cells. Except in MEFs, either rapamycin or AZD8055 increased eIF4E phosphorylation in these cell lines (Fig. 5.5). As rapamycin and AZD8055 possessed contrary effects towards Akt phosphorylation (shown in Chapter 3), it is unlikely that the increased level of eIF4E phosphorylation caused by these inhibitors was due to Akt activation. Akt phosphorylation at Thr308 was not significantly affected by AZ'9224 in PC3 and ZR75 cells (Fig. 5.5). Other investigators reported that silencing p70 S6K had no effect on rapamycin-induced eIF4E phosphorylation (Wang *et al.*, 2007b). Therefore, it appears that inhibition of mTORC1 activity increases eIF4E phosphorylation independently of Akt and p70 S6K.

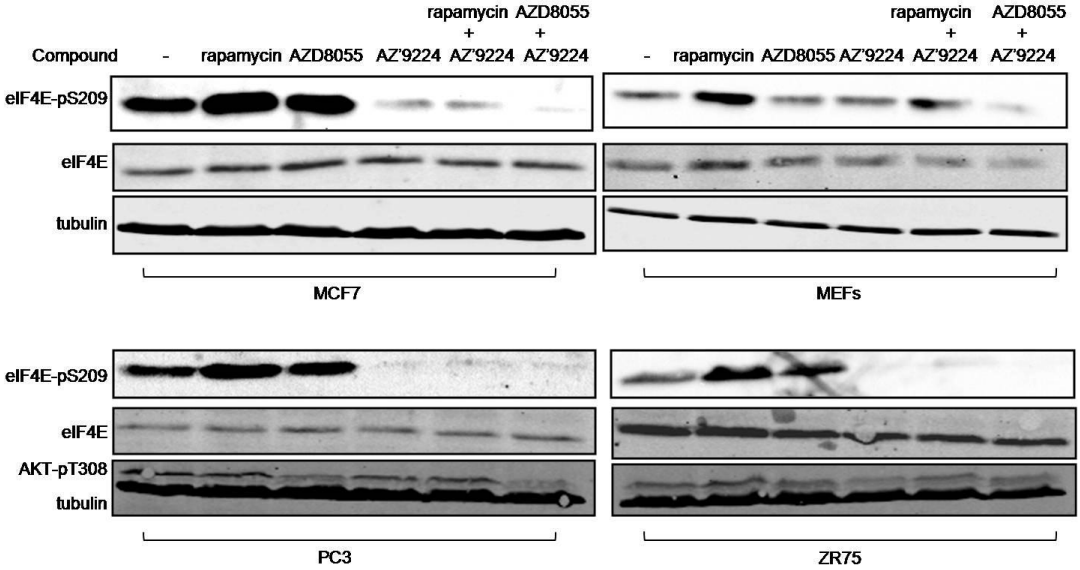


Figure 5.5. Effects of AZ'9224 and mTOR inhibitors on eIF4E phosphorylation in various cell types.

Serum-fed MCF7, MEFs, PC3 and ZR75 cells were treated with rapamycin (100 nM), AZD8055 (100 nM), AZ'9224 (1 μM), or co-treated with rapamycin/AZ'9224 or AZD8055/AZ'9224. Whole cell lysates were subjected to western blots using the indicated antibodies.

5.2.2 The inhibition by AZ'9224 of the formation of eIF4F in MCF7 and PC3 cells may be due to its off-target effects on other kinases.

The effect of AZ'9224 on eIF4E phosphorylation appears very promising. Therefore, it is of high importance to assess its specificity, i.e. whether AZ'9224 shows inhibitory effects against other protein kinases/signalling pathways.

Higher concentrations of the compond (up to 10 μM) were used in the following experiment. As shown in Fig. 5.6, AZ'9224 at 10 μM had a mild effect on the binding of eIF4GI to eIF4E without inhibiting 4E-BP1 phosphorylation at Thr37/46 in MCF7 and PC3 cells. If AZ'9224 only inhibits Mnks without side effects on other kinases, the decreased amount of bound eIF4GI could suggest that phosphorylation of eIF4E at Ser209 has an effect on eIF4GI/eIF4E binding.

However, subsequent experiments showed that AZ'9224 also inhibited the phosphorylation of Erk1/2, which is upstream of Mnks. Fig. 5.7 demonstrated that AZ'9224 at 10 μM decreased the serum-induced phosphorylation of Erk1/2 when inhibiting eIF4G binding in both MCF7 and PC3 cells. Furthermore, phosphorylation of the PDK1 site Thr308 in Akt was also impaired by AZ'9224 at 10 μM. Although AZ'9224 did not exhibit any inhibitory effect on the phosphorylation of 4E-BP1 in MCF7 and PC3 growing in serum (Fig. 5.6), it did block the phosphorylation of rpS6 (Fig. 5.7). Serum-induced S6 phosphorylation at Ser240/244 was slightly decreased in MCF7 but substantially in PC3 cells.

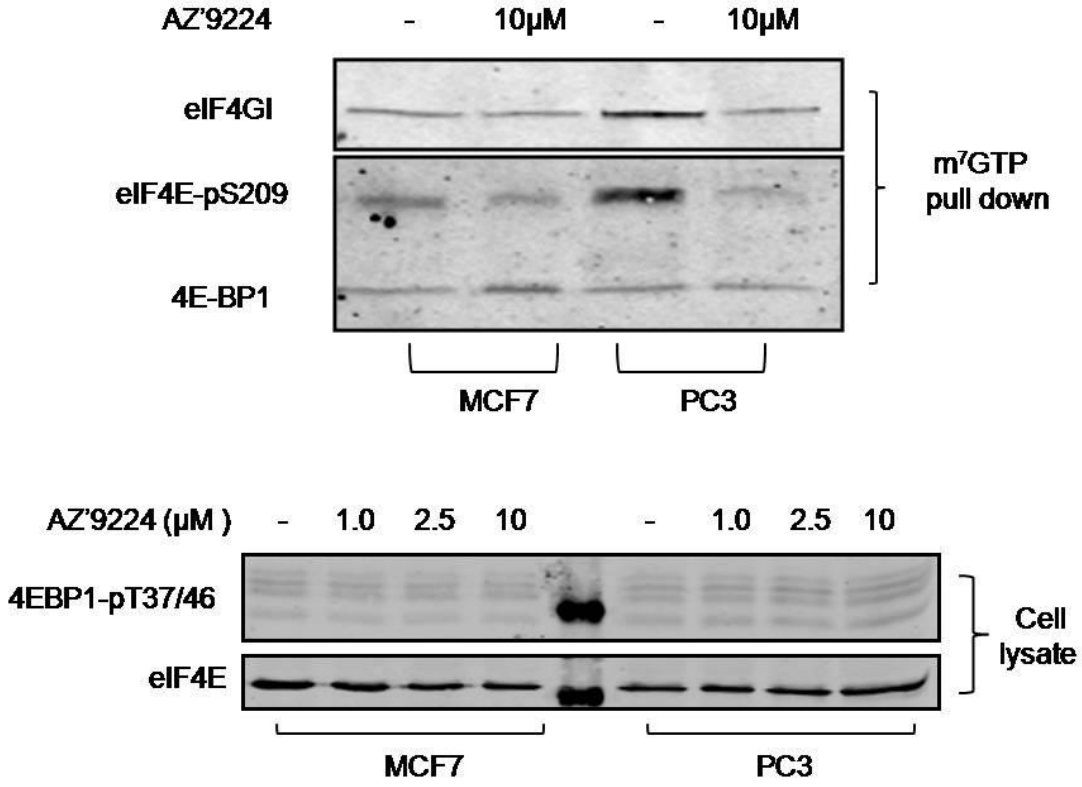


Figure 5.6. Effect of AZ'9224 on the binding of eIF4GI to eIF4E in MCF7 and PC3 cells.

MCF7 and PC3 cells growing in serum-maintained medium were treated with AZ'9224 at the indicated concentrations for 1 h. Equal amounts of lysates were subjected to affinity chromatography on m^7GTP-Sepharose. Bound proteins were analysed by western blot using the indicated antibodies. The bottom blot of eIF4E from whole cell lysate was used as an input control. The phosphorylation of 4E-BP1 at Thr37/46 was also examined.

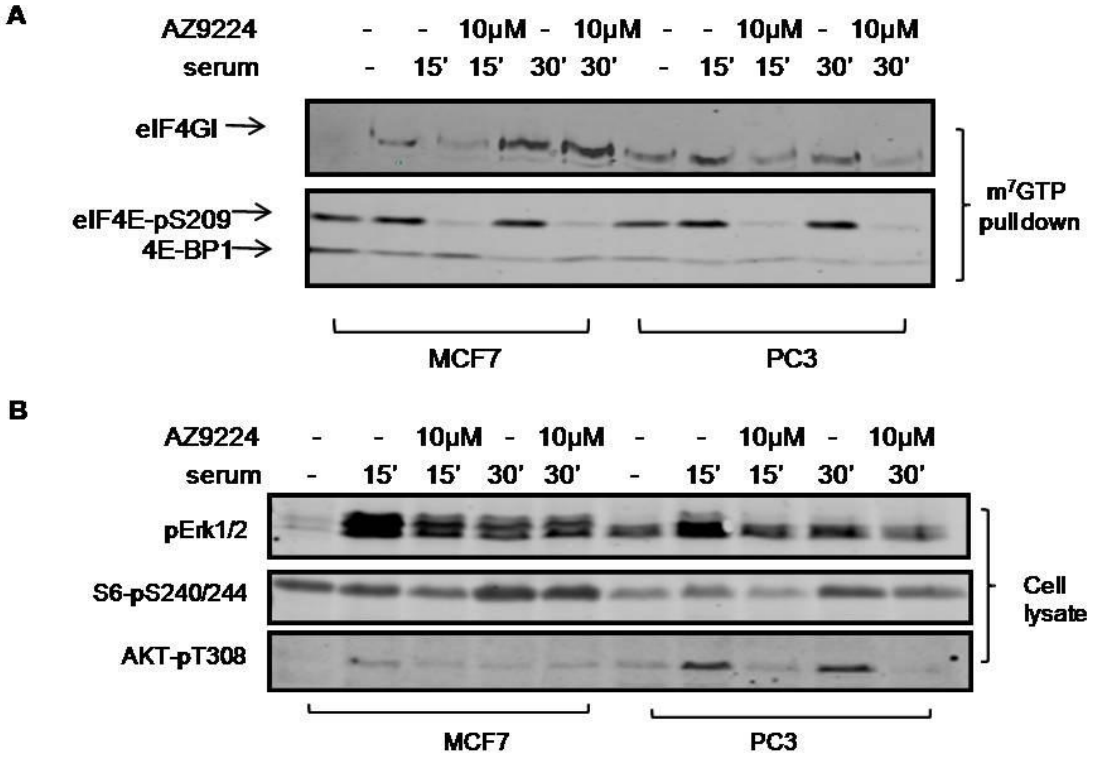


Figure 5.7. Effect of AZ'9224 on the serum-induced binding of eIF4GI to eIF4E in MCF7 and PC3 cells.

MCF7 and PC3 cells were starved of serum overnight (>16 h) and were then treated with AZ'9224 at the indicated concentrations for 1 h prior to serum stimulation for the indicated times. (A) Equal amounts of lysates were subjected to affinity chromatography on m^7GTP-Sepharose. Bound proteins were analysed by western blot using the indicated antibodies. (B) Whole lysates were examined by western blot using the indicated antibodies.

5.3 Summary and Discussion

In summary, despite the striking effects of AZ'9224 on eIF4E phosphorylation at Ser209 in various cell types, it unfortunately exhibits some off-target effects, i.e. it inhibits protein kinases or other effects which are either upstream of the Mnks or up/downstream of mTOR. All these studies indicate that AZ'9224 is not a specific inhibitor of Mnk isoforms and the results from this compound should be difficult to interpret. Therefore, the generation of a novel specific Mnk inhibitor or Mnk1/2 double knockout animals is expected to provide more valuable information about the mechanism of Mnks in the future (Ueda *et al.*, 2004).

mTOR inhibitors, particularly rapamycin, increased the phosphorylation of eIF4E phosphorylation on Ser209, which has been reported before (Wang *et al.*, 2007b). The phosphorylation of eIF4E has been shown to be regulated by Mnks through modulation of Mnk1-eIF4G interaction (Shveygert *et al.*, 2010, Pyronnet *et al.*, 1999). Given that the distinct effects of rapamycin and mTOR-KIs on the binding of eIF4GI/eIF4E (Fig. 3.9-3.10), the reason accounting for their apparently similar effects on eIF4E phosphorylation remains unclear. Indeed, my finding that rapamycin increases the binding of eIF4G/eIF4E provides a potential explanation to its ability to increase eIF4E phosphorylation, since eIF4E phosphorylation is partially modulated by Mnk1-eIF4G interaction (it is also controlled by Mnk activity) (Shveygert et al., 2010, Knauf et al., 2001, Pyronnet et al., 1999). Wang *et al.* (2007b) concluded that inhibition of mTOR by rapamycin increases eIF4E phosphorylation through a PI3K-dependent and Mnk-mediated mechanism. However, the mechanism by which AZD8055 increased eIF4E phosphorylation remains unclear. Overall, these data suggest simultaneous inhibition of mTOR and Mnk may provide better results in tumour therapy, especially in those which are not sensitive to rapamycin (Bianchini *et al.*, 2008, Wang *et al.*, 2007b).

CHAPTER 6
STUDIES ON THE ROLE OF PHOSPHORYLATION OF eIF4E

6.1 Introduction

The cap-binding protein eIF4E may act as a convergence point for the PI3K and MAPK signalling pathways. eIF4E is partially activated via phosphorylation at Ser209 by Mnks (eIF4E is also controlled by the interaction with 4E-BP1; Knauf et al., 2001, Waskiewicz et al., 1997, Wang et al., 1998, Pause et al., 1994). However, the physiological significance of eIF4E phosphorylation has not been clearly defined so far.

6.1.1 Ser209 is the only phosphorylation site on eIF4E

Ser209 is located near the C-terminus of eIF4E (Flynn and Proud, 1995, Joshi *et al.*, 1995). It is now clear that eIF4E is phosphorylated by the Mnks at a single site Ser209 in mammals, although Ser53 was initially identified as the phosphorylation site (Rychlik *et al.*, 1987). Multiple lines of evidence have shown that the Ser53Ala mutant still underwent phosphorylation (Flynn and Proud, 1995, Lazaris-Karatzas *et al.*, 1990, Kaufman *et al.*, 1993). It is likely that the mutation of Ser53 to Ala affects the function of eIF4E via the interference with the structure of eIF4E, rather than the phosphorylation state of eIF4E (Scheper and Proud, 2002). Ser209 was first identified as the major phosphorylation site by Joshi *et al.* (1995) and Flynn *et al.* (1995). Recently, Sonenberg *et al.* determined that Ser209 is the only phosphorylation site on eIF4E using an orthophosphate labelling approach (Furic *et al.*, 2010). Using MEFs isolated from Wild-Type (WT) and KI (in which Ser209 was mutated to Ala) littermate embryos, ^{32}P-radiolabelled eIF4E was detected by immunoprecipitation in only WT MEFs, which underwent a two-fold increase by PMA treatment (Furic *et al.*, 2010).

6.1.2 Effects of phosphorylation of eIF4E on its affinity for capped mRNA

Given the fact that phosphorylation of eIF4E is increased by extracellular stimuli that activate protein synthesis (Wang et al., 1998), it was generally thought phosphorylation of eIF4E increased its affinity to cap structure. To address this, Minich *et al.* developed a method for the separation of phosphorylated and nonphosphorylated eIF4E from rabbit reticulocytes by chromatography on rRNA-Sepharose (Minich *et al.*, 1994). Using this method, they found that phosphorylated eIF4E had three- to four-fold greater affinity for cap analogs and capped mRNA than nonphosphorylated eIF-4E. However, it is possible that contamination with other proteins such as 4E-BP1 in Minich *et al.*'s method would influence their results.

Subsequently, with the discovery of the Mnks, other investigators raised controversial opinions. For instance, Proud's group pointed out that phosphorylation of eIF4E actually diminished its ability to bind cap analogue or capped mRNA (Scheper *et al.*, 2002). Using fluorescence spectroscopy and surface plasmon resonance techniques, it was clear that phosphorylated eIF4E bound with lower affinity than the nonphosphorylated form to cap analog and to capped RNA (Scheper *et al.*, 2002). The reduction of the cap affinity for phosphorylated eIF4E was observed by Zuberek *et al.* (2003). Proud's group have proposed a new model of eIF4E phosphorylation during translation initiation. In this model, phosphorylation of eIF4E occurs immediately after the formation of eIF4F complex. Reduced affinity of eIF4E for the cap-structure results in its dissociation from the cap, allowing the recruitment of a second eIF4E molecule and associated proteins, plus the 40S subunit. Proud's group have also demonstrated that the phosphorylation of eIF4E has no effect on its binding to eIF4G/4E-BP1, which is consistent with the 3D structure of eIF4E: the region that binds eIF4G/4E-BP1 is far away from the phosphorylation site Ser209 (reviewed in Scheper and Proud, 2002).

6.1.3 Physiological role of eIF4E phosphorylation

The biological significance of eIF4E phosphorylation remains controversial. It seems critical for normal development in *Drosophila,* since Lachance *et al.* (2002) found that transgenic *Drosophila* organisms expressing eIF4E-Ser251Ala (in *Drosophila*, Ser251 corresponds to Ser209 of mammalian eIF4E) in an eIF4E mutant background had decreased viability (35% lethality), were delayed in development and smaller than control siblings. When Ser251 was mutated to Asp to mimic constitutive phosphorylation, the lethality was fully rescued in these transgenic lines. In contrast, mice with deletion in Mnk1/2 kinases developed normally in the absence of detectable phosphorylated eIF4E (Ueda *et al.*, 2004). Wendel *et al.* (2004) showed that eIF4E was a potent oncogene *in vivo* using the Eμ-*Myc* transgenic mouse lymphoma model. Using the same model, they further demonstrated that the oncogenic activity of eIF4E was connected with its ability to activate translation and become phosphorylated on Ser209 (Wendel *et al.*, 2007). In human prostate carcinomas (PCas), inactivation of *PTEN* caused an increase of basal level of PI3K and constitutive activation of the Akt pathway. Bianchini *et al.* (2008) found that the mTOR and the Mnk/eIF4E pathway

were balanced to maintain the translation of mRNAs for proteins required for cell proliferation and stress response in PCas. Analysis by Wheater *et al.* in breast cancer cell lines provided evidence that the sensitivity of breast cancer cells to Mnk inhibition correlated with basal levels of eIF4E phosphorylation. They suggested that the presence of phosphorylated eIF4E could provide a biomarker for the identification of tumours which are responsive to Mnk inhibition (Wheater *et al.*, 2010). In primary central nervous system lymphoma cells (PCNSL), Muta *et al.* (2011) showed that eIF4E phosphorylation played an important role in proliferation and inhibition of the Mnk/eIF4E pathway could be a promising therapeutic target in patients with PCNSL. Studies by Furic and colleagues (2010) showed that knock-in mice expressing a nonphosphorylable form of eIF4E-Ser209Ala (eIF4E-KI) were resistant to tumourigenesis in a prostate cancer model. When they investigated the molecular basis that underlies the eIF4E-KI mice to tumourigenesis using polysome profiling and DNA microarray, a subset of mRNAs of KI MEFs were found to shift to lighter fractions relative to WT MEFs, such as the chemokine (C-C motif) ligand 2 and 7 (Ccl2 and Ccl7); the matrix metalloproteinases (MMPs) MMP3 and MMP9 and baculoviral IAP repeat-containing protein 2 (BIRC2). Their results indicated that some mRNAs involved in tumour progression differed in translational level between WT and KI MEFs. Overall, these data suggest the oncogenic activity of phospho-eIF4E and its potential as a target in cancer therapy.

6.1.4 Sumoylation of eIF4E

Protein Sumoylation which is mediated by activating (E1), conjugating (E2) and ligating (E3) enzymes is an important regulator of protein function in a wide range of cellular processes (Di Bacco and Gill, 2006). In mammals, there are four SUMO (small ubiquitin-like modifier) molecules, designated SUMO-1 to SUMO-4. These four SUMO molecules exhibit variable sequence identity and expression pattern (Wilkinson and Henley, 2010). SUMO-1 is also known as UBL1 (Shen *et al.*, 1996), PIC1 (Boddy *et al.*, 1996), sentrin (Okura *et al.*, 1996), GMP1 (Matunis *et al.*, 1996) and Smt3c (Lapenta *et al.*, 1997). SUMO-1 was first reported as a covalent protein modifier of RanGAP1 (Ran-GTPase-activating protein) (Matunis *et al.*, 1996). The sequence of SUMO-2 is highly similar to SUMO-3. However, the sequences of SUMO-2/3 share only about 50% similarity with that of SUMO-1. SUMO-4 isoform

has been predicted as a 95-residue protein, although endogenous SUMO-4 protein has not been detected. SUMO-1/2/3 are expressed ubiquitously (reviewed in Wilkinson and Henley, 2010).

The functional outcomes of sumoylation are immensely diverse but its molecular consequences on the substrate protein can be summarized into three aspects. First, sumoylation may block the interaction between kinases and substrate proteins via "masking" the binding site. Secondly, after attachment to the substrate, SUMO may create a new binding site and recruit new binding partners in a SUMO-dependent pattern. Finally, SUMO modification can lead to a conformational change of the sumoylated protein, regulating its activity or exposing previously masked binding faces (reviewed in Geiss-Friedlander and Melchior, 2007, Wilkinson and Henley, 2010).

A study in *Drosophila* confirmed eIF4E as a sumoylation substrate (Nie *et al.*, 2009). Given the importance of SUMO modification in protein regulation, it is of high interest to investigate the role of mammalian eIF4E sumoylation in mRNA translation and protein synthesis. Recently, Xu *et al.* (2010) reported that eIF4E was sumoylated on lysines 36, 49, 162, 206 and 212 by SUMO-1 and the sumoylation promoted the formation of eIF4F complex. However, they only detected a very small proportion of eIF4E which was sumoylated both *in vivo* and *in vitro.*

6.1.5 The control of TNFα biosynthesis by the Mnks.

TNFα (tumour necrosis factor) is a cytokine involved in the control of inflammatory phenomena. Its synthesis is under complex regulation. Through AU-rich elements (AREs) in the 3'UTR of its mRNA, the expression of TNFα is tightly controlled at the posttranscriptional level, including transport (Dumitru *et al.*, 2000), stabilization (Kontoyiannis *et al.*, 1999) and translation (Han *et al.*, 1990). However, the mechanism remains poorly understood. Both Erk and p38 MAPK pathways are known to regulate the production of TNFα (Ballester *et al.*, 1998, Buxade *et al.*, 2001). It has been reported that MAP kinase-activated protein kinase-2 (MK-2) is essential for biosynthesis of TNFα without affecting its mRNA level and secretion (Kotlyarov *et al.*, 1999). Buxade *et al.* (2005) identified Mnks/eIF4E pathway as a novel player in the post-transcriptional regulation of TNFα in T cells. They showed that Mnks regulate TNFα expression in T cells via the 3'UTR of its mRNA. A Mnk inhibitor (CGP57380) or siRNA-mediated knockdown of Mnk1 decreased TNFα production, while

overexpression of Mnk1 enhanced protein expression of a reporter containing the TNFα 3'UTR. Moreover, novel substrates for the Mnks that bind to the AREs of TNFα mRNA have been identified, including hnRNP A1 and the PSF-p54^{nrb} complex (Buxade et al., 2008, Buxade et al., 2005). The phosphorylation mediated by Mnks reduced the binding of hnRNP A1to TNFα mRNA *in vivo.*

6.2 Results

6.2 .1 Effects of AZ'9224 on the sumoylation of eIF4E

The report that phosphorylation of eIF4E is required for its sumoylation may provide new insights into the control of eIF4E translational activity (Xu *et al.*, 2010). Thus, it was important to study effects of the Mnk inhibitor AZ'9224 on eIF4E sumoylation. Xu *et al.* (2010) utilized a vector for eIF4E with mutations of all the five lysines, which may interfere with the phosphorylation site at Ser209, whereas the use of the Mnk inhibitor circumvents this issue.

As shown in Fig. 6.1, multiple bands were detected in the m^{7}GTP pull down assay after immunoblotted with anti-SUMO-1. However, in the presence of a large amount of eIF4E in the bound material, only a minimal fraction (less than 1%) was apparently modified by SUMO-1. Adding NEM (N-ethylmaleimide; the isopeptidase inhibitor) in the lysis buffer did not significantly enhance the SUMO-1 signal. Unlike the dramatic effect of eIF4E phosphorylation on its sumoylation found by Xu *et al.* (2010), the inhibition of eIF4E phosphorylation by the Mnk inhibitor AZ'9224 did not affect its sumoylation (Fig. 6.1). One reason may be that only a tiny amount of eIF4E was modified by SUMO-1, resulting in the difficulty of detection. Subsequently, the expression of eIF4E was increased by introducing wild type eIF4E into HeLa cells. Again, only a tiny proportion of eIF4E which might be modified by SUMO-1 was detected (data not shown). Therefore, my data using the Mnk inhibitor AZ'9224 in HeLa cells did not confirm Xu *et al.*'s findings.

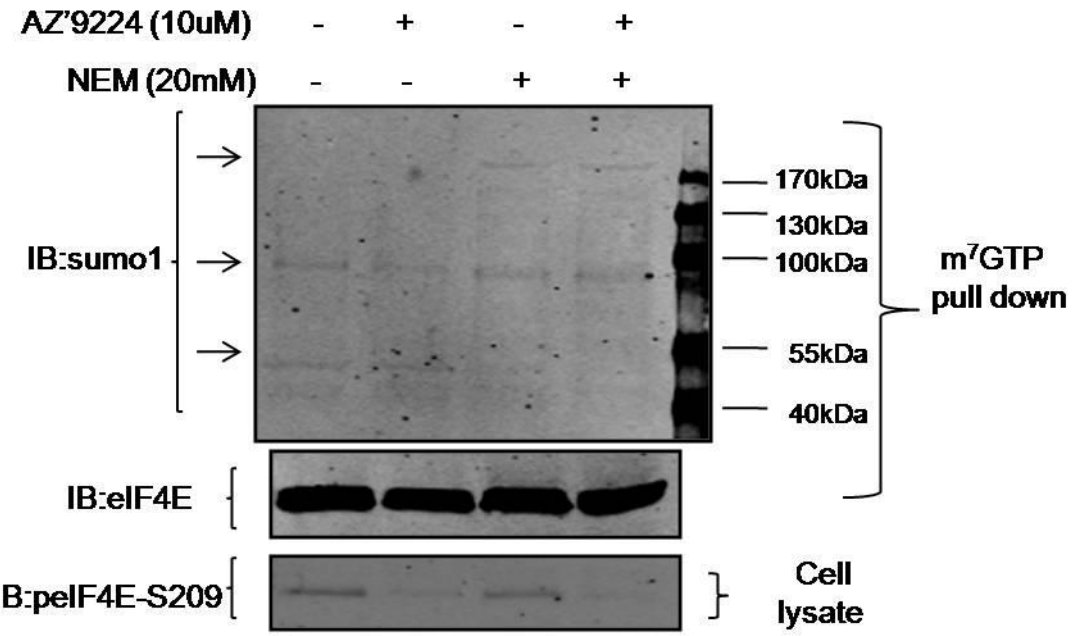


Figure 6.1. Effects of AZ'9224 on sumoylation of eIF4E. HeLa cells were cultured in regular complete medium and then treated with AZ'9224 for 1 h.

After harvesting, the cells were lysed in the presence or absence of 20 mM NEM (the isopeptidase inhibitor). Cell lysates were subjected to m^7GTP pull down assay and the bound material was analysed by western blot using anti-SUMO-1 or anti-eIF4E. A 10% amount of input lysates was resolved by SDS-PAGE and immunoblotted with anti-p-eIF4E-S209. Arrows indicate possible signals for SUMO-1-eIF4E.

6.2.2 Studies on the role of Mnk/eIF4E on the synthesis of TNFα.

Buxade *et al.* (2005) already demonstrated a role for the Mnks in the control of TNFα biosynthesis in T cells. Indeed, TNFα is primarily produced by activated macrophages. Here, I was interested in investigating the regulation of TNFα in mouse leukaemic monocyte macrophage cell line, RAW cells.

LPS (lipopolysaccharide) induces TNFα biosynthesis in murine macrophages (Kotlyarov *et al.*, 1999, Dumitru *et al.*, 2000). Thus, RAW cells were treated with various kinase inhibitors prior to LPS stimulation. As shown in Fig. 6.2, the potent inhibitor of p38 MAPK, SB203580 did not significantly inhibit the phosphorylation of p38 MAPK, as it inhibits its catalytic activity (Kumar *et al.*, 1999). However, SB203580 caused a reduction in eIF4E phosphorylation, suggesting that it worked (Fig. 6.2). The LPS-stimulated expression of TNFα obtained from whole RAW cells lysate was substantially diminished by treatment with PD098059 (an inhibitor of MEK1) but remained unchanged by either SB203580 or AZ'9224 (Fig. 6.2). These results show that TNFα synthesis depends much more on MEK/Erk than p38 MAPK signalling in RAW cells.

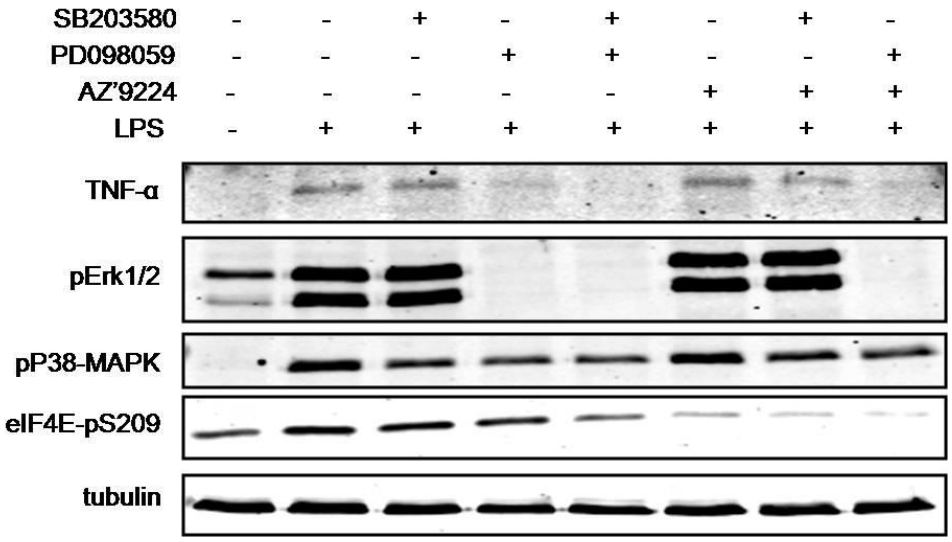


Figure 6.2. Effects of MAPK pathway on the synthesis of TNFα in RAW cells. RAW cells were treated as indicated for 1 h with 10 μM SB203580, 10 μM PD098059 or 1 μM AZ'9224 prior to LPS stimulation. After harvesting, the lysates were subjected to western blotting using the indicated antibodies.

6.2.3 Studies on the role of eIF4E phosphorylation on the binding with specific mRNAs using RIP assay.

Based on the results in (Furic *et al.*, 2010*)*, it would be interesting to examine whether eIF4E phosphorylation plays a role in the binding of specific mRNAs to eIF4E. The method RIP (RNA-protein interactions) assay, which was inspired by the chromatin immunoprecipitaion (ChIP) approach, made it possible to study the interaction of proteins such as eIF4E with specific messages (Niranjanakumari *et al.*, 2002). The protocol for this method has been described in *Materials and Methods section 2.4.6.* Briefly, live cells were crosslinked with formaldehyde and then quenched by glycine. The fixed cells were sonicated and a specific antibody against the target protein was used to immunoprecipitate crosslinked complexes. Following stringent washes, the crosslinks were reversed and isolated RNA was analysed by RT-PCR using specific primers (Niranjanakumari et al., 2002).

Based on the list of mRNAs which were more actively translated in WT compared to KI MEFs (in which eIF4E Ser209 was mutated to Ala; Furic et al., 2010), 6 candidates were chosen for RIP analysis, including *Birc2, Ccl7, Mmp3, Vegfc, Rbmc, Mcl1*. The functions of the proteins encoded by these messages are described in Table 6.1. HeLa cells were transfected with HA-eIF4E, HA-eIF4E-Ser209A or the empty vector (EV). After 2 days, cells were harvested and crosslinked as described in *Methods and Materials.* The supernatant (unbound fraction after immunoprecipitation) and the pellet (immunoprecipitated fraction) samples were first analysed using β-actin primers (Fig. 6.3). β-actin was amplified in all the supernatant samples (EV, HA-eIF4E and HA-eIF4E-S209A). With respect to the pellet samples, unfortunately, a small amount of PCR products was still detected for the EV sample. This may be due to the nonspecific binding during the immunoprecipitation and/or contamination with other samples. Interestingly, β-actin mRNA binds to both HA-eIF4E and HA-eIF4E - Ser209A (Fig. 6.3).

Table 6.1. Candidates of RIP assay analysis and the brief description of their functions.

Gene symbol	Description of Functions (provided by NCBI database)
Birc2	baculoviral IAP repeat containing 2 The protein encoded by this gene is a member of a family of proteins that inhibits apoptosis by binding to tumour necrosis factor receptor-associated factors TRaf1 and TRaf2.
Ccl7	chemokine (C-C motif) ligand 7 This gene encodes monocyte chemotactic protein 3, a secreted chemokine which attracts macrophages during inflammation and metastasis.
Mmp3	matrix metallopeptidase 3 Proteins of the matrix metalloproteinase (MMP) family are involved in the breakdown of extracellular matrix in normal physiological processes.
Vegfc	vascular endothelial growth factor C The protein encoded by this gene is a member of the platelet-derived growth factor/vascular endothelial growth factor (PDGF/VEGF) family.
Rbmx	RNA binding motif protein This gene, an active X chromosome homolog of the Y chromosome RBMY gene, is widely expressed whereas the RBMY gene evolved a male-specific function in spermatogenesis.
Mcl1	myeloid cell leukemia sequence 1 This gene encodes an anti-apoptotic protein, which is a member of the Bcl-2 family. It has been identified as a target of eIF4E. In human lymphoma, enhanced translation of Mcl1 likely contributes to eIF4E-mediated oncogenesis (Wendel *et al.*, 2007).

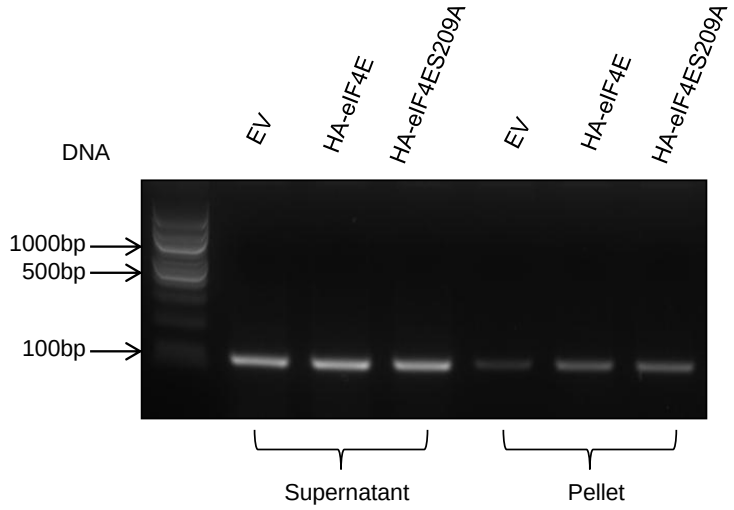


Figure 6.3. RIP results for β-actin mRNA.

Serum-replete HeLa cells transfected with HA-eIF4E or HA-eIF4E-S209A are crosslinked, immunoprecipitated and analysed by RT-PCR using β-actin primers (length of amplification product, ~100bp).

The interaction of eIF4E with other mRNAs was then examined using RIP assay (Fig. 6.4). No PCR products were visible for Mmp3 either in the supernatant or the pellet samples (Fig. 6.4, Mmp3). According to studies from (2008), *MMP3* mRNA expression was deficient in HeLa cells. Although the amplification of *Mcl1, Vegfc, Rbmx, Birc2, Ccl7* is very strong in the supernatant samples, only very weak signals were detected in the pellet samples (Fig. 6.4; no signal detectable for *Ccl7)*. Nevertheless, both HA-eIF4E and HA-eIF4E-S209A are able to interact with these mRNAs, although the detectable signal is very weak.

There may be several reasons accounting for the low level of PCR products in the pellet sample. First, the efficiency of transfection may be too low. However, both the vectors gave excellent expression in HeLa cells (Fig. 6.5).

Secondly, the efficiency of immunoprecipitation may be too low, i.e. a large of amount of over-expressed HA-eIF4E/eIF4E-S209A remained in the supernatant rather than pulled down into the pellets. To assess this, the supernatant and the pellet samples from the RIP assay were subjected to HA immunoprecipitation and western blot (Fig. 6.6). Although only a small proportion of supernatant was loaded onto SDS-PAGE for analysis, a significant amount of exogenous eIF4E was detected. Therefore, incomplete immunoprecipitation probably contributes to the low amplification of the PCR products in the pellets.

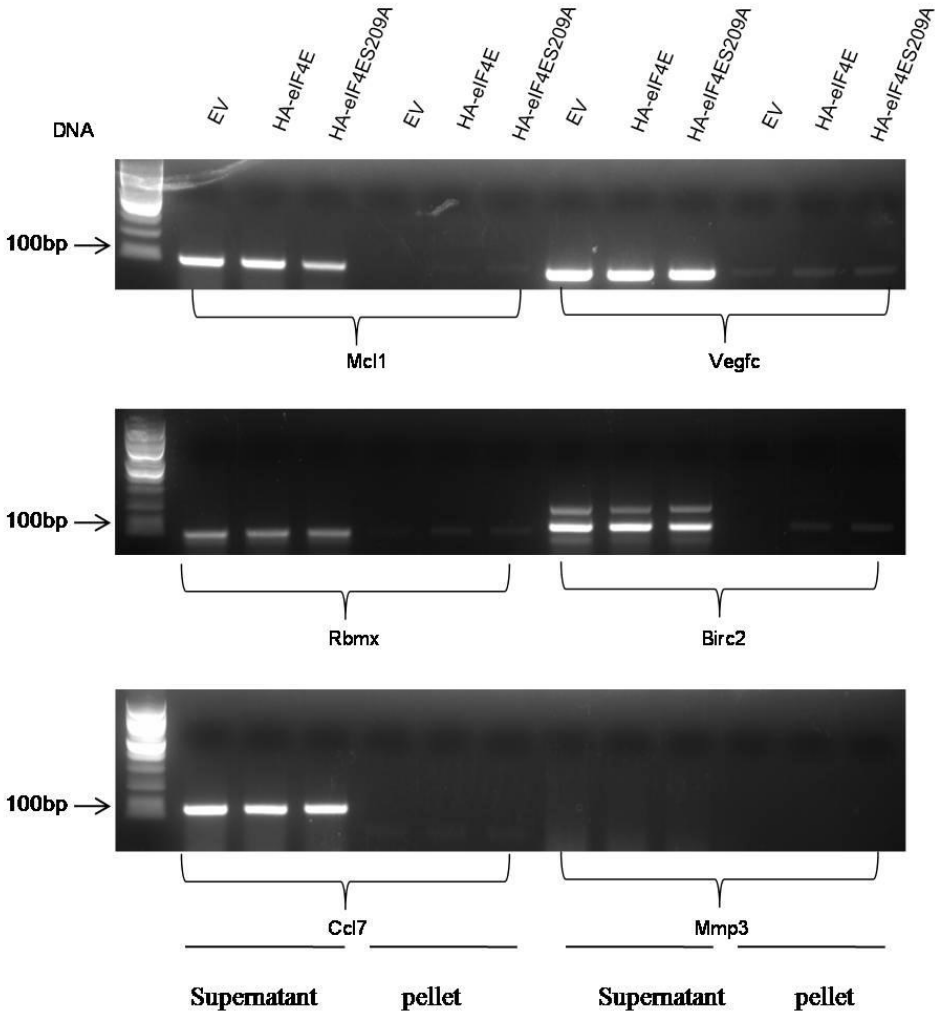


Figure 6.4. RIP results of Mcl1, Vegfc, Rbmx, Birc2, Ccl7.

Serum-replete HeLa cells transfected with HA-eIF4E or HA-eIF4E-S209A are crosslinked, immunoprecipitated and analysed by RT-PCR using specific primers. Primer details are described in *Materials and Methods section 2.4.6.*

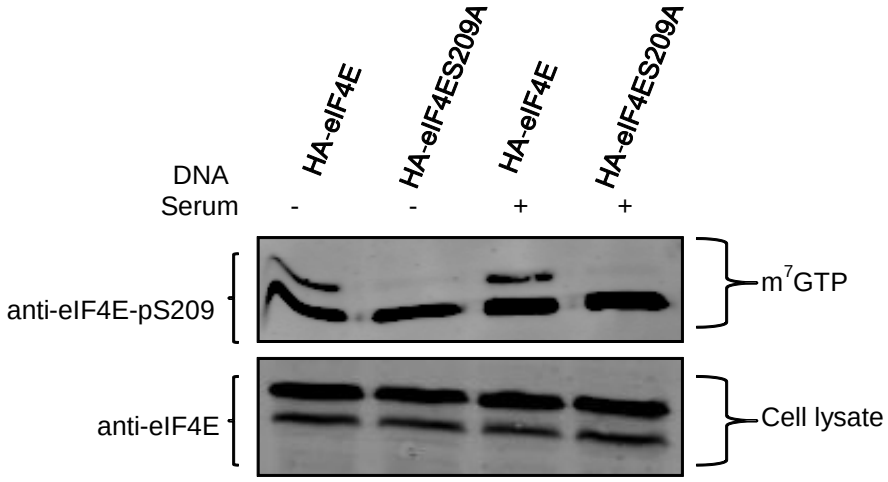


Figure 6.5. The expression of HA-eIF4E and HA-eIF4E-S209A in HeLa cells. After transfected with the indicated DNAs, HeLa cells were starved of serum overnight and then stimulated with fresh serum (10%) for 30 min. The phosphorylation of eIF4E in the bound material was tested using anti-eIF4E-pS209 antibody. The level of exogenous and endogenous total eIF4E in whole cell lysate was examined using anti-eIF4E.

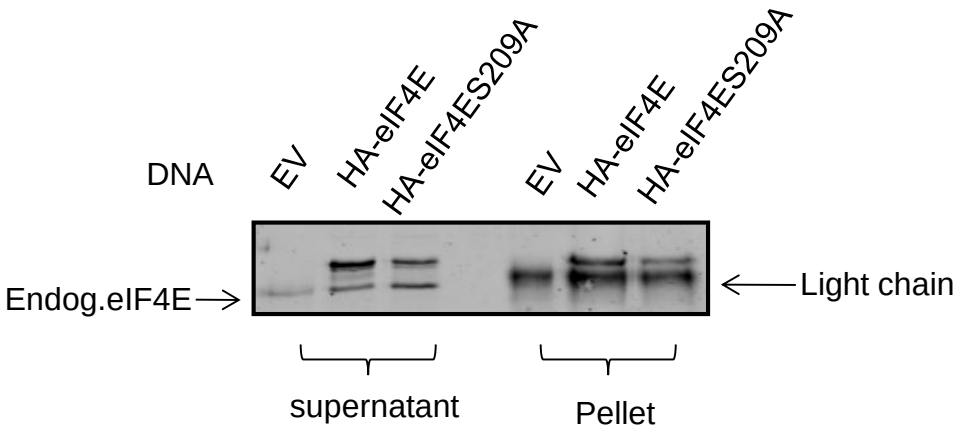


Figure 6.6. Efficiency of HA immunoprecipitation.

HeLa cells transfected with the indicated vectors were lysed and the lysates were subjected to immunoprecipitation using anti-HA; one tenth of supernatant samples were analysed by western blotting. The positions of endogenous eIF4E and the light chain of the anti-HA antibody are indicated. EV: empty vector.

6.3 Discussion

Despite the obligatory role of eIF4E in cap-dependent translation, it remains unclear how its activity is controlled. Although Xu *et al.* suggested the importance of eIF4E sumoylation for its oncogenic activity (Xu *et al.*, 2010), I only detected a tiny proportion of eIF4E which might be modified by SUMO-1 (even when exogenous eIF4E was introduced). Moreover, the Mnk inhibitor AZ'9224 did not have an effect on these possible sumoylated eIF4E. My data do not support findings of Xu *et al.* (2010), suggesting that the interplay between eIF4E phosphorylation and sumoylation needs to be further investigated.

I also studied the role of the Mnks in TNFα synthesis in RAW cells using the Mnk inhibitor AZ'9224. Previous work has been done to define how p38 MAP kinase controls TNFα production in macrophages (Salituro *et al.*, 1999). Mnks have been identified as novel components in the control of TNFα biosynthesis in T cells (Buxade *et al.*, 2005). However, TNFα expression in RAW cells was not affected by treatment with the Mnk inhibitor AZ'9224 even when eIF4E phosphorylation was inhibited. It may be that, unlike in T-cells, Mnks play a less important role in TNFα synthesis in RAW cells due to the reduced dependency on p38 MAPK.

eIF4E phosphorylation has also been implicated in promoting tumourigenesis, possibly through its control on the translation efficiency of a subset of mRNAs encoding protumourigenic factors (Furic *et al.*, 2010, Wendel *et al.*, 2007). However, the mechanisms that render the translation of these mRNAs more sensitive to eIF4E phosphorylation are still undefined. By examining the interaction of different forms of eIF4E with a subset of mRNAs using RIP assays, both wild-type HA-eIF4E and HA-eIF4E-S209A were found to bind to these mRNAs. It should be noted that since the PCR performed in the RIP assay is not quantitative, any differences between the association of eIF4E with HA-eIF4E and that with HA-eIF4E-S209A could not be assessed. Nevertheless, the RIP assay can be a more powerful tool to identify RNA-protein interactions *in vivo*, for example, with the help of real-time quantitative RT-PCR.

CHAPTER 7

FINAL DISCUSSION

Distinct effects of rapamycin and mTOR-KIs on protein turnover

The success of rapamycin and rapalogs on transplantation and some cancer therapies (e.g. kidney) has suggested that mTOR is an exciting target in the treatment of cancer and other diseases (Yuan et al., 2009). However, due to the mode of action of rapamycin and rapalogs (since they do not block the kinase activity of mTOR), they have relatively limited clinical utility, leading to the development of mTOR-KIs, which directly target the mTOR catalytic site. My study gives a detailed comparison of the effects of rapamycin and the newly-developed mTOR-KIs AZD8055 and PP242 (Chresta et al., 2010, Feldman et al., 2009) on the mTOR signalling pathway, protein synthesis and degradation.

Compared with rapamycin, mTOR-KIs exerted significantly distinct effects on both mTORC1 and mTORC2. In HeLa cells, mTOR-KIs inhibited the phosphorylation of 4E-BP1 at multiple sites which are rather insensitive to rapamycin. With respect to the readouts of mTORC2 activity, mTOR-KIs substantially decreased the phosphorylation of AKT at Ser473 whereas rapamycin actually promoted this event. The hyperactivation of Akt caused by rapamycin is a reflection of the relief of the negative feedback loop from S6K to IRS1 (Harrington et al., 2004, Um et al., 2004) as well as its lack of effect on mTORC2. Rapamycin and mTOR-KIs also displayed different effects on the critical step of cap-dependent translation initiation, the formation of eIF4F. Treating HeLa cells with either rapamycin or mTOR-KIs increased the association of eIF4E and 4E-BP1, although mTOR-KIs did this to a much greater extent. However, the binding of eIF4G (and eIF4A) to eIF4E was modestly enhanced by rapamycin when it was largely abolished by mTOR-KIs. Consistent with this, treatment with rapamycin for 2h or 6h has much less impact on the rate of [^{35}S]methionine incorporation than mTOR-KIs. The ability of rapamycin in causing superactivated Akt and increased eIF4G binding provides useful insights into the limited application of rapamycin and rapalogs in anti-cancer therapy.

To have a better understanding of effects of mTOR inhibition caused by these inhibitors, a novel approach called pSILAC was applied to study this on a proteomic scale. Grolleau and colleagues (2002) have combined microarray and [^{35}S]methionine labelling to study specific translational control by rapamycin in T cells. Their results have uncovered that the polysome-associated RNA levels of most of the expressed genes are partially reduced following rapamycin treatment, including a large number of ribosomal proteins and elongation factors. Their findings provided a comprehensive

understanding of rapamycin's molecular mechanisms in T cells. However, the approaches employed by them have some disadvantages. For example, microarray only examines behaviours of mRNAs, and thus is not able to reflect the actual levels of individual proteins. pSILAC is a simple but powerful approach as it detects actual rates of synthesis of individual proteins. Our pSILAC data revealed novel features of expression of mRNAs especially those containing a pyrimidine tract at their 5'-end. The expression of these mRNAs, which are termed as 5'-TOPs, was more sensitive to mTOR inhibition caused by mTOR-KIs than by rapamycin. Polysomal association of these mRNAs under such conditions confirmed the increased sensitivity of their translation to control by mTOR-KIs (the total transcript levels encoding these proteins did not change as measured by Dr Valentina Iadevaia). Moreover, this feature was shared by a subset of mRNAs which were found to contain a tract of pyrimidines at their 5'-UTR after searching the DBTSS database (http://dbtss.hgc.jp), although the status of these mRNAs as possible 5'-TOPs is yet to be determined.

The bigger effects of mTOR-KIs on general protein synthesis and the synthesis of 5'-TOP mRNAs might be a reflection of inhibition of mTORC2 functions. However, Torin1, another mTOR-KI, has been shown to suppress global protein synthesis through an mTORC2-independent manner (Thoreen et al., 2009). Therefore, it is possible that rapamycin-resistant functions of mTORC1 (e.g. 4E-BP1 phosphorylation) are required for the stronger inhibition of protein synthesis by mTOR-KIs. To test this hypothesis, wild type eIF4E was overexpressed in HeLa cells in order to introduce more "free eIF4E" to eliminate effects of increased levels of hypophosphorylated 4E-BP1. But eIF4E overexpression had no significant impact on inhibition of either general protein synthesis or synthesis of specific mRNAs (Dr Valentina Iadevaia's data), demonstrating that eIF4E/4E-BP1 binding is not the main event resulting in the distinct effects of rapamycin and mTOR-KIs. Hence, it seems that the greater effects of mTOR-KIs are reflections of additional unidentified rapamycin-resistant functions of mTORC1 and/or a novel complex rather than the canonical mTORC1 and mTORC2 (mTORC3? Fig. 7.1).

Interestingly, it was found that the stability of ribosomal proteins was increased upon mTOR inhibition, even though their synthesis was substantially impaired. This finding is in line with the recent results of Schwanhausser *et al.* (2011) who also employed pSILAC approach to study gene expression control in mouse fibroblasts. They showed that under energy constraints, abundant proteins, such as ribosomal proteins

were significantly more stable than less abundant ones. Moreover, the length of these abundant proteins is significantly shorter, which makes sense since protein half-lives and lengths are tightly related to theoretical energy consumption (Schwanhausser et al., 2011).

Physiological role of eIF4E phosphorylation

eIF4E phosphorylation has been established as a key event in tumorigenesis, thus raising the possibility that chemical compounds which prevent eIF4E phosphorylation (for example, Mnk inhibitors) could be used in cancer therapy (Furic et al., 2010, Ueda et al., 2010). Unfortunately, AZ'9224 was found not to be a specific Mnk inhibitor in various mammalian cell lines.

It has been suggested that sumoylation of eIF4E is an important regulatory mechanism for cap-dependent translation and oncogenic transformation and eIF4E phosphorylation is required for its sumoylation in NIH-3T3 cells (Xu *et al.,* 2010). However, AZ'9224 failed to have an effect on eIF4E sumoylation when its phosphorylation was abolished in HeLa cells, suggesting that the interplay between these two events needs to be further clarified in a wide range of cell types. With respect to the role of Mnks on TNFα synthesis, they may be less important in RAW cells than in T cells (Buxade *et al.*, 2005). The effects of eIF4E phosphorylation on its interaction with specific mRNA were studied using RIP assay. The data are interesting since they showed that loss of eIF4E phosphorylation did not abolish its ability to bind to several transcripts.

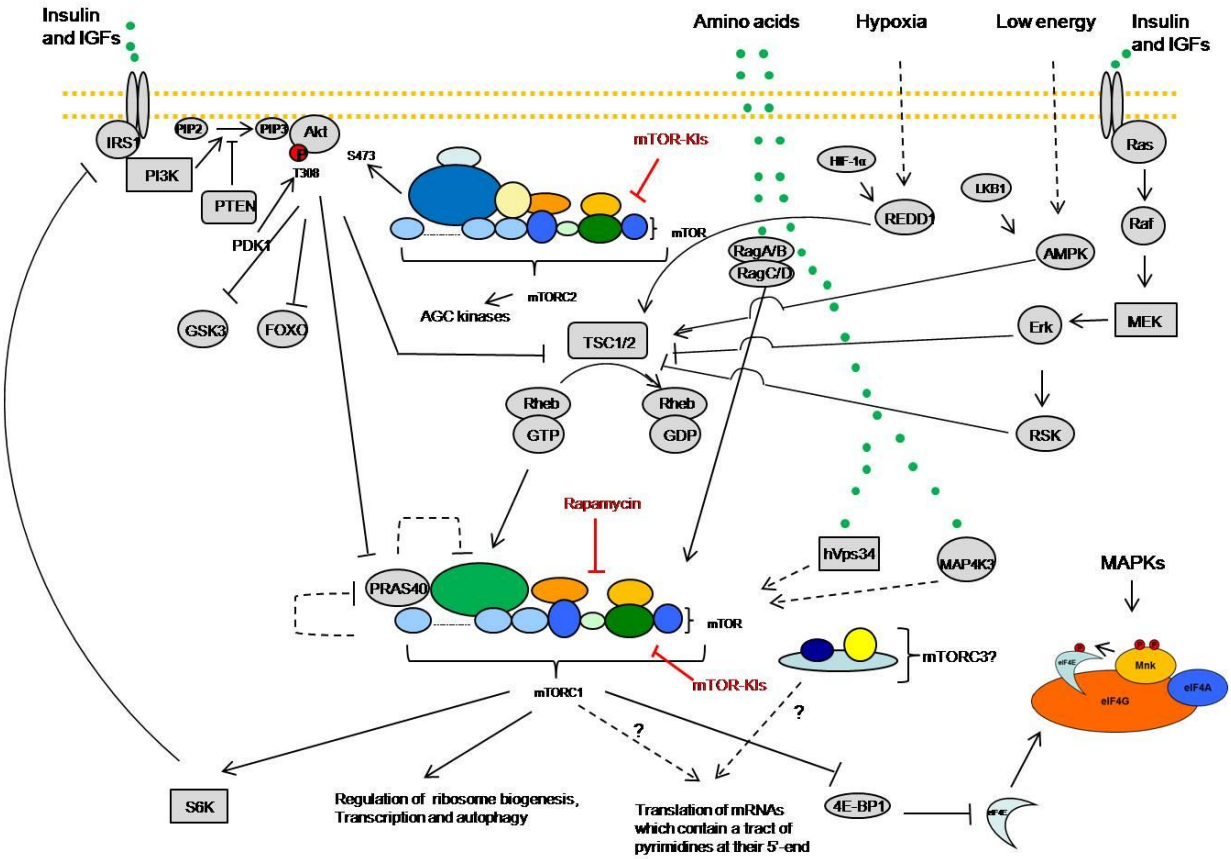


Figure 7.1. The mTOR signalling network.

Multiple signals including growth factors, nutrients, energy and stress can regulate the activity of mTORC1 through the central regulator TSC1/2. The activation of mTORC1 targets various downstream effectors which are involved in the control of ribosome biogenesis, transcription, translation and autophagy. mTORC2 is involved in the phosphorylation of HM site of many AGC kinases (for example, Ser473 in Akt). Besides regulated by 4E-BP1, eIF4E is also phosphorylated by Mnks which are recruited to eIF4G. A more detailed summary of the mTOR signalling network is given below.

As shown in Fig. 7.1, two complexes, mTORC1 and mTORC2, exist in mammals and the regulation and functions of mTORC1 are much better understood than those of mTORC2. mTORC1 regulates ribosome biogenesis, transcription, mRNA translation and autophagy by integrating signals that are generated by insulin and IGFs, amino acids, energy levels and various stressors such as hypoxia. TSC1/2 is the point where signal integration occurs. As a tumour suppressor, TSC1/2 negatively regulates mTORC1 through its GAP (GTPase activating protein) activity towards Rheb, converting Rheb to its inactive, GDP-bound form. Multiple upstream signalling inputs from PI3K–Akt, Ras–Erk–RSK, LKB1–AMPK, REDD1 and Rag proteins either positively or negatively regulate mTORC1 signalling when stimulated by extracellular signals. Several kinases, including AKT, Erk and RSK phosphorylate TSC2 and result in the inhibition of the GAP activity of TSC2. In contrast, AMPK-mediated phosphorylation positively regulates the GAP activity of TSC2 (where REDD1 was thought to sequester 14-3-3 proteins away from TSC2). Moreover, some of these kinases modulate mTORC1 independently of TSC1/2, such as Akt-mediated phosphorylation of the mTORC1 inhibitory factor, PRAS40. In addition, Rag proteins mediate translocation of mTORC1 to lysosomal membranes through the help of a trimeric complex, Ragulator, in response to amino acid sufficiency (not depicted in this figure). The activation of mTORC1 serves to control various downstream targets such as S6K and 4E-BP1. 4E-BP1 competes with eIF4G to bind to eIF4E, leading to inhibition of eIF4F formation. eIF4E activity is also regulated by phosphorylation by Mnks. S6K exerts a negative feedback loop towards IRS1, downregulating the activity of components of the insulin/PI3k/Akt pathway. mTORC2 has been demonstrated roles in the regulation of the actin cytoskeleton and the phosphorylation of some AGC kinases, including Akt, PKC and SGK. The mode of action of mTOR-KIs is distinguished from that of rapamycin, i.e. they directly target the kinase domain of mTOR and thus block both mTORC1 and mTOC2 activities. MAPK signalling promotes phosphorylation of eIF4E through Mnk1/2, which occurs when Mnks are recruited to eIF4G.

The future directions of mTOR-KIs in cancer therapy

mTOR controls many aspects of cellular physiology by coordinating an adequate response to changes in growth factors, nutrients and environmental stresses (a

summary of the mTOR signalling network is presented in Fig. 7.1). Since it is frequently dysregulated in many diseases such as cancer and metabolic disorders, mTOR has been considered an exciting target in cancer therapy. My studies showed that mTOR KIs, AZD8055 and PP242, had stronger effects on mTOR complexes, mRNA translation and protein degradation compared with rapamycin. In addition, data from Sini *et al.* (2010) showed that AZD8055 also had greater impacts on the induction of autophagy and cell death than rapamycin did in some cell lines. PP242 has also been demonstrated to have a marked therapeutic response in rapamycin-resistant tumours (Hsieh et al., 2010). As such, mTOR-KIs, which inhibit both mTOR complexes, may offer therapeutic advantages to rapamycin and its analogs. In the recent years, the field of the development of mTOR-KIs has matured with several compounds currently in the early stages of clinical trials, including AZD8055 (Chresta et al., 2010), OSI-027 (Bhagwat et al., 2011) and CCI-223 (Zask et al., 2011).

It is note worthy that, besides the mTOR pathway, oncogenic activation can also induce other signalling pathways such as MAPKs. Therefore, combined treatments which target multiple pathways may maximize the inhibition of the oncogenic activity and tumorigenesis.

REFERENCES

ALESSI, D. R., ANDJELKOVIC, M., CAUDWELL, B., CRON, P., MORRICE, N., COHEN, P. & HEMMINGS, B. A. 1996. Mechanism of activation of protein kinase B by insulin and IGF-1. EMBO J, 15, 6541-51.

ALESSI, D. R., DEAK, M., CASAMAYOR, A., CAUDWELL, F. B., MORRICE, N., NORMAN, D. G., GAFFNEY, P., REESE, C. B., MACDOUGALL, C. N., HARBISON, D., ASHWORTH, A. & BOWNES, M. 1997a. 3-Phosphoinositide-dependent protein kinase-1 (PDK1): structural and functional homology with the Drosophila DSTPK61 kinase. Curr Biol, 7, 776-89.

ALESSI, D. R., JAMES, S. R., DOWNES, C. P., HOLMES, A. B., GAFFNEY, P. R., REESE, C. B. & COHEN, P. 1997b. Characterization of a 3-phosphoinositide-dependent protein kinase which phosphorylates and activates protein kinase Balpha. Curr Biol, 7, 261-9.

AMRANI, N., MINET, M., LE GOUAR, M., LACROUTE, F. & WYERS, F. 1997. Yeast Pab1 interacts with Rna15 and participates in the control of the poly(A) tail length in vitro. Mol Cell Biol, 17, 3694-701.

ANDJELKOVIC, M., ALESSI, D. R., MEIER, R., FERNANDEZ, A., LAMB, N. J., FRECH, M., CRON, P., COHEN, P., LUCOCQ, J. M. & HEMMINGS, B. A. 1997. Role of translocation in the activation and function of protein kinase B. J Biol Chem, 272, 31515-24.

ANDRADE, M. A. & BORK, P. 1995. HEAT repeats in the Huntington's disease protein. Nat Genet, 11, 115-6.

ARSHAM, A. M., HOWELL, J. J. & SIMON, M. C. 2003. A novel hypoxia-inducible factor-independent hypoxic response regulating mammalian target of rapamycin and its targets. J Biol Chem, 278, 29655-60.

AVEROUS, J., FONSECA, B. D. & PROUD, C. G. 2008. Regulation of cyclin D1 expression by mTORC1 signaling requires eukaryotic initiation factor 4E-binding protein 1. Oncogene, 27, 1106-13.

AVNI, D., BIBERMAN, Y. & MEYUHAS, O. 1997. The 5' terminal oligopyrimidine tract confers translational control on TOP mRNAs in a cell type- and sequence context-dependent manner. Nucleic Acids Res, 25, 995-1001.

BAIN, J., PLATER, L., ELLIOTT, M., SHPIRO, N., HASTIE, C. J., MCLAUCHLAN, H., KLEVERNIC, I., ARTHUR, J. S., ALESSI, D. R. & COHEN, P. 2007. The selectivity of protein kinase inhibitors: a further update. Biochem J, 408, 297-315.

BALLESTER, A., VELASCO, A., TOBENA, R. & ALEMANY, S. 1998. Cot kinase activates tumor necrosis factor-alpha gene expression in a cyclosporin A-resistant manner. J Biol Chem, 273, 14099-106.

BALLIF, B. A., ROUX, P. P., GERBER, S. A., MACKEIGAN, J. P., BLENIS, J. & GYGI, S. P. 2005. Quantitative phosphorylation profiling of the ERK/p90 ribosomal S6 kinase-signaling cassette and its targets, the tuberous sclerosis tumor suppressors. Proc Natl Acad Sci U S A, 102, 667-72.

BANDI, H. R., FERRARI, S., KRIEG, J., MEYER, H. E. & THOMAS, G. 1993. Identification of 40 S ribosomal protein S6 phosphorylation sites in Swiss mouse 3T3 fibroblasts stimulated with serum. J Biol Chem, 268, 4530-3.

BARTH-BAUS, D., STRATTON, C. A., PARROTT, L., MYERSON, H., MEYUHAS, O., TEMPLETON, D. J., LANDRETH, G. E. & HENSOLD, J. O. 2002. S6 phosphorylation-independent pathways regulate translation of 5'-terminal oligopyrimidine tract-containing mRNAs in differentiating hematopoietic cells. Nucleic Acids Res, 30, 1919-28.

BASERGA, R. 2005. The insulin-like growth factor-I receptor as a target for cancer therapy. Expert Opin Ther Targets, 9, 753-68.

BAYASCAS, J. R. 2008. Dissecting the role of the 3-phosphoinositide-dependent protein kinase-1 (PDK1) signalling pathways. Cell Cycle, 7, 2978-82.

BAYASCAS, J. R. & ALESSI, D. R. 2005. Regulation of Akt/PKB Ser473 phosphorylation. Mol Cell, 18, 143-5.

BAYASCAS, J. R., WULLSCHLEGER, S., SAKAMOTO, K., GARCIA-MARTINEZ, J. M., CLACHER, C., KOMANDER, D., VAN AALTEN, D. M., BOINI, K. M., LANG, F., LIPINA, C., LOGIE, L., SUTHERLAND, C., CHUDEK, J. A., VAN DIEPEN, J. A., VOSHOL, P. J., LUCOCQ, J. M. & ALESSI, D. R. 2008. Mutation of the PDK1 PH domain inhibits protein kinase B/Akt, leading to small size and insulin resistance. Mol Cell Biol, 28, 3258-72.

BERNAL, A. & KIMBRELL, D. A. 2000. Drosophila Thor participates in host immune defense and connects a translational regulator with innate immunity. Proc Natl Acad Sci U S A, 97, 6019-24.

BEUGNET, A., WANG, X. & PROUD, C. G. 2003. Target of rapamycin (TOR)-signaling and RAIP motifs play distinct roles in the mammalian TOR-dependent phosphorylation of initiation factor 4E-binding protein 1. J Biol Chem, 278, 40717-22.

BHAGWAT, S. V. & CREW, A. P. 2010. Novel inhibitors of mTORC1 and mTORC2. Curr Opin Investig Drugs, 11, 638-45.

BHAGWAT, S. V., GOKHALE, P. C., CREW, A. P., COOKE, A., YAO, Y., MANTIS, C., KAHLER, J., WORKMAN, J., BITTNER, M., DUDKIN, L., EPSTEIN, D. M., GIBSON, N. W., WILD, R., ARNOLD, L. D., HOUGHTON, P. J. & PACHTER, J. A. 2011. Preclinical characterization of OSI-027, a potent and selective inhibitor of mTORC1 and mTORC2: distinct from rapamycin. Mol Cancer Ther, 10, 1394-406.

BIANCHINI, A., LOIARRO, M., BIELLI, P., BUSA, R., PARONETTO, M. P., LORENI, F., GEREMIA, R. & SETTE, C. 2008. Phosphorylation of eIF4E by MNKs supports protein synthesis, cell cycle progression and proliferation in prostate cancer cells. Carcinogenesis, 29, 2279-88.

BIONDI, R. M. 2004. Phosphoinositide-dependent protein kinase 1, a sensor of protein conformation. Trends Biochem Sci, 29, 136-42.

BIONDI, R. M., KIELOCH, A., CURRIE, R. A., DEAK, M. & ALESSI, D. R. 2001. The PIF-binding pocket in PDK1 is essential for activation of S6K and SGK, but not PKB. EMBO J, 20, 4380-90.

BODDY, M. N., HOWE, K., ETKIN, L. D., SOLOMON, E. & FREEMONT, P. S. 1996. PIC 1, a novel ubiquitin-like protein which interacts with the PML component of a multiprotein complex that is disrupted in acute promyelocytic leukaemia. Oncogene, 13, 971-82.

BOLSTER, D. R., CROZIER, S. J., KIMBALL, S. R. & JEFFERSON, L. S. 2002. AMP-activated protein kinase suppresses protein synthesis in rat skeletal muscle through down-regulated mammalian target of rapamycin (mTOR) signaling. J Biol Chem, 277, 23977-80.

BORNANCIN, F. & PARKER, P. J. 1996. Phosphorylation of threonine 638 critically controls the dephosphorylation and inactivation of protein kinase Calpha. Curr Biol, 6, 1114-23.

BRODBECK, D., CRON, P. & HEMMINGS, B. A. 1999. A human protein kinase Bgamma with regulatory phosphorylation sites in the activation loop and in the C-terminal hydrophobic domain. J Biol Chem, 274, 9133-6.

BROWN, E. J., ALBERS, M. W., SHIN, T. B., ICHIKAWA, K., KEITH, C. T., LANE, W. S. & SCHREIBER, S. L. 1994. A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature, 369, 756-8.

BROWNE, G. J. & PROUD, C. G. 2002. Regulation of peptide-chain elongation in mammalian cells. Eur J Biochem, 269, 5360-8.

BRUGAROLAS, J., LEI, K., HURLEY, R. L., MANNING, B. D., REILING, J. H., HAFEN, E., WITTERS, L. A., ELLISEN, L. W. & KAELIN, W. G., JR. 2004. Regulation of mTOR function in response to hypoxia by REDD1 and the TSC1/TSC2 tumor suppressor complex. Genes Dev, 18, 2893-904.

BUNDSCHU, K., WALTER, U. & SCHUH, K. 2006. The VASP-Spred-Sprouty domain puzzle. J Biol Chem, 281, 36477-81.

BURNETT, P. E., BARROW, R. K., COHEN, N. A., SNYDER, S. H. & SABATINI, D. M. 1998. RAFT1 phosphorylation of the translational regulators p70 S6 kinase and 4E-BP1. Proc Natl Acad Sci U S A, 95, 1432-7.

BUSHELL, M., STONELEY, M., KONG, Y. W., HAMILTON, T. L., SPRIGGS, K. A., DOBBYN, H. C., QIN, X., SARNOW, P. & WILLIS, A. E. 2006. Polypyrimidine tract binding protein regulates IRES-mediated gene expression during apoptosis. Mol Cell, 23, 401-12.

BUSHELL, M., WOOD, W., CARPENTER, G., PAIN, V. M., MORLEY, S. J. & CLEMENS, M. J. 2001. Disruption of the interaction of mammalian protein synthesis eukaryotic initiation factor 4B with the poly(A)-binding protein by caspase- and viral protease-mediated cleavages. J Biol Chem, 276, 23922-8.

BUXADE, M., MORRICE, N., KREBS, D. L. & PROUD, C. G. 2008. The PSF.p54nrb complex is a novel Mnk substrate that binds the mRNA for tumor necrosis factor alpha. J Biol Chem, 283, 57-65.

BUXADE, M., PARRA, J. L., ROUSSEAU, S., SHPIRO, N., MARQUEZ, R., MORRICE, N., BAIN, J., ESPEL, E. & PROUD, C. G. 2005. The Mnks are novel components in the control of TNF alpha biosynthesis and phosphorylate and regulate hnRNP A1. Immunity, 23, 177-89.

BUXADE, M., RAMIREZ-ALVARADO, M., FERNANDEZ-TROY, N., MACKENZIE, S., CASAROLI-MARANO, R. P., VILELLA, R. & ESPEL, E. 2001. Integrating signals from T-cell receptor and serum by T cells enhance translation of tumour necrosis factor-alpha. Immunology, 102, 416-25.

CAI, S. L., TEE, A. R., SHORT, J. D., BERGERON, J. M., KIM, J., SHEN, J., GUO, R., JOHNSON, C. L., KIGUCHI, K. & WALKER, C. L. 2006. Activity of TSC2 is inhibited by AKT-mediated phosphorylation and membrane partitioning. J Cell Biol, 173, 279-89.

CAM, H., EASTON, J. B., HIGH, A. & HOUGHTON, P. J. 2010. mTORC1 signaling under hypoxic conditions is controlled by ATM-dependent phosphorylation of HIF-1alpha. Mol Cell, 40, 509-20.

CAMACHO-VANEGAS, O., WEIGHARDT, F., GHIGNA, C., AMALDI, F., RIVA, S. & BIAMONTI, G. 1997. Growth-dependent and growth-independent translation of messengers for heterogeneous nuclear ribonucleoproteins. Nucleic Acids Res, 25, 3950-4.

CAMPBELL, L. E., WANG, X. & PROUD, C. G. 1999. Nutrients differentially regulate multiple translation factors and their control by insulin. Biochem J, 344 Pt 2, 433-41.

CARON, S., CHARON, M., CRAMER, E., SONENBERG, N. & DUSANTER-FOURT, I. 2004. Selective modification of eukaryotic initiation factor 4F (eIF4F) at the onset of cell differentiation: recruitment of eIF4GII and long-lasting phosphorylation of eIF4E. Mol Cell Biol, 24, 4920-8.

CARRACEDO, A., MA, L., TERUYA-FELDSTEIN, J., ROJO, F., SALMENA, L., ALIMONTI, A., EGIA, A., SASAKI, A. T., THOMAS, G., KOZMA, S. C., PAPA, A., NARDELLA, C., CANTLEY, L. C., BASELGA, J. & PANDOLFI, P. P. 2008. Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer. J Clin Invest, 118, 3065-74.

CARRIERE, A., CARGNELLO, M., JULIEN, L. A., GAO, H., BONNEIL, E., THIBAULT, P. & ROUX, P. P. 2008. Oncogenic MAPK signaling stimulates mTORC1 activity by promoting RSK-mediated raptor phosphorylation. Curr Biol, 18, 1269-77.

CASTELLO, A., ALVAREZ, E. & CARRASCO, L. 2006. Differential cleavage of eIF4GI and eIF4GII in mammalian cells. Effects on translation. J Biol Chem, 281, 33206-16.

CASTRO, A. F., REBHUN, J. F., CLARK, G. J. & QUILLIAM, L. A. 2003. Rheb binds tuberous sclerosis complex 2 (TSC2) and promotes S6 kinase activation in a rapamycin- and farnesylation-dependent manner. J Biol Chem, 278, 32493-6.

CHEADLE, J. P., REEVE, M. P., SAMPSON, J. R. & KWIATKOWSKI, D. J. 2000. Molecular genetic advances in tuberous sclerosis. Hum Genet, 107, 97-114.

CHEN, W. S., XU, P. Z., GOTTLOB, K., CHEN, M. L., SOKOL, K., SHIYANOVA, T., RONINSON, I., WENG, W., SUZUKI, R., TOBE, K., KADOWAKI, T. & HAY, N. 2001. Growth retardation and increased apoptosis in mice with homozygous disruption of the Akt1 gene. Genes Dev, 15, 2203-8.

CHENG, J., KANG, X., ZHANG, S. & YEH, E. T. 2007. SUMO-specific protease 1 is essential for stabilization of HIF1alpha during hypoxia. Cell, 131, 584-95.

CHIU, M. I., KATZ, H. & BERLIN, V. 1994. RAPT1, a mammalian homolog of yeast Tor, interacts with the FKBP12/rapamycin complex. Proc Natl Acad Sci U S A, 91, 12574-8.

CHO, P. F., POULIN, F., CHO-PARK, Y. A., CHO-PARK, I. B., CHICOINE, J. D., LASKO, P. & SONENBERG, N. 2005. A new paradigm for translational control: inhibition via 5'-3' mRNA tethering by Bicoid and the eIF4E cognate 4EHP. Cell, 121, 411-23.

CHOI, K. M., MCMAHON, L. P. & LAWRENCE, J. C., JR. 2003. Two motifs in the translational repressor PHAS-I required for efficient phosphorylation by mammalian target of rapamycin and for recognition by raptor. J Biol Chem, 278, 19667-73.

CHONG-KOPERA, H., INOKI, K., LI, Y., ZHU, T., GARCIA-GONZALO, F. R., ROSA, J. L. & GUAN, K. L. 2006. TSC1 stabilizes TSC2 by inhibiting the interaction between TSC2 and the HERC1 ubiquitin ligase. J Biol Chem, 281, 8313-6.

CHRESTA, C. M., DAVIES, B. R., HICKSON, I., HARDING, T., COSULICH, S., CRITCHLOW, S. E., VINCENT, J. P., ELLSTON, R., JONES, D., SINI, P., JAMES, D., HOWARD, Z., DUDLEY, P., HUGHES, G., SMITH, L., MAGUIRE, S., HUMMERSONE, M., MALAGU, K., MENEAR, K., JENKINS, R., JACOBSEN, M., SMITH, G. C., GUICHARD, S. & PASS, M. 2010. AZD8055 is a potent, selective, and orally bioavailable ATP-competitive mammalian target of rapamycin kinase inhibitor with in vitro and in vivo antitumor activity. Cancer Res, 70, 288-98.

COCKMAN, M. E., MASSON, N., MOLE, D. R., JAAKKOLA, P., CHANG, G. W., CLIFFORD, S. C., MAHER, E. R., PUGH, C. W., RATCLIFFE, P. J. & MAXWELL, P. H. 2000. Hypoxia inducible factor-alpha binding and ubiquitylation by the von Hippel-Lindau tumor suppressor protein. J Biol Chem, 275, 25733-41.

COLLER, J. M., GRAY, N. K. & WICKENS, M. P. 1998. mRNA stabilization by poly(A) binding protein is independent of poly(A) and requires translation. Genes Dev, 12, 3226-35.

CORRADETTI, M. N. & GUAN, K. L. 2006. Upstream of the mammalian target of rapamycin: do all roads pass through mTOR? Oncogene, 25, 6347-60.

CRACKOWER, M. A., OUDIT, G. Y., KOZIERADZKI, I., SARAO, R., SUN, H., SASAKI, T., HIRSCH, E., SUZUKI, A., SHIOI, T., IRIE-SASAKI, J., SAH, R., CHENG, H. Y., RYBIN, V. O., LEMBO, G., FRATTA, L., OLIVEIRA-DOS-SANTOS, A. J., BENOVIC, J. L., KAHN, C. R., IZUMO, S., STEINBERG, S. F., WYMANN, M. P., BACKX, P. H. & PENNINGER, J. M. 2002. Regulation of myocardial contractility and cell size by distinct PI3K-PTEN signaling pathways. Cell, 110, 737-49.

CRAIG, A. W., HAGHIGHAT, A., YU, A. T. & SONENBERG, N. 1998. Interaction of polyadenylate-binding protein with the eIF4G homologue PAIP enhances translation. Nature, 392, 520-3.

CROSIO, C., BOYL, P. P., LORENI, F., PIERANDREI-AMALDI, P. & AMALDI, F. 2000. La protein has a positive effect on the translation of TOP mRNAs in vivo. Nucleic Acids Res, 28, 2927-34.

CROSS, D. A., ALESSI, D. R., COHEN, P., ANDJELKOVICH, M. & HEMMINGS, B. A. 1995. Inhibition of glycogen synthase kinase-3 by insulin mediated by protein kinase B. Nature, 378, 785-9.

CURRIE, R. A., WALKER, K. S., GRAY, A., DEAK, M., CASAMAYOR, A., DOWNES, C. P., COHEN, P., ALESSI, D. R. & LUCOCQ, J. 1999. Role of phosphatidylinositol 3,4,5-trisphosphate in regulating the activity and localization of 3-phosphoinositide-dependent protein kinase-1. Biochem J, 337 (Pt 3), 575-83.

DAMES, S. A., MULET, J. M., RATHGEB-SZABO, K., HALL, M. N. & GRZESIEK, S. 2005. The solution structure of the FATC domain of the protein kinase target of rapamycin suggests a role for redox-dependent structural and cellular stability. J Biol Chem, 280, 20558-64.

DAMGAARD, C. K. & LYKKE-ANDERSEN, J. 2011. Translational coregulation of 5'TOP mRNAs by TIA-1 and TIAR. Genes Dev, 25, 2057-68.

DAN, H. C., SUN, M., YANG, L., FELDMAN, R. I., SUI, X. M., OU, C. C., NELLIST, M., YEUNG, R. S., HALLEY, D. J., NICOSIA, S. V., PLEDGER, W. J. & CHENG, J. Q. 2002. Phosphatidylinositol 3-kinase/Akt pathway regulates tuberous sclerosis tumor suppressor complex by phosphorylation of tuberin. J Biol Chem, 277, 35364-70.

DASILVA, J., XU, L., KIM, H. J., MILLER, W. T. & BAR-SAGI, D. 2006. Regulation of sprouty stability by Mnk1-dependent phosphorylation. Mol Cell Biol, 26, 1898-907.

DATTA, S. R., DUDEK, H., TAO, X., MASTERS, S., FU, H., GOTOH, Y. & GREENBERG, M. E. 1997. Akt phosphorylation of BAD couples survival signals to the cell-intrinsic death machinery. Cell, 91, 231-41.

DATTA, S. R., KATSOV, A., HU, L., PETROS, A., FESIK, S. W., YAFFE, M. B. & GREENBERG, M. E. 2000. 14-3-3 proteins and survival kinases cooperate to inactivate BAD by BH3 domain phosphorylation. Mol Cell, 6, 41-51.

DAVIS, R. J. 1993. The mitogen-activated protein kinase signal transduction pathway. J Biol Chem, 268, 14553-6.

DEL PESO, L., GONZALEZ-GARCIA, M., PAGE, C., HERRERA, R. & NUNEZ, G. 1997. Interleukin-3-induced phosphorylation of BAD through the protein kinase Akt. Science, 278, 687-9.

DENNIS, P. B., JAESCHKE, A., SAITOH, M., FOWLER, B., KOZMA, S. C. & THOMAS, G. 2001. Mammalian TOR: a homeostatic ATP sensor. Science, 294, 1102-5.

DEYOUNG, M. P., HORAK, P., SOFER, A., SGROI, D. & ELLISEN, L. W. 2008. Hypoxia regulates TSC1/2-mTOR signaling and tumor suppression through REDD1-mediated 14-3-3 shuttling. Genes Dev, 22, 239-51.

DI BACCO, A. & GILL, G. 2006. SUMO-specific proteases and the cell cycle. An essential role for SENP5 in cell proliferation. Cell Cycle, 5, 2310-3.

DMITRIEV, S. E., TERENIN, I. M., DUNAEVSKY, Y. E., MERRICK, W. C. & SHATSKY, I. N. 2003. Assembly of 48S translation initiation complexes from purified components with mRNAs that have some base pairing within their 5' untranslated regions. Mol Cell Biol, 23, 8925-33.

DOHERTY, M. K., WHITEHEAD, C., MCCORMACK, H., GASKELL, S. J. & BEYNON, R. J. 2005. Proteome dynamics in complex organisms: using stable isotopes to monitor individual protein turnover rates. Proteomics, 5, 522-33.

DORRELLO, N. V., PESCHIAROLI, A., GUARDAVACCARO, D., COLBURN, N. H., SHERMAN, N. E. & PAGANO, M. 2006. S6K1- and betaTRCP-mediated degradation of PDCD4 promotes protein translation and cell growth. Science, 314, 467-71.

DOSTIE, J., FERRAIUOLO, M., PAUSE, A., ADAM, S. A. & SONENBERG, N. 2000. A novel shuttling protein, 4E-T, mediates the nuclear import of the mRNA 5' cap-binding protein, eIF4E. EMBO J, 19, 3142-56.

DOWLING, R. J., TOPISIROVIC, I., ALAIN, T., BIDINOSTI, M., FONSECA, B. D., PETROULAKIS, E., WANG, X., LARSSON, O., SELVARAJ, A., LIU, Y., KOZMA, S. C., THOMAS, G. & SONENBERG, N. 2010. mTORC1-mediated cell proliferation, but not cell growth, controlled by the 4E-BPs. Science, 328, 1172-6.

DUMITRU, C. D., CECI, J. D., TSATSANIS, C., KONTOYIANNIS, D., STAMATAKIS, K., LIN, J. H., PATRIOTIS, C., JENKINS, N. A., COPELAND, N. G., KOLLIAS, G. & TSICHLIS, P. N. 2000. TNF-alpha induction by LPS is regulated posttranscriptionally via a Tpl2/ERK-dependent pathway. Cell, 103, 1071-83.

EGUCHI, T., KUBOTA, S., KAWATA, K., MUKUDAI, Y., UEHARA, J., OHGAWARA, T., IBARAGI, S., SASAKI, A., KUBOKI, T. & TAKIGAWA, M. 2008. Novel transcription-factor-like function of human matrix metalloproteinase 3 regulating the CTGF/CCN2 gene. Mol Cell Biol, 28, 2391-413.

FACCHINETTI, V., OUYANG, W., WEI, H., SOTO, N., LAZORCHAK, A., GOULD, C., LOWRY, C., NEWTON, A. C., MAO, Y., MIAO, R. Q., SESSA, W. C., QIN, J., ZHANG, P., SU, B. & JACINTO, E. 2008. The mammalian target of rapamycin complex 2 controls folding and stability of Akt and protein kinase C. EMBO J, 27, 1932-43.

FADDEN, P., HAYSTEAD, T. A. & LAWRENCE, J. C., JR. 1997. Identification of phosphorylation sites in the translational regulator, PHAS-I, that are controlled by insulin and rapamycin in rat adipocytes. J Biol Chem, 272, 10240-7.

FELDMAN, M. E., APSEL, B., UOTILA, A., LOEWITH, R., KNIGHT, Z. A., RUGGERO, D. & SHOKAT, K. M. 2009. Active-site inhibitors of mTOR target rapamycin-resistant outputs of mTORC1 and mTORC2. PLoS Biol, 7, e38.

FERRARI, S., BANDI, H. R., HOFSTEENGE, J., BUSSIAN, B. M. & THOMAS, G. 1991. Mitogen-activated 70K S6 kinase. Identification of in vitro 40 S ribosomal S6 phosphorylation sites. J Biol Chem, 266, 22770-5.

FINDLAY, G. M., YAN, L., PROCTER, J., MIEULET, V. & LAMB, R. F. 2007. A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling. Biochem J, 403, 13-20.

FINGAR, D. C., RICHARDSON, C. J., TEE, A. R., CHEATHAM, L., TSOU, C. & BLENIS, J. 2004. mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/eukaryotic translation initiation factor 4E. Mol Cell Biol, 24, 200-16.

FINGAR, D. C., SALAMA, S., TSOU, C., HARLOW, E. & BLENIS, J. 2002. Mammalian cell size is controlled by mTOR and its downstream targets S6K1 and 4EBP1/eIF4E. Genes Dev, 16, 1472-87.

FLYNN, A. & PROUD, C. G. 1995. Serine 209, not serine 53, is the major site of phosphorylation in initiation factor eIF-4E in serum-treated Chinese hamster ovary cells. J Biol Chem, 270, 21684-8.

FONSECA, B. D., ALAIN, T., FINESTONE, L. K., HUANG, B. P., ROLFE, M., JIANG, T., YAO, Z., HERNANDEZ, G., BENNETT, C. F. & PROUD, C. G. 2011. Pharmacological and genetic evaluation of proposed roles of mitogen-activated protein kinase/extracellular signal-regulated kinase kinase (MEK), extracellular signal-regulated kinase (ERK), and p90(RSK) in the control of mTORC1 protein signaling by phorbol esters. J Biol Chem, 286, 27111-22.

FONSECA, B. D., LEE, V. H. & PROUD, C. G. 2008. The binding of PRAS40 to 14-3-3 proteins is not required for activation of mTORC1 signalling by phorbol esters/ERK. Biochem J, 411, 141-9.

FONSECA, B. D., SMITH, E. M., LEE, V. H., MACKINTOSH, C. & PROUD, C. G. 2007. PRAS40 is a target for mammalian target of rapamycin complex 1 and is required for signaling downstream of this complex. J Biol Chem, 282, 24514-24.

FUKUNAGA, R. & HUNTER, T. 1997. MNK1, a new MAP kinase-activated protein kinase, isolated by a novel expression screening method for identifying protein kinase substrates. EMBO J, 16, 1921-33.

FURIC, L., RONG, L., LARSSON, O., KOUMAKPAYI, I. H., YOSHIDA, K., BRUESCHKE, A., PETROULAKIS, E., ROBICHAUD, N., POLLAK, M., GABOURY, L. A., PANDOLFI, P. P., SAAD, F. & SONENBERG, N. 2010. eIF4E phosphorylation promotes tumorigenesis and is associated with prostate cancer progression. Proc Natl Acad Sci U S A, 107, 14134-9.

GACHET, Y., TOURNIER, S., LEE, M., LAZARIS-KARATZAS, A., POULTON, T. & BOMMER, U. A. 1999. The growth-related, translationally controlled protein P23 has properties of a

*tubulin binding protein and associates transiently with microtubules during the cell cycle. J Cell Sci, 112 (Pt 8)**,** 1257-71.*

*GANLEY, I. G., LAM DU, H., WANG, J., DING, X., CHEN, S. & JIANG, X. 2009. ULK1.ATG13.FIP200 complex mediates mTOR signaling and is essential for autophagy. J Biol Chem, 284**,** 12297-305.*

*GAO, X. & PAN, D. 2001. TSC1 and TSC2 tumor suppressors antagonize insulin signaling in cell growth. Genes Dev, 15**,** 1383-92.*

*GARAMI, A., ZWARTKRUIS, F. J., NOBUKUNI, T., JOAQUIN, M., ROCCIO, M., STOCKER, H., KOZMA, S. C., HAFEN, E., BOS, J. L. & THOMAS, G. 2003. Insulin activation of Rheb, a mediator of mTOR/S6K/4E-BP signaling, is inhibited by TSC1 and 2. Mol Cell, 11**,** 1457-66.*

*GARCIA-MARTINEZ, J. M. & ALESSI, D. R. 2008. mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1). Biochem J, 416**,** 375-85.*

*GARCIA-MARTINEZ, J. M., MORAN, J., CLARKE, R. G., GRAY, A., COSULICH, S. C., CHRESTA, C. M. & ALESSI, D. R. 2009. Ku-0063794 is a specific inhibitor of the mammalian target of rapamycin (mTOR). Biochem J, 421**,** 29-42.*

*GAROFALO, R. S., ORENA, S. J., RAFIDI, K., TORCHIA, A. J., STOCK, J. L., HILDEBRANDT, A. L., COSKRAN, T., BLACK, S. C., BREES, D. J., WICKS, J. R., MCNEISH, J. D. & COLEMAN, K. G. 2003. Severe diabetes, age-dependent loss of adipose tissue, and mild growth deficiency in mice lacking Akt2/PKB beta. J Clin Invest, 112**,** 197-208.*

*GEISS-FRIEDLANDER, R. & MELCHIOR, F. 2007. Concepts in sumoylation: a decade on. Nat Rev Mol Cell Biol, 8**,** 947-56.*

*GILDEA, J. J., HERLEVSEN, M., HARDING, M. A., GULDING, K. M., MOSKALUK, C. A., FRIERSON, H. F. & THEODORESCU, D. 2004. PTEN can inhibit in vitro organotypic and in vivo orthotopic invasion of human bladder cancer cells even in the absence of its lipid phosphatase activity. Oncogene, 23**,** 6788-97.*

*GINGRAS, A. C., GYGI, S. P., RAUGHT, B., POLAKIEWICZ, R. D., ABRAHAM, R. T., HOEKSTRA, M. F., AEBERSOLD, R. & SONENBERG, N. 1999. Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism. Genes Dev, 13**,** 1422-37.*

*GINGRAS, A. C., RAUGHT, B., GYGI, S. P., NIEDZWIECKA, A., MIRON, M., BURLEY, S. K., POLAKIEWICZ, R. D., WYSLOUCH-CIESZYNSKA, A., AEBERSOLD, R. & SONENBERG, N. 2001. Hierarchical phosphorylation of the translation inhibitor 4E-BP1. Genes Dev, 15**,** 2852-64.*

*GOKE, A., GOKE, R., KNOLLE, A., TRUSHEIM, H., SCHMIDT, H., WILMEN, A., CARMODY, R., GOKE, B. & CHEN, Y. H. 2002. DUG is a novel homologue of translation initiation factor 4G that binds eIF4A. Biochem Biophys Res Commun, 297**,** 78-82.*

*GOUT, I., MINAMI, T., HARA, K., TSUJISHITA, Y., FILONENKO, V., WATERFIELD, M. D. & YONEZAWA, K. 1998. Molecular cloning and characterization of a novel p70 S6 kinase, p70 S6 kinase beta containing a proline-rich region. J Biol Chem, 273**,** 30061-4.*

*GRIFO, J. A., ABRAMSON, R. D., SATLER, C. A. & MERRICK, W. C. 1984. RNA-stimulated ATPase activity of eukaryotic initiation factors. J Biol Chem, 259**,** 8648-54.*

*GROLLEAU, A., BOWMAN, J., PRADET-BALADE, B., PURAVS, E., HANASH, S., GARCIA-SANZ, J. A. & BERETTA, L. 2002. Global and specific translational control by rapamycin in T cells uncovered by microarrays and proteomics. J Biol Chem, 277**,** 22175-84.*

*GROVE, J. R., BANERJEE, P., BALASUBRAMANYAM, A., COFFER, P. J., PRICE, D. J., AVRUCH, J. & WOODGETT, J. R. 1991. Cloning and expression of two human p70 S6 kinase polypeptides differing only at their amino termini. Mol Cell Biol, 11**,** 5541-50.*

*GRUHLER, A., SCHULZE, W. X., MATTHIESEN, R., MANN, M. & JENSEN, O. N. 2005. Stable isotope labeling of Arabidopsis thaliana cells and quantitative proteomics by mass spectrometry. Mol Cell Proteomics, 4**,** 1697-709.*

GSTAIGER, M., LUKE, B., HESS, D., OAKELEY, E. J., WIRBELAUER, C., BLONDEL, M., VIGNERON, M., PETER, M. & KREK, W. 2003. Control of nutrient-sensitive transcription programs by the unconventional prefoldin URI. Science, 302, 1208-12.

GUERTIN, D. A., STEVENS, D. M., THOREEN, C. C., BURDS, A. A., KALAANY, N. Y., MOFFAT, J., BROWN, M., FITZGERALD, K. J. & SABATINI, D. M. 2006. Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKCalpha, but not S6K1. Dev Cell, 11, 859-71.

GWINN, D. M., SHACKELFORD, D. B., EGAN, D. F., MIHAYLOVA, M. M., MERY, A., VASQUEZ, D. S., TURK, B. E. & SHAW, R. J. 2008. AMPK phosphorylation of raptor mediates a metabolic checkpoint. Mol Cell, 30, 214-26.

HAGHIGHAT, A., MADER, S., PAUSE, A. & SONENBERG, N. 1995. Repression of cap-dependent translation by 4E-binding protein 1: competition with p220 for binding to eukaryotic initiation factor-4E. EMBO J, 14, 5701-9.

HAMILTON, H. K. & TONKOVIC-CAPIN, V. 2011. Images in clinical medicine. Facial angiofibromas associated with tuberous sclerosis. N Engl J Med, 364, 1061.

HAN, J., BROWN, T. & BEUTLER, B. 1990. Endotoxin-responsive sequences control cachectin/tumor necrosis factor biosynthesis at the translational level. J Exp Med, 171, 465-75.

HANNAN, K. M., BRANDENBURGER, Y., JENKINS, A., SHARKEY, K., CAVANAUGH, A., ROTHBLUM, L., MOSS, T., POORTINGA, G., MCARTHUR, G. A., PEARSON, R. B. & HANNAN, R. D. 2003. mTOR-dependent regulation of ribosomal gene transcription requires S6K1 and is mediated by phosphorylation of the carboxy-terminal activation domain of the nucleolar transcription factor UBF. Mol Cell Biol, 23, 8862-77.

HARA, K., MARUKI, Y., LONG, X., YOSHINO, K., OSHIRO, N., HIDAYAT, S., TOKUNAGA, C., AVRUCH, J. & YONEZAWA, K. 2002. Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell, 110, 177-89.

HARA, K., YONEZAWA, K., WENG, Q. P., KOZLOWSKI, M. T., BELHAM, C. & AVRUCH, J. 1998. Amino acid sufficiency and mTOR regulate p70 S6 kinase and eIF-4E BP1 through a common effector mechanism. J Biol Chem, 273, 14484-94.

HARADA, H., ANDERSEN, J. S., MANN, M., TERADA, N. & KORSMEYER, S. J. 2001. p70S6 kinase signals cell survival as well as growth, inactivating the pro-apoptotic molecule BAD. Proc Natl Acad Sci U S A, 98, 9666-70.

HARDIE, D. G., SALT, I. P., HAWLEY, S. A. & DAVIES, S. P. 1999. AMP-activated protein kinase: an ultrasensitive system for monitoring cellular energy charge. Biochem J, 338 (Pt 3), 717-22.

HARRINGTON, L. S., FINDLAY, G. M., GRAY, A., TOLKACHEVA, T., WIGFIELD, S., REBHOLZ, H., BARNETT, J., LESLIE, N. R., CHENG, S., SHEPHERD, P. R., GOUT, I., DOWNES, C. P. & LAMB, R. F. 2004. The TSC1-2 tumor suppressor controls insulin-PI3K signaling via regulation of IRS proteins. J Cell Biol, 166, 213-23.

HAUGE, C., ANTAL, T. L., HIRSCHBERG, D., DOEHN, U., THORUP, K., IDRISSOVA, L., HANSEN, K., JENSEN, O. N., JORGENSEN, T. J., BIONDI, R. M. & FRODIN, M. 2007. Mechanism for activation of the growth factor-activated AGC kinases by turn motif phosphorylation. EMBO J, 26, 2251-61.

HAWLEY, S. A., BOUDEAU, J., REID, J. L., MUSTARD, K. J., UDD, L., MAKELA, T. P., ALESSI, D. R. & HARDIE, D. G. 2003. Complexes between the LKB1 tumor suppressor, STRAD alpha/beta and MO25 alpha/beta are upstream kinases in the AMP-activated protein kinase cascade. J Biol, 2, 28.

HAY, N. & SONENBERG, N. 2004. Upstream and downstream of mTOR. Genes Dev, 18, 1926-45.

*HEESOM, K. J., AVISON, M. B., DIGGLE, T. A. & DENTON, R. M. 1998. Insulin-stimulated kinase from rat fat cells that phosphorylates initiation factor 4E-binding protein 1 on the rapamycin-insensitive site (serine-111). Biochem J, 336 (Pt 1)**,** 39-48.*

*HEFNER, Y., BORSCH-HAUBOLD, A. G., MURAKAMI, M., WILDE, J. I., PASQUET, S., SCHIELTZ, D., GHOMASHCHI, F., YATES, J. R., 3RD, ARMSTRONG, C. G., PATERSON, A., COHEN, P., FUKUNAGA, R., HUNTER, T., KUDO, I., WATSON, S. P. & GELB, M. H. 2000. Serine 727 phosphorylation and activation of cytosolic phospholipase A2 by MNK1-related protein kinases. J Biol Chem, 275**,** 37542-51.*

*HELLIWELL, S. B., WAGNER, P., KUNZ, J., DEUTER-REINHARD, M., HENRIQUEZ, R. & HALL, M. N. 1994. TOR1 and TOR2 are structurally and functionally similar but not identical phosphatidylinositol kinase homologues in yeast. Mol Biol Cell, 5**,** 105-18.*

*HERMAN, P. K. & EMR, S. D. 1990. Characterization of VPS34, a gene required for vacuolar protein sorting and vacuole segregation in Saccharomyces cerevisiae. Mol Cell Biol, 10**,** 6742-54.*

*HIDALGO, M. & ROWINSKY, E. K. 2000. The rapamycin-sensitive signal transduction pathway as a target for cancer therapy. Oncogene, 19**,** 6680-6.*

HIRAOKA, D., OKUMURA, E. & KISHIMOTO, T. 2011. Turn motif phosphorylation negatively regulates activation loop phosphorylation in Akt. Oncogene.

*HOHEISEL, J. D. 2006. Microarray technology: beyond transcript profiling and genotype analysis. Nat Rev Genet, 7**,** 200-10.*

*HORNSTEIN, E., GIT, A., BRAUNSTEIN, I., AVNI, D. & MEYUHAS, O. 1999. The expression of poly(A)-binding protein gene is translationally regulated in a growth-dependent fashion through a 5'-terminal oligopyrimidine tract motif. J Biol Chem, 274**,** 1708-14.*

*HOSHINO, S., IMAI, M., KOBAYASHI, T., UCHIDA, N. & KATADA, T. 1999. The eukaryotic polypeptide chain releasing factor (eRF3/GSPT) carrying the translation termination signal to the 3'-Poly(A) tail of mRNA. Direct association of erf3/GSPT with polyadenylate-binding protein. J Biol Chem, 274**,** 16677-80.*

*HSIEH, A. C., COSTA, M., ZOLLO, O., DAVIS, C., FELDMAN, M. E., TESTA, J. R., MEYUHAS, O., SHOKAT, K. M. & RUGGERO, D. 2010. Genetic dissection of the oncogenic mTOR pathway reveals druggable addiction to translational control via 4EBP-eIF4E. Cancer Cell, 17**,** 249-61.*

*HUMPHREY, E. L., SHAMJI, A. F., BERNSTEIN, B. E. & SCHREIBER, S. L. 2004. Rpd3p relocation mediates a transcriptional response to rapamycin in yeast. Chem Biol, 11**,** 295-9.*

*IADEVAIA, V., CALDAROLA, S., BIONDINI, L., GISMONDI, A., KARLSSON, S., DIANZANI, I. & LORENI, F. 2010. PIM1 kinase is destabilized by ribosomal stress causing inhibition of cell cycle progression. Oncogene, 29**,** 5490-9.*

*IADEVAIA, V., CALDAROLA, S., TINO, E., AMALDI, F. & LORENI, F. 2008. All translation elongation factors and the e, f, and h subunits of translation initiation factor 3 are encoded by 5'-terminal oligopyrimidine (TOP) mRNAs. RNA, 14**,** 1730-6.*

*IKENOUE, T., INOKI, K., YANG, Q., ZHOU, X. & GUAN, K. L. 2008. Essential function of TORC2 in PKC and Akt turn motif phosphorylation, maturation and signalling. EMBO J, 27**,** 1919-31.*

*IM, E., VON LINTIG, F. C., CHEN, J., ZHUANG, S., QUI, W., CHOWDHURY, S., WORLEY, P. F., BOSS, G. R. & PILZ, R. B. 2002. Rheb is in a high activation state and inhibits B-Raf kinase in mammalian cells. Oncogene, 21**,** 6356-65.*

*IMATAKA, H., GRADI, A. & SONENBERG, N. 1998. A newly identified N-terminal amino acid sequence of human eIF4G binds poly(A)-binding protein and functions in poly(A)-dependent translation. EMBO J, 17**,** 7480-9.*

*INOKI, K., CORRADETTI, M. N. & GUAN, K. L. 2005. Dysregulation of the TSC-mTOR pathway in human disease. Nat Genet, 37**,** 19-24.*

INOKI, K., LI, Y., XU, T. & GUAN, K. L. 2003a. Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling. Genes Dev, 17, 1829-34.

INOKI, K., LI, Y., ZHU, T., WU, J. & GUAN, K. L. 2002. TSC2 is phosphorylated and inhibited by Akt and suppresses mTOR signalling. Nat Cell Biol, 4, 648-57.

INOKI, K., OUYANG, H., ZHU, T., LINDVALL, C., WANG, Y., ZHANG, X., YANG, Q., BENNETT, C., HARADA, Y., STANKUNAS, K., WANG, C. Y., HE, X., MACDOUGALD, O. A., YOU, M., WILLIAMS, B. O. & GUAN, K. L. 2006. TSC2 integrates Wnt and energy signals via a coordinated phosphorylation by AMPK and GSK3 to regulate cell growth. Cell, 126, 955-68.

INOKI, K., ZHU, T. & GUAN, K. L. 2003b. TSC2 mediates cellular energy response to control cell growth and survival. Cell, 115, 577-90.

ISOTANI, S., HARA, K., TOKUNAGA, C., INOUE, H., AVRUCH, J. & YONEZAWA, K. 1999. Immunopurified mammalian target of rapamycin phosphorylates and activates p70 S6 kinase alpha in vitro. J Biol Chem, 274, 34493-8.

IVANOV, V. N., ZHOU, H., PARTRIDGE, M. A. & HEI, T. K. 2009. Inhibition of ataxia telangiectasia mutated kinase activity enhances TRAIL-mediated apoptosis in human melanoma cells. Cancer Res, 69, 3510-9.

JACINTO, E., FACCHINETTI, V., LIU, D., SOTO, N., WEI, S., JUNG, S. Y., HUANG, Q., QIN, J. & SU, B. 2006. SIN1/MIP1 maintains rictor-mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell, 127, 125-37.

JACINTO, E., LOEWITH, R., SCHMIDT, A., LIN, S., RUEGG, M. A., HALL, A. & HALL, M. N. 2004. Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol, 6, 1122-8.

JACINTO, E. & LORBERG, A. 2008. TOR regulation of AGC kinases in yeast and mammals. Biochem J, 410, 19-37.

JAUCH, R., CHO, M. K., JAKEL, S., NETTER, C., SCHREITER, K., AICHER, B., ZWECKSTETTER, M., JACKLE, H. & WAHL, M. C. 2006. Mitogen-activated protein kinases interacting kinases are autoinhibited by a reprogrammed activation segment. EMBO J, 25, 4020-32.

JAUCH, R., JAKEL, S., NETTER, C., SCHREITER, K., AICHER, B., JACKLE, H. & WAHL, M. C. 2005. Crystal structures of the Mnk2 kinase domain reveal an inhibitory conformation and a zinc binding site. Structure, 13, 1559-68.

JEFFERIES, H. B., FUMAGALLI, S., DENNIS, P. B., REINHARD, C., PEARSON, R. B. & THOMAS, G. 1997. Rapamycin suppresses 5'TOP mRNA translation through inhibition of p70s6k. EMBO J, 16, 3693-704.

JEFFERIES, H. B., REINHARD, C., KOZMA, S. C. & THOMAS, G. 1994. Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family. Proc Natl Acad Sci U S A, 91, 4441-5.

JEFFERIES, H. B. & THOMAS, G. 1994. Elongation factor-1 alpha mRNA is selectively translated following mitogenic stimulation. J Biol Chem, 269, 4367-72.

JIANG, H. & ENGLISH, A. M. 2002. Quantitative analysis of the yeast proteome by incorporation of isotopically labeled leucine. J Proteome Res, 1, 345-50.

JONES, A. C., SHYAMSUNDAR, M. M., THOMAS, M. W., MAYNARD, J., IDZIASZCZYK, S., TOMKINS, S., SAMPSON, J. R. & CHEADLE, J. P. 1999. Comprehensive mutation analysis of TSC1 and TSC2-and phenotypic correlations in 150 families with tuberous sclerosis. Am J Hum Genet, 64, 1305-15.

JONES, P. F., JAKUBOWICZ, T. & HEMMINGS, B. A. 1991a. Molecular cloning of a second form of rac protein kinase. Cell Regul, 2, 1001-9.

JONES, P. F., JAKUBOWICZ, T., PITOSSI, F. J., MAURER, F. & HEMMINGS, B. A. 1991b. Molecular cloning and identification of a serine/threonine protein kinase of the second-messenger subfamily. Proc Natl Acad Sci U S A, 88, 4171-5.

JORGENSEN, P., RUPES, I., SHAROM, J. R., SCHNEPER, L., BROACH, J. R. & TYERS, M. 2004. A dynamic transcriptional network communicates growth potential to ribosome synthesis and critical cell size. Genes Dev, 18, 2491-505.

JOSHI, B., CAI, A. L., KEIPER, B. D., MINICH, W. B., MENDEZ, R., BEACH, C. M., STEPINSKI, J., STOLARSKI, R., DARZYNKIEWICZ, E. & RHOADS, R. E. 1995. Phosphorylation of eukaryotic protein synthesis initiation factor 4E at Ser-209. J Biol Chem, 270, 14597-603.

JOSHI, B., CAMERON, A. & JAGUS, R. 2004. Characterization of mammalian eIF4E-family members. Eur J Biochem, 271, 2189-203.

JUHASZ, G., HILL, J. H., YAN, Y., SASS, M., BAEHRECKE, E. H., BACKER, J. M. & NEUFELD, T. P. 2008. The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila. J Cell Biol, 181, 655-66.

JUNG, C. H., JUN, C. B., RO, S. H., KIM, Y. M., OTTO, N. M., CAO, J., KUNDU, M. & KIM, D. H. 2009. ULK-Atg13-FIP200 complexes mediate mTOR signaling to the autophagy machinery. Mol Biol Cell, 20, 1992-2003.

JUNG, C. H., RO, S. H., CAO, J., OTTO, N. M. & KIM, D. H. 2010. mTOR regulation of autophagy. FEBS Lett, 584, 1287-95.

KABEYA, Y., NODA, N. N., FUJIOKA, Y., SUZUKI, K., INAGAKI, F. & OHSUMI, Y. 2009. Characterization of the Atg17-Atg29-Atg31 complex specifically required for starvation-induced autophagy in Saccharomyces cerevisiae. Biochem Biophys Res Commun, 389, 612-5.

KABUTA, T., HAKUNO, F., CHO, Y., YAMANAKA, D., CHIDA, K., ASANO, T., WADA, K. & TAKAHASHI, S. 2010. Insulin receptor substrate-3, interacting with Bcl-3, enhances p50 NF-kappaB activity. Biochem Biophys Res Commun, 394, 697-702.

KAKEGAWA, T., OHUCHI, N., HAYAKAWA, A., HIRATA, S., MATSUDA, M., KOGURE, K., KOBAYASHI, H., INOUE, A. & KASPAR, R. L. 2007. Identification of AUF1 as a rapamycin-responsive binding protein to the 5'-terminal oligopyrimidine element of mRNAs. Arch Biochem Biophys, 465, 274-81.

KAMADA, Y., FUJIOKA, Y., SUZUKI, N. N., INAGAKI, F., WULLSCHLEGER, S., LOEWITH, R., HALL, M. N. & OHSUMI, Y. 2005. Tor2 directly phosphorylates the AGC kinase Ypk2 to regulate actin polarization. Mol Cell Biol, 25, 7239-48.

KAMADA, Y., FUNAKOSHI, T., SHINTANI, T., NAGANO, K., OHSUMI, M. & OHSUMI, Y. 2000. Tor-mediated induction of autophagy via an Apg1 protein kinase complex. J Cell Biol, 150, 1507-13.

KARIM, M. M., HUGHES, J. M., WARWICKER, J., SCHEPER, G. C., PROUD, C. G. & MCCARTHY, J. E. 2001. A quantitative molecular model for modulation of mammalian translation by the eIF4E-binding protein 1. J Biol Chem, 276, 20750-7.

KAUFMAN, R. J., MURTHA-RIEL, P., PITTMAN, D. D. & DAVIES, M. V. 1993. Characterization of wild-type and Ser53 mutant eukaryotic initiation factor 4E overexpression in mammalian cells. J Biol Chem, 268, 11902-9.

KAWAMATA, T., KAMADA, Y., KABEYA, Y., SEKITO, T. & OHSUMI, Y. 2008. Organization of the pre-autophagosomal structure responsible for autophagosome formation. Mol Biol Cell, 19, 2039-50.

KAWASOME, H., PAPST, P., WEBB, S., KELLER, G. M., JOHNSON, G. L., GELFAND, E. W. & TERADA, N. 1998. Targeted disruption of p70(s6k) defines its role in protein synthesis and rapamycin sensitivity. Proc Natl Acad Sci U S A, 95, 5033-8.

KERNER, M. J., NAYLOR, D. J., ISHIHAMA, Y., MAIER, T., CHANG, H. C., STINES, A. P., GEORGOPOULOS, C., FRISHMAN, D., HAYER-HARTL, M., MANN, M. & HARTL, F. U. 2005. Proteome-wide analysis of chaperonin-dependent protein folding in Escherichia coli. Cell, 122, 209-20.

KESHWANI, M. M., VON DAAKE, S., NEWTON, A. C., HARRIS, T. K. & TAYLOR, S. S. 2011. Hydrophobic motif phosphorylation is not required for activation loop phosphorylation of p70 ribosomal protein S6 kinase 1 (S6K1). J Biol Chem, 286, 23552-8.

KIEFT, J. S. 2008. Viral IRES RNA structures and ribosome interactions. Trends Biochem Sci, 33, 274-83.

KIHARA, A., NODA, T., ISHIHARA, N. & OHSUMI, Y. 2001. Two distinct Vps34 phosphatidylinositol 3-kinase complexes function in autophagy and carboxypeptidase Y sorting in Saccharomyces cerevisiae. J Cell Biol, 152, 519-30.

KIM, D. H., SARBASSOV, D. D., ALI, S. M., KING, J. E., LATEK, R. R., ERDJUMENT-BROMAGE, H., TEMPST, P. & SABATINI, D. M. 2002. mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell, 110, 163-75.

KIM, D. H., SARBASSOV, D. D., ALI, S. M., LATEK, R. R., GUNTUR, K. V., ERDJUMENT-BROMAGE, H., TEMPST, P. & SABATINI, D. M. 2003. GbetaL, a positive regulator of the rapamycin-sensitive pathway required for the nutrient-sensitive interaction between raptor and mTOR. Mol Cell, 11, 895-904.

KIMURA, N., TOKUNAGA, C., DALAL, S., RICHARDSON, C., YOSHINO, K., HARA, K., KEMP, B. E., WITTERS, L. A., MIMURA, O. & YONEZAWA, K. 2003. A possible linkage between AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) signalling pathway. Genes Cells, 8, 65-79.

KNAUF, U., TSCHOPP, C. & GRAM, H. 2001. Negative regulation of protein translation by mitogen-activated protein kinase-interacting kinases 1 and 2. Mol Cell Biol, 21, 5500-11.

KOBAYASHI, T. & COHEN, P. 1999. Activation of serum- and glucocorticoid-regulated protein kinase by agonists that activate phosphatidylinositide 3-kinase is mediated by 3-phosphoinositide-dependent protein kinase-1 (PDK1) and PDK2. Biochem J, 339 (Pt 2), 319-28.

KOH, H., JEE, K., LEE, B., KIM, J., KIM, D., YUN, Y. H., KIM, J. W., CHOI, H. S. & CHUNG, J. 1999. Cloning and characterization of a nuclear S6 kinase, S6 kinase-related kinase (SRK); a novel nuclear target of Akt. Oncogene, 18, 5115-9.

KOMAR, A. A. & HATZOGLOU, M. 2005. Internal ribosome entry sites in cellular mRNAs: mystery of their existence. J Biol Chem, 280, 23425-8.

KOMAR, A. A. & HATZOGLOU, M. 2011. Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. Cell Cycle, 10, 229-40.

KONISHI, H., KURODA, S., TANAKA, M., MATSUZAKI, H., ONO, Y., KAMEYAMA, K., HAGA, T. & KIKKAWA, U. 1995. Molecular cloning and characterization of a new member of the RAC protein kinase family: association of the pleckstrin homology domain of three types of RAC protein kinase with protein kinase C subspecies and beta gamma subunits of G proteins. Biochem Biophys Res Commun, 216, 526-34.

KONTOYIANNIS, D., PASPARAKIS, M., PIZARRO, T. T., COMINELLI, F. & KOLLIAS, G. 1999. Impaired on/off regulation of TNF biosynthesis in mice lacking TNF AU-rich elements: implications for joint and gut-associated immunopathologies. Immunity, 10, 387-98.

KOTLYAROV, A., NEININGER, A., SCHUBERT, C., ECKERT, R., BIRCHMEIER, C., VOLK, H. D. & GAESTEL, M. 1999. MAPKAP kinase 2 is essential for LPS-induced TNF-alpha biosynthesis. Nat Cell Biol, 1, 94-7.

KOVACINA, K. S., PARK, G. Y., BAE, S. S., GUZZETTA, A. W., SCHAEFER, E., BIRNBAUM, M. J. & ROTH, R. A. 2003. Identification of a proline-rich Akt substrate as a 14-3-3 binding partner. J Biol Chem, 278, 10189-94.

KRAUSE, U., BERTRAND, L. & HUE, L. 2002. Control of p70 ribosomal protein S6 kinase and acetyl-CoA carboxylase by AMP-activated protein kinase and protein phosphatases in isolated hepatocytes. Eur J Biochem, 269, 3751-9.

KRIEG, J., HOFSTEENGE, J. & THOMAS, G. 1988. Identification of the 40 S ribosomal protein S6 phosphorylation sites induced by cycloheximide. J Biol Chem, 263, 11473-7.

KRISTOF, A. S., MARKS-KONCZALIK, J., BILLINGS, E. & MOSS, J. 2003. Stimulation of signal transducer and activator of transcription-1 (STAT1)-dependent gene transcription by lipopolysaccharide and interferon-gamma is regulated by mammalian target of rapamycin. J Biol Chem, 278, 33637-44.

KUMAR, S., JIANG, M. S., ADAMS, J. L. & LEE, J. C. 1999. Pyridinylimidazole compound SB 203580 inhibits the activity but not the activation of p38 mitogen-activated protein kinase. Biochem Biophys Res Commun, 263, 825-31.

KUNZ, J., HENRIQUEZ, R., SCHNEIDER, U., DEUTER-REINHARD, M., MOVVA, N. R. & HALL, M. N. 1993. Target of rapamycin in yeast, TOR2, is an essential phosphatidylinositol kinase homolog required for G1 progression. Cell, 73, 585-96.

LACHANCE, P. E., MIRON, M., RAUGHT, B., SONENBERG, N. & LASKO, P. 2002. Phosphorylation of eukaryotic translation initiation factor 4E is critical for growth. Mol Cell Biol, 22, 1656-63.

LAND, S. C. & TEE, A. R. 2007. Hypoxia-inducible factor 1alpha is regulated by the mammalian target of rapamycin (mTOR) via an mTOR signaling motif. J Biol Chem, 282, 20534-43.

LAPENTA, V., CHIURAZZI, P., VAN DER SPEK, P., PIZZUTI, A., HANAOKA, F. & BRAHE, C. 1997. SMT3A, a human homologue of the S. cerevisiae SMT3 gene, maps to chromosome 21qter and defines a novel gene family. Genomics, 40, 362-6.

LASKEY, R. A., HONDA, B. M., MILLS, A. D. & FINCH, J. T. 1978. Nucleosomes are assembled by an acidic protein which binds histones and transfers them to DNA. Nature, 275, 416-20.

LAURING, B., SAKAI, H., KREIBICH, G. & WIEDMANN, M. 1995. Nascent polypeptide-associated complex protein prevents mistargeting of nascent chains to the endoplasmic reticulum. Proc Natl Acad Sci U S A, 92, 5411-5.

LAVAN, B. E., FANTIN, V. R., CHANG, E. T., LANE, W. S., KELLER, S. R. & LIENHARD, G. E. 1997. A novel 160-kDa phosphotyrosine protein in insulin-treated embryonic kidney cells is a new member of the insulin receptor substrate family. J Biol Chem, 272, 21403-7.

LAVIN, M. F. 1999. ATM: the product of the gene mutated in ataxia-telangiectasia. Int J Biochem Cell Biol, 31, 735-40.

LAWLOR, M. A., MORA, A., ASHBY, P. R., WILLIAMS, M. R., MURRAY-TAIT, V., MALONE, L., PRESCOTT, A. R., LUCOCQ, J. M. & ALESSI, D. R. 2002. Essential role of PDK1 in regulating cell size and development in mice. EMBO J, 21, 3728-38.

LAZARIS-KARATZAS, A., MONTINE, K. S. & SONENBERG, N. 1990. Malignant transformation by a eukaryotic initiation factor subunit that binds to mRNA 5' cap. Nature, 345, 544-7.

LEE, J. H., PESTOVA, T. V., SHIN, B. S., CAO, C., CHOI, S. K. & DEVER, T. E. 2002. Initiation factor eIF5B catalyzes second GTP-dependent step in eukaryotic translation initiation. Proc Natl Acad Sci U S A, 99, 16689-94.

LEONARD, S., PLANTE, D., WITTMANN, S., DAIGNEAULT, N., FORTIN, M. G. & LALIBERTE, J. F. 2000. Complex formation between potyvirus VPg and translation eukaryotic initiation factor 4E correlates with virus infectivity. J Virol, 74, 7730-7.

LESLIE, N. R. & DOWNES, C. P. 2002. PTEN: The down side of PI 3-kinase signalling. Cell Signal, 14, 285-95.

LESLIE, N. R., YANG, X., DOWNES, C. P. & WEIJER, C. J. 2007. PtdIns(3,4,5)P(3)-dependent and -independent roles for PTEN in the control of cell migration. Curr Biol, 17, 115-25.

LI, J., YEN, C., LIAW, D., PODSYPANINA, K., BOSE, S., WANG, S. I., PUC, J., MILIARESIS, C., RODGERS, L., MCCOMBIE, R., BIGNER, S. H., GIOVANELLA, B. C., ITTMANN, M., TYCKO, B., HIBSHOOSH, H., WIGLER, M. H. & PARSONS, R. 1997. PTEN, a putative protein tyrosine phosphatase gene mutated in human brain, breast, and prostate cancer. Science, 275, 1943-7.

LIN, T. A., KONG, X., HAYSTEAD, T. A., PAUSE, A., BELSHAM, G., SONENBERG, N. & LAWRENCE, J. C., JR. 1994. PHAS-I as a link between mitogen-activated protein kinase and translation initiation. Science, 266, 653-6.

LOFFING, J., FLORES, S. Y. & STAUB, O. 2006. Sgk kinases and their role in epithelial transport. Annu Rev Physiol, 68, 461-90.

LYNCH, E. D., OSTERMEYER, E. A., LEE, M. K., ARENA, J. F., JI, H., DANN, J., SWISSHELM, K., SUCHARD, D., MACLEOD, P. M., KVINNSLAND, S., GJERTSEN, B. T., HEIMDAL, K., LUBS, H., MOLLER, P. & KING, M. C. 1997. Inherited mutations in PTEN that are associated with breast cancer, cowden disease, and juvenile polyposis. Am J Hum Genet, 61, 1254-60.

MA, L., CHEN, Z., ERDJUMENT-BROMAGE, H., TEMPST, P. & PANDOLFI, P. P. 2005. Phosphorylation and functional inactivation of TSC2 by Erk implications for tuberous sclerosis and cancer pathogenesis. Cell, 121, 179-93.

MADER, S., LEE, H., PAUSE, A. & SONENBERG, N. 1995. The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol Cell Biol, 15, 4990-7.

MAHALINGAM, M. & COOPER, J. A. 2001. Phosphorylation of mammalian eIF4E by Mnk1 and Mnk2: tantalizing prospects for a role in translation. Prog Mol Subcell Biol, 27, 132-42.

MAHIMAINATHAN, L. & CHOUDHURY, G. G. 2004. Inactivation of platelet-derived growth factor receptor by the tumor suppressor PTEN provides a novel mechanism of action of the phosphatase. J Biol Chem, 279, 15258-68.

MAMANE, Y., PETROULAKIS, E., LEBACQUER, O. & SONENBERG, N. 2006. mTOR, translation initiation and cancer. Oncogene, 25, 6416-22.

MANNING, B. D. & CANTLEY, L. C. 2003. Rheb fills a GAP between TSC and TOR. Trends Biochem Sci, 28, 573-6.

MANNING, B. D. & CANTLEY, L. C. 2007. AKT/PKB signaling: navigating downstream. Cell, 129, 1261-74.

MANNING, B. D., TEE, A. R., LOGSDON, M. N., BLENIS, J. & CANTLEY, L. C. 2002. Identification of the tuberous sclerosis complex-2 tumor suppressor gene product tuberin as a target of the phosphoinositide 3-kinase/akt pathway. Mol Cell, 10, 151-62.

MARCOTRIGIANO, J., GINGRAS, A. C., SONENBERG, N. & BURLEY, S. K. 1997. Cocrystal structure of the messenger RNA 5' cap-binding protein (eIF4E) bound to 7-methyl-GDP. Cell, 89, 951-61.

MARCOTRIGIANO, J., GINGRAS, A. C., SONENBERG, N. & BURLEY, S. K. 1999. Cap-dependent translation initiation in eukaryotes is regulated by a molecular mimic of eIF4G. Mol Cell, 3, 707-16.

MARDILOVICH, K., PANKRATZ, S. L. & SHAW, L. M. 2009. Expression and function of the insulin receptor substrate proteins in cancer. Cell Commun Signal, 7, 14.

MARION, R. M., REGEV, A., SEGAL, E., BARASH, Y., KOLLER, D., FRIEDMAN, N. & O'SHEA, E. K. 2004. Sfp1 is a stress- and nutrient-sensitive regulator of ribosomal protein gene expression. Proc Natl Acad Sci U S A, 101, 14315-22.

MARTIN, D. E., SOULARD, A. & HALL, M. N. 2004. TOR regulates ribosomal protein gene expression via PKA and the Forkhead transcription factor FHL1. Cell, 119, 969-79.

MARTINEAU, Y., DERRY, M. C., WANG, X., YANAGIYA, A., BERLANGA, J. J., SHYU, A. B., IMATAKA, H., GEHRING, K. & SONENBERG, N. 2008. Poly(A)-binding protein-interacting protein 1 binds to eukaryotic translation initiation factor 3 to stimulate translation. Mol Cell Biol, 28, 6658-67.

MATUNIS, M. J., COUTAVAS, E. & BLOBEL, G. 1996. A novel ubiquitin-like modification modulates the partitioning of the Ran-GTPase-activating protein RanGAP1 between the cytosol and the nuclear pore complex. J Cell Biol, 135, 1457-70.

MAXWELL, P. H., WIESENER, M. S., CHANG, G. W., CLIFFORD, S. C., VAUX, E. C., COCKMAN, M. E., WYKOFF, C. C., PUGH, C. W., MAHER, E. R. & RATCLIFFE, P. J. 1999. The tumour suppressor protein VHL targets hypoxia-inducible factors for oxygen-dependent proteolysis. Nature, 399, 271-5.

MAYER, C. & GRUMMT, I. 2006. Ribosome biogenesis and cell growth: mTOR coordinates transcription by all three classes of nuclear RNA polymerases. Oncogene, 25, 6384-91.

MAYER, C., ZHAO, J., YUAN, X. & GRUMMT, I. 2004. mTOR-dependent activation of the transcription factor TIF-IA links rRNA synthesis to nutrient availability. Genes Dev, 18, 423-34.

MCMANUS, E. J., COLLINS, B. J., ASHBY, P. R., PRESCOTT, A. R., MURRAY-TAIT, V., ARMIT, L. J., ARTHUR, J. S. & ALESSI, D. R. 2004. The in vivo role of PtdIns(3,4,5)P3 binding to PDK1 PH domain defined by knockin mutation. EMBO J, 23, 2071-82.

METHOT, N., PICKETT, G., KEENE, J. D. & SONENBERG, N. 1996a. In vitro RNA selection identifies RNA ligands that specifically bind to eukaryotic translation initiation factor 4B: the role of the RNA remotif. RNA, 2, 38-50.

METHOT, N., SONG, M. S. & SONENBERG, N. 1996b. A region rich in aspartic acid, arginine, tyrosine, and glycine (DRYG) mediates eukaryotic initiation factor 4B (eIF4B) self-association and interaction with eIF3. Mol Cell Biol, 16, 5328-34.

MEYUHAS, O. 2000. Synthesis of the translational apparatus is regulated at the translational level. Eur J Biochem, 267, 6321-30.

MILLER, G., PANOV, K. I., FRIEDRICH, J. K., TRINKLE-MULCAHY, L., LAMOND, A. I. & ZOMERDIJK, J. C. 2001. hRRN3 is essential in the SL1-mediated recruitment of RNA Polymerase I to rRNA gene promoters. EMBO J, 20, 1373-82.

MILNER, E., BARNEA, E., BEER, I. & ADMON, A. 2006. The turnover kinetics of major histocompatibility complex peptides of human cancer cells. Mol Cell Proteomics, 5, 357-65.

MINICH, W. B., BALASTA, M. L., GOSS, D. J. & RHOADS, R. E. 1994. Chromatographic resolution of in vivo phosphorylated and nonphosphorylated eukaryotic translation initiation factor eIF-4E: increased cap affinity of the phosphorylated form. Proc Natl Acad Sci U S A, 91, 7668-72.

MINVIELLE-SEBASTIA, L., PREKER, P. J., WIEDERKEHR, T., STRAHM, Y. & KELLER, W. 1997. The major yeast poly(A)-binding protein is associated with cleavage factor IA and functions in premessenger RNA 3'-end formation. Proc Natl Acad Sci U S A, 94, 7897-902.

MIRON, M., VERDU, J., LACHANCE, P. E., BIRNBAUM, M. J., LASKO, P. F. & SONENBERG, N. 2001. The translational inhibitor 4E-BP is an effector of PI(3)K/Akt signalling and cell growth in Drosophila. Nat Cell Biol, 3, 596-601.

MOKREJS, M., VOPALENSKY, V., KOLENATY, O., MASEK, T., FEKETOVA, Z., SEKYROVA, P., SKALOUDOVA, B., KRIZ, V. & POSPISEK, M. 2006. IRESite: the database of experimentally verified IRES structures (www.iresite.org). Nucleic Acids Res, 34, D125-30.

MOTHE-SATNEY, I., BRUNN, G. J., MCMAHON, L. P., CAPALDO, C. T., ABRAHAM, R. T. & LAWRENCE, J. C., JR. 2000. Mammalian target of rapamycin-dependent phosphorylation of PHAS-I in four (S/T)P sites detected by phospho-specific antibodies. J Biol Chem, 275, 33836-43.

MUTA, D., MAKINO, K., NAKAMURA, H., YANO, S., KUDO, M. & KURATSU, J. 2011. Inhibition of eIF4E phosphorylation reduces cell growth and proliferation in primary central nervous system lymphoma cells. J Neurooncol, 101, 33-9.

NAGLE, J. A., MA, Z., BYRNE, M. A., WHITE, M. F. & SHAW, L. M. 2004. Involvement of insulin receptor substrate 2 in mammary tumor metastasis. Mol Cell Biol, 24, 9726-35.

NAKAJO, T., YAMATSUJI, T., BAN, H., SHIGEMITSU, K., HAISA, M., MOTOKI, T., NOMA, K., NOBUHISA, T., MATSUOKA, J., GUNDUZ, M., YONEZAWA, K., TANAKA, N. & NAOMOTO, Y. 2005. Glutamine is a key regulator for amino acid-controlled cell growth through the mTOR signaling pathway in rat intestinal epithelial cells. Biochem Biophys Res Commun, 326, 174-80.

NAPOLI, I., MERCALDO, V., BOYL, P. P., ELEUTERI, B., ZALFA, F., DE RUBEIS, S., DI MARINO, D., MOHR, E., MASSIMI, M., FALCONI, M., WITKE, W., COSTA-MATTIOLI, M., SONENBERG, N., ACHSEL, T. & BAGNI, C. 2008. The fragile X syndrome protein represses activity-dependent translation through CYFIP1, a new 4E-BP. Cell, 134, 1042-54.

NEER, E. J., SCHMIDT, C. J., NAMBUDRIPAD, R. & SMITH, T. F. 1994. The ancient regulatory-protein family of WD-repeat proteins. Nature, 371, 297-300.

NIE, M., XIE, Y., LOO, J. A. & COUREY, A. J. 2009. Genetic and proteomic evidence for roles of Drosophila SUMO in cell cycle control, Ras signaling, and early pattern formation. PLoS One, 4, e5905.

NIRANJANAKUMARI, S., LASDA, E., BRAZAS, R. & GARCIA-BLANCO, M. A. 2002. Reversible cross-linking combined with immunoprecipitation to study RNA-protein interactions in vivo. Methods, 26, 182-90.

NOBUKUNI, T., JOAQUIN, M., ROCCIO, M., DANN, S. G., KIM, S. Y., GULATI, P., BYFIELD, M. P., BACKER, J. M., NATT, F., BOS, J. L., ZWARTKRUIS, F. J. & THOMAS, G. 2005. Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase. Proc Natl Acad Sci U S A, 102, 14238-43.

NODA, T. & OHSUMI, Y. 1998. Tor, a phosphatidylinositol kinase homologue, controls autophagy in yeast. J Biol Chem, 273, 3963-6.

O'FARRELL, P. H. 1975. High resolution two-dimensional electrophoresis of proteins. J Biol Chem, 250, 4007-21.

O'HAGAN, A. R., ELLSWORTH, R., SECIC, M., ROTHNER, A. D. & BROUHARD, B. H. 1996. Renal manifestations of tuberous sclerosis complex. Clin Pediatr (Phila), 35, 483-9.

O'LOGHLEN, A., GONZALEZ, V. M., JURADO, T., SALINAS, M. & MARTIN, M. E. 2007. Characterization of the activity of human MAP kinase-interacting kinase Mnk1b. Biochim Biophys Acta, 1773, 1416-27.

O'REILLY, K. E., ROJO, F., SHE, Q. B., SOLIT, D., MILLS, G. B., SMITH, D., LANE, H., HOFMANN, F., HICKLIN, D. J., LUDWIG, D. L., BASELGA, J. & ROSEN, N. 2006. mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt. Cancer Res, 66, 1500-8.

OH, W. J., WU, C. C., KIM, S. J., FACCHINETTI, V., JULIEN, L. A., FINLAN, M., ROUX, P. P., SU, B. & JACINTO, E. 2010. mTORC2 can associate with ribosomes to promote cotranslational phosphorylation and stability of nascent Akt polypeptide. EMBO J, 29, 3939-51.

OKURA, T., GONG, L., KAMITANI, T., WADA, T., OKURA, I., WEI, C. F., CHANG, H. M. & YEH, E. T. 1996. Protection against Fas/APO-1- and tumor necrosis factor-mediated cell death by a novel protein, sentrin. J Immunol, 157, 4277-81.

ONG, S. E., BLAGOEV, B., KRATCHMAROVA, I., KRISTENSEN, D. B., STEEN, H., PANDEY, A. & MANN, M. 2002. Stable isotope labeling by amino acids in cell culture, SILAC, as a simple and accurate approach to expression proteomics. Mol Cell Proteomics, 1, 376-86.

OROM, U. A., NIELSEN, F. C. & LUND, A. H. 2008. MicroRNA-10a binds the 5'UTR of ribosomal protein mRNAs and enhances their translation. Mol Cell, 30, 460-71.

PARRA-PALAU, J. L., SCHEPER, G. C., WILSON, M. L. & PROUD, C. G. 2003. Features in the N and C termini of the MAPK-interacting kinase Mnk1 mediate its nucleocytoplasmic shuttling. J Biol Chem, 278, 44197-204.

PARRA, J. L., BUXADE, M. & PROUD, C. G. 2005. Features of the catalytic domains and C termini of the MAPK signal-integrating kinases Mnk1 and Mnk2 determine their differing activities and regulatory properties. J Biol Chem, 280, 37623-33.

PATURSKY-POLISCHUK, I., STOLOVICH-RAIN, M., HAUSNER-HANOCHI, M., KASIR, J., CYBULSKI, N., AVRUCH, J., RUEGG, M. A., HALL, M. N. & MEYUHAS, O. 2009. The TSC-mTOR pathway mediates translational activation of TOP mRNAs by insulin largely in a raptor- or rictor-independent manner. Mol Cell Biol, 29, 640-9.

PAUSE, A., BELSHAM, G. J., GINGRAS, A. C., DONZE, O., LIN, T. A., LAWRENCE, J. C., JR. & SONENBERG, N. 1994. Insulin-dependent stimulation of protein synthesis by phosphorylation of a regulator of 5'-cap function. Nature, 371, 762-7.

PEARCE, L. R., HUANG, X., BOUDEAU, J., PAWLOWSKI, R., WULLSCHLEGER, S., DEAK, M., IBRAHIM, A. F., GOURLAY, R., MAGNUSON, M. A. & ALESSI, D. R. 2007. Identification of Protor as a novel Rictor-binding component of mTOR complex-2. Biochem J, 405, 513-22.

PEARCE, L. R., SOMMER, E. M., SAKAMOTO, K., WULLSCHLEGER, S. & ALESSI, D. R. 2011. Protor-1 is required for efficient mTORC2-mediated activation of SGK1 in the kidney. Biochem J, 436, 169-79.

PELLETIER, J. & SONENBERG, N. 1988. Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. Nature, 334, 320-5.

PELLIZZONI, L., CARDINALI, B., LIN-MARQ, N., MERCANTI, D. & PIERANDREI-AMALDI, P. 1996. A Xenopus laevis homologue of the La autoantigen binds the pyrimidine tract of the 5' UTR of ribosomal protein mRNAs in vitro: implication of a protein factor in complex formation. J Mol Biol, 259, 904-15.

PELLIZZONI, L., LOTTI, F., MARAS, B. & PIERANDREI-AMALDI, P. 1997. Cellular nucleic acid binding protein binds a conserved region of the 5' UTR of Xenopus laevis ribosomal protein mRNAs. J Mol Biol, 267, 264-75.

PELLIZZONI, L., LOTTI, F., RUTJES, S. A. & PIERANDREI-AMALDI, P. 1998. Involvement of the Xenopus laevis Ro60 autoantigen in the alternative interaction of La and CNBP proteins with the 5'UTR of L4 ribosomal protein mRNA. J Mol Biol, 281, 593-608.

PENDE, M., UM, S. H., MIEULET, V., STICKER, M., GOSS, V. L., MESTAN, J., MUELLER, M., FUMAGALLI, S., KOZMA, S. C. & THOMAS, G. 2004. S6K1(-/-)/S6K2(-/-) mice exhibit perinatal lethality and rapamycin-sensitive 5'-terminal oligopyrimidine mRNA translation and reveal a mitogen-activated protein kinase-dependent S6 kinase pathway. Mol Cell Biol, 24, 3112-24.

PENG, T., GOLUB, T. R. & SABATINI, D. M. 2002. The immunosuppressant rapamycin mimics a starvation-like signal distinct from amino acid and glucose deprivation. Mol Cell Biol, 22, 5575-84.

PEREZ-TENORIO, G. & STAL, O. 2002. Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br J Cancer, 86, 540-5.

PESTOVA, T. V., BORUKHOV, S. I. & HELLEN, C. U. 1998. Eukaryotic ribosomes require initiation factors 1 and 1A to locate initiation codons. Nature, 394, 854-9.

PESTOVA, T. V. & KOLUPAEVA, V. G. 2002. The roles of individual eukaryotic translation initiation factors in ribosomal scanning and initiation codon selection. Genes Dev, 16, 2906-22.

PETERSON, R. T., BEAL, P. A., COMB, M. J. & SCHREIBER, S. L. 2000. FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. J Biol Chem, 275, 7416-23.

PETERSON, T. R., LAPLANTE, M., THOREEN, C. C., SANCAK, Y., KANG, S. A., KUEHL, W. M., GRAY, N. S. & SABATINI, D. M. 2009. DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell, 137, 873-86.

POTTER, C. J., HUANG, H. & XU, T. 2001. Drosophila Tsc1 functions with Tsc2 to antagonize insulin signaling in regulating cell growth, cell proliferation, and organ size. Cell, 105, 357-68.

POTTER, C. J., PEDRAZA, L. G. & XU, T. 2002. Akt regulates growth by directly phosphorylating Tsc2. Nat Cell Biol, 4, 658-65.

POULIN, F., GINGRAS, A. C., OLSEN, H., CHEVALIER, S. & SONENBERG, N. 1998. 4E-BP3, a new member of the eukaryotic initiation factor 4E-binding protein family. J Biol Chem, 273, 14002-7.

PRATT, J. M., PETTY, J., RIBA-GARCIA, I., ROBERTSON, D. H., GASKELL, S. J., OLIVER, S. G. & BEYNON, R. J. 2002. Dynamics of protein turnover, a missing dimension in proteomics. Mol Cell Proteomics, 1, 579-91.

PROUD, C. G. 2005. eIF2 and the control of cell physiology. Semin Cell Dev Biol, 16, 3-12.

PULLEN, N., DENNIS, P. B., ANDJELKOVIC, M., DUFNER, A., KOZMA, S. C., HEMMINGS, B. A. & THOMAS, G. 1998. Phosphorylation and activation of p70s6k by PDK1. Science, 279, 707-10.

PYRONNET, S., DOSTIE, J. & SONENBERG, N. 2001. Suppression of cap-dependent translation in mitosis. Genes Dev, 15, 2083-93.

PYRONNET, S., IMATAKA, H., GINGRAS, A. C., FUKUNAGA, R., HUNTER, T. & SONENBERG, N. 1999. Human eukaryotic translation initiation factor 4G (eIF4G) recruits mnk1 to phosphorylate eIF4E. EMBO J, 18, 270-9.

QIN, X. & SARNOW, P. 2004. Preferential translation of internal ribosome entry site-containing mRNAs during the mitotic cycle in mammalian cells. J Biol Chem, 279, 13721-8.

QUELO, I., AKHOUAYRI, O., PRUD'HOMME, J. & ST-ARNAUD, R. 2004. GSK3 beta-dependent phosphorylation of the alpha NAC coactivator regulates its nuclear translocation and proteasome-mediated degradation. Biochemistry, 43, 2906-14.

RAFTOPOULOU, M., ETIENNE-MANNEVILLE, S., SELF, A., NICHOLLS, S. & HALL, A. 2004. Regulation of cell migration by the C2 domain of the tumor suppressor PTEN. Science, 303, 1179-81.

RAPPSILBER, J., ISHIHAMA, Y. & MANN, M. 2003. Stop and go extraction tips for matrix-assisted laser desorption/ionization, nanoelectrospray, and LC/MS sample pretreatment in proteomics. Anal Chem, 75, 663-70.

RAUGHT, B., GINGRAS, A. C., GYGI, S. P., IMATAKA, H., MORINO, S., GRADI, A., AEBERSOLD, R. & SONENBERG, N. 2000. Serum-stimulated, rapamycin-sensitive phosphorylation sites in the eukaryotic translation initiation factor 4GI. EMBO J, 19, 434-44.

RAUGHT, B., PEIRETTI, F., GINGRAS, A. C., LIVINGSTONE, M., SHAHBAZIAN, D., MAYEUR, G. L., POLAKIEWICZ, R. D., SONENBERG, N. & HERSHEY, J. W. 2004. Phosphorylation of eucaryotic translation initiation factor 4B Ser422 is modulated by S6 kinases. EMBO J, 23, 1761-9.

RAYASAM, G. V., TULASI, V. K., SODHI, R., DAVIS, J. A. & RAY, A. 2009. Glycogen synthase kinase 3: more than a namesake. Br J Pharmacol, 156, 885-98.

REDPATH, N. T., PRICE, N. T., SEVERINOV, K. V. & PROUD, C. G. 1993. Regulation of elongation factor-2 by multisite phosphorylation. Eur J Biochem, 213, 689-99.

RHEI, E., KANG, L., BOGOMOLNIY, F., FEDERICI, M. G., BORGEN, P. I. & BOYD, J. 1997. Mutation analysis of the putative tumor suppressor gene PTEN/MMAC1 in primary breast carcinomas. Cancer Res, 57, 3657-9.

RIDLER, K., SUCKLING, J., HIGGINS, N., BOLTON, P. & BULLMORE, E. 2004. Standardized whole brain mapping of tubers and subependymal nodules in tuberous sclerosis complex. J Child Neurol, 19, 658-65.

RISINGER, J. I., HAYES, A. K., BERCHUCK, A. & BARRETT, J. C. 1997. PTEN/MMAC1 mutations in endometrial cancers. Cancer Res, 57, 4736-8.

ROCCIO, M., BOS, J. L. & ZWARTKRUIS, F. J. 2006. Regulation of the small GTPase Rheb by amino acids. Oncogene, 25, 657-64.

ROGERS, G. W., JR., RICHTER, N. J. & MERRICK, W. C. 1999. Biochemical and kinetic characterization of the RNA helicase activity of eukaryotic initiation factor 4A. J Biol Chem, 274, 12236-44.

ROGERS, L. D. & FOSTER, L. J. 2007. The dynamic phagosomal proteome and the contribution of the endoplasmic reticulum. Proc Natl Acad Sci U S A, 104, 18520-5.

ROHDE, J. R. & CARDENAS, M. E. 2003. The tor pathway regulates gene expression by linking nutrient sensing to histone acetylation. Mol Cell Biol, 23, 629-35.

ROLFE, M., MCLEOD, L. E., PRATT, P. F. & PROUD, C. G. 2005. Activation of protein synthesis in cardiomyocytes by the hypertrophic agent phenylephrine requires the activation of ERK and involves phosphorylation of tuberous sclerosis complex 2 (TSC2). Biochem J, 388, 973-84.

ROSNER, M. & HENGSTSCHLAGER, M. 2008. Cytoplasmic and nuclear distribution of the protein complexes mTORC1 and mTORC2: rapamycin triggers dephosphorylation and delocalization of the mTORC2 components rictor and sin1. Hum Mol Genet, 17, 2934-48.

ROSNER, M. & HENGSTSCHLAGER, M. 2011a. Nucleocytoplasmic localization of p70 S6K1, but not of its isoforms p85 and p31, is regulated by TSC2/mTOR. Oncogene.

ROSNER, M. & HENGSTSCHLAGER, M. 2011b. Nucleocytoplasmic localization of p70 S6K1, but not of its isoforms p85 and p31, is regulated by TSC2/mTOR. Oncogene, 30, 4509-22.

ROUX, P. P., BALLIF, B. A., ANJUM, R., GYGI, S. P. & BLENIS, J. 2004. Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase. Proc Natl Acad Sci U S A, 101, 13489-94.

ROUX, P. P., SHAHBAZIAN, D., VU, H., HOLZ, M. K., COHEN, M. S., TAUNTON, J., SONENBERG, N. & BLENIS, J. 2007. RAS/ERK signaling promotes site-specific ribosomal protein S6 phosphorylation via RSK and stimulates cap-dependent translation. J Biol Chem, 282, 14056-64.

ROZEN, F., EDERY, I., MEEROVITCH, K., DEVER, T. E., MERRICK, W. C. & SONENBERG, N. 1990. Bidirectional RNA helicase activity of eucaryotic translation initiation factors 4A and 4F. Mol Cell Biol, 10, 1134-44.

ROZOVSKY, N., BUTTERWORTH, A. C. & MOORE, M. J. 2008. Interactions between eIF4AI and its accessory factors eIF4B and eIF4H. RNA, 14, 2136-48.

RUVINSKY, I., KATZ, M., DREAZEN, A., GIELCHINSKY, Y., SAADA, A., FREEDMAN, N., MISHANI, E., ZIMMERMAN, G., KASIR, J. & MEYUHAS, O. 2009. Mice deficient in ribosomal protein S6 phosphorylation suffer from muscle weakness that reflects a growth defect and energy deficit. PLoS One, 4, e5618.

RUVINSKY, I., SHARON, N., LERER, T., COHEN, H., STOLOVICH-RAIN, M., NIR, T., DOR, Y., ZISMAN, P. & MEYUHAS, O. 2005. Ribosomal protein S6 phosphorylation is a determinant of cell size and glucose homeostasis. Genes Dev, 19, 2199-211.

RYAZANOV, A. G. & DAVYDOVA, E. K. 1989. Mechanism of elongation factor 2 (EF-2) inactivation upon phosphorylation. Phosphorylated EF-2 is unable to catalyze translocation. FEBS Lett, 251, 187-90.

RYCHLIK, W., DOMIER, L. L., GARDNER, P. R., HELLMANN, G. M. & RHOADS, R. E. 1987. Amino acid sequence of the mRNA cap-binding protein from human tissues. Proc Natl Acad Sci U S A, 84, 945-9.

SABATINI, D. M., ERDJUMENT-BROMAGE, H., LUI, M., TEMPST, P. & SNYDER, S. H. 1994. RAFT1: a mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell, 78, 35-43.

SACHDEV, D., HARTELL, J. S., LEE, A. V., ZHANG, X. & YEE, D. 2004. A dominant negative type I insulin-like growth factor receptor inhibits metastasis of human cancer cells. J Biol Chem, 279, 5017-24.

SALITURO, F. G., GERMANN, U. A., WILSON, K. P., BEMIS, G. W., FOX, T. & SU, M. S. 1999. Inhibitors of p38 MAP kinase: therapeutic intervention in cytokine-mediated diseases. Curr Med Chem, 6, 807-23.

SALMENA, L., CARRACEDO, A. & PANDOLFI, P. P. 2008. Tenets of PTEN tumor suppression. Cell, 133, 403-14.

SANCAK, Y., BAR-PELED, L., ZONCU, R., MARKHARD, A. L., NADA, S. & SABATINI, D. M. 2010. Ragulator-Rag complex targets mTORC1 to the lysosomal surface and is necessary for its activation by amino acids. Cell, 141, 290-303.

SANCAK, Y., PETERSON, T. R., SHAUL, Y. D., LINDQUIST, R. A., THOREEN, C. C., BAR-PELED, L. & SABATINI, D. M. 2008. The Rag GTPases bind raptor and mediate amino acid signaling to mTORC1. Science, 320, 1496-501.

SANCAK, Y., THOREEN, C. C., PETERSON, T. R., LINDQUIST, R. A., KANG, S. A., SPOONER, E., CARR, S. A. & SABATINI, D. M. 2007. PRAS40 is an insulin-regulated inhibitor of the mTORC1 protein kinase. Mol Cell, 25, 903-15.

SARBASSOV, D. D., ALI, S. M., KIM, D. H., GUERTIN, D. A., LATEK, R. R., ERDJUMENT-BROMAGE, H., TEMPST, P. & SABATINI, D. M. 2004. Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptor-independent pathway that regulates the cytoskeleton. Curr Biol, 14, 1296-302.

SARBASSOV, D. D., ALI, S. M., SENGUPTA, S., SHEEN, J. H., HSU, P. P., BAGLEY, A. F., MARKHARD, A. L. & SABATINI, D. M. 2006. Prolonged rapamycin treatment inhibits mTORC2 assembly and Akt/PKB. Mol Cell, 22, 159-68.

SARBASSOV, D. D., GUERTIN, D. A., ALI, S. M. & SABATINI, D. M. 2005. Phosphorylation and regulation of Akt/PKB by the rictor-mTOR complex. Science, 307, 1098-101.

SAUCEDO, L. J., GAO, X., CHIARELLI, D. A., LI, L., PAN, D. & EDGAR, B. A. 2003. Rheb promotes cell growth as a component of the insulin/TOR signalling network. Nat Cell Biol, 5, 566-71.

SCHALM, S. S. & BLENIS, J. 2002. Identification of a conserved motif required for mTOR signaling. Curr Biol, 12, 632-9.

SCHALM, S. S., FINGAR, D. C., SABATINI, D. M. & BLENIS, J. 2003. TOS motif-mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. Curr Biol, 13, 797-806.

SCHEPER, G. C., MORRICE, N. A., KLEIJN, M. & PROUD, C. G. 2001. The mitogen-activated protein kinase signal-integrating kinase Mnk2 is a eukaryotic initiation factor 4E kinase with high levels of basal activity in mammalian cells. Mol Cell Biol, 21, 743-54.

SCHEPER, G. C., PARRA, J. L., WILSON, M., VAN KOLLENBURG, B., VERTEGAAL, A. C., HAN, Z. G. & PROUD, C. G. 2003. The N and C termini of the splice variants of the human mitogen-activated protein kinase-interacting kinase Mnk2 determine activity and localization. Mol Cell Biol, 23, 5692-705.

SCHEPER, G. C. & PROUD, C. G. 2002. Does phosphorylation of the cap-binding protein eIF4E play a role in translation initiation? Eur J Biochem, 269, 5350-9.

SCHEPER, G. C., VAN KOLLENBURG, B., HU, J., LUO, Y., GOSS, D. J. & PROUD, C. G. 2002. Phosphorylation of eukaryotic initiation factor 4E markedly reduces its affinity for capped mRNA. J Biol Chem, 277, 3303-9.

SCHIBLER, U., KELLEY, D. E. & PERRY, R. P. 1977. Comparison of methylated sequences in messenger RNA and heterogeneous nuclear RNA from mouse L cells. J Mol Biol, 115, 695-714.

SCHMELZLE, T. & HALL, M. N. 2000. TOR, a central controller of cell growth. Cell, 103, 253-62.

SCHUBERT, M., BRAZIL, D. P., BURKS, D. J., KUSHNER, J. A., YE, J., FLINT, C. L., FARHANG-FALLAH, J., DIKKES, P., WAROT, X. M., RIO, C., CORFAS, G. & WHITE, M. F. 2003. Insulin receptor substrate-2 deficiency impairs brain growth and promotes tau phosphorylation. J Neurosci, 23, 7084-92.

SCHULTZ, H., ENGEL, K. & GAESTEL, M. 1997. PMA-induced activation of the p42/44ERK- and p38RK-MAP kinase cascades in HL-60 cells is PKC dependent but not essential for differentiation to the macrophage-like phenotype. J Cell Physiol, 173, 310-8.

SCHWANHAUSSER, B., BUSSE, D., LI, N., DITTMAR, G., SCHUCHHARDT, J., WOLF, J., CHEN, W. & SELBACH, M. 2011. Global quantification of mammalian gene expression control. Nature, 473, 337-42.

SCHWANHAUSSER, B., GOSSEN, M., DITTMAR, G. & SELBACH, M. 2009. Global analysis of cellular protein translation by pulsed SILAC. Proteomics, 9, 205-9.

SCHWARTZ, E. I., INTINE, R. V. & MARAIA, R. J. 2004. CK2 is responsible for phosphorylation of human La protein serine-366 and can modulate rpL37 5'-terminal oligopyrimidine mRNA metabolism. Mol Cell Biol, 24, 9580-91.

SEKIGUCHI, T., HIROSE, E., NAKASHIMA, N., II, M. & NISHIMOTO, T. 2001. Novel G proteins, Rag C and Rag D, interact with GTP-binding proteins, Rag A and Rag B. J Biol Chem, 276, 7246-57.

SHAH, O. J., WANG, Z. & HUNTER, T. 2004. Inappropriate activation of the TSC/Rheb/mTOR/S6K cassette induces IRS1/2 depletion, insulin resistance, and cell survival deficiencies. Curr Biol, 14, 1650-6.

SHAHBAZIAN, D., ROUX, P. P., MIEULET, V., COHEN, M. S., RAUGHT, B., TAUNTON, J., HERSHEY, J. W., BLENIS, J., PENDE, M. & SONENBERG, N. 2006. The mTOR/PI3K and MAPK pathways converge on eIF4B to control its phosphorylation and activity. EMBO J, 25, 2781-91.

SHAV-TAL, Y. & ZIPORI, D. 2002. PSF and p54(nrb)/NonO--multi-functional nuclear proteins. FEBS Lett, 531, 109-14.

SHEN, Z., PARDINGTON-PURTYMUN, P. E., COMEAUX, J. C., MOYZIS, R. K. & CHEN, D. J. 1996. UBL1, a human ubiquitin-like protein associating with human RAD51/RAD52 proteins. Genomics, 36, 271-9.

SHIMA, H., PENDE, M., CHEN, Y., FUMAGALLI, S., THOMAS, G. & KOZMA, S. C. 1998. Disruption of the p70(s6k)/p85(s6k) gene reveals a small mouse phenotype and a new functional S6 kinase. EMBO J, 17, 6649-59.

SHIN, B. S., MAAG, D., ROLL-MECAK, A., AREFIN, M. S., BURLEY, S. K., LORSCH, J. R. & DEVER, T. E. 2002. Uncoupling of initiation factor eIF5B/IF2 GTPase and translational activities by mutations that lower ribosome affinity. Cell, 111, 1015-25.

SHIOI, T., MCMULLEN, J. R., KANG, P. M., DOUGLAS, P. S., OBATA, T., FRANKE, T. F., CANTLEY, L. C. & IZUMO, S. 2002. Akt/protein kinase B promotes organ growth in transgenic mice. Mol Cell Biol, 22, 2799-809.

SHOSHANI, T., FAERMAN, A., METT, I., ZELIN, E., TENNE, T., GORODIN, S., MOSHEL, Y., ELBAZ, S., BUDANOV, A., CHAJUT, A., KALINSKI, H., KAMER, I., ROZEN, A., MOR, O., KESHET, E., LESHKOWITZ, D., EINAT, P., SKALITER, R. & FEINSTEIN, E. 2002. Identification of a novel hypoxia-inducible factor 1-responsive gene, RTP801, involved in apoptosis. Mol Cell Biol, 22, 2283-93.

SHUMWAY, S. D., LI, Y. & XIONG, Y. 2003. 14-3-3beta binds to and negatively regulates the tuberous sclerosis complex 2 (TSC2) tumor suppressor gene product, tuberin. J Biol Chem, 278, 2089-92.

SHVEYGERT, M., KAISER, C., BRADRICK, S. S. & GROMEIER, M. 2010. Regulation of eukaryotic initiation factor 4E (eIF4E) phosphorylation by mitogen-activated protein kinase occurs through modulation of Mnk1-eIF4G interaction. Mol Cell Biol, 30, 5160-7.

SINI, P., JAMES, D., CHRESTA, C. & GUICHARD, S. 2010. Simultaneous inhibition of mTORC1 and mTORC2 by mTOR kinase inhibitor AZD8055 induces autophagy and cell death in cancer cells. Autophagy, 6.

SMITH, E. M., FINN, S. G., TEE, A. R., BROWNE, G. J. & PROUD, C. G. 2005. The tuberous sclerosis protein TSC2 is not required for the regulation of the mammalian target of rapamycin by amino acids and certain cellular stresses. J Biol Chem, 280, 18717-27.

SOFER, A., LEI, K., JOHANNESSEN, C. M. & ELLISEN, L. W. 2005. Regulation of mTOR and cell growth in response to energy stress by REDD1. Mol Cell Biol, 25, 5834-45.

SONENBERG, N. & DEVER, T. E. 2003. Eukaryotic translation initiation factors and regulators. Curr Opin Struct Biol, 13, 56-63.

SONG, G., OUYANG, G. & BAO, S. 2005. The activation of Akt/PKB signaling pathway and cell survival. J Cell Mol Med, 9, 59-71.

STEELMAN, L. S., BERTRAND, F. E. & MCCUBREY, J. A. 2004. The complexity of PTEN: mutation, marker and potential target for therapeutic intervention. Expert Opin Ther Targets, 8, 537-50.

STILO, R., LIGUORO, D., DI JESO, B., LEONARDI, A. & VITO, P. 2003. The alpha-chain of the nascent polypeptide-associated complex binds to and regulates FADD function. Biochem Biophys Res Commun, 303, 1034-41.

STOCKER, H., RADIMERSKI, T., SCHINDELHOLZ, B., WITTWER, F., BELAWAT, P., DARAM, P., BREUER, S., THOMAS, G. & HAFEN, E. 2003. Rheb is an essential regulator of S6K in controlling cell growth in Drosophila. Nat Cell Biol, 5, 559-65.

STOKOE, D., STEPHENS, L. R., COPELAND, T., GAFFNEY, P. R., REESE, C. B., PAINTER, G. F., HOLMES, A. B., MCCORMICK, F. & HAWKINS, P. T. 1997. Dual role of phosphatidylinositol-3,4,5-trisphosphate in the activation of protein kinase B. Science, 277, 567-70.

STOLOVICH, M., TANG, H., HORNSTEIN, E., LEVY, G., COHEN, R., BAE, S. S., BIRNBAUM, M. J. & MEYUHAS, O. 2002. Transduction of growth or mitogenic signals into translational activation of TOP mRNAs is fully reliant on the phosphatidylinositol 3-kinase-mediated pathway but requires neither S6K1 nor rpS6 phosphorylation. Mol Cell Biol, 22, 8101-13.

SUDHAKAR, A., RAMACHANDRAN, A., GHOSH, S., HASNAIN, S. E., KAUFMAN, R. J. & RAMAIAH, K. V. 2000. Phosphorylation of serine 51 in initiation factor 2 alpha (eIF2 alpha) promotes complex formation between eIF2 alpha(P) and eIF2B and causes inhibition in the guanine nucleotide exchange activity of eIF2B. Biochemistry, 39, 12929-38.

SULIS, M. L. & PARSONS, R. 2003. PTEN: from pathology to biology. Trends Cell Biol, 13, 478-83.

SUN, X. J., PONS, S., WANG, L. M., ZHANG, Y., YENUSH, L., BURKS, D., MYERS, M. G., JR., GLASHEEN, E., COPELAND, N. G., JENKINS, N. A., PIERCE, J. H. & WHITE, M. F. 1997. The IRS-2 gene on murine chromosome 8 encodes a unique signaling adapter for insulin and cytokine action. Mol Endocrinol, 11, 251-62.

SUZUKI, Y., YAMASHITA, R., NAKAI, K. & SUGANO, S. 2002. DBTSS: DataBase of human Transcriptional Start Sites and full-length cDNAs. Nucleic Acids Res, 30, 328-31.

SVITKIN, Y. V., PAUSE, A., HAGHIGHAT, A., PYRONNET, S., WITHERELL, G., BELSHAM, G. J. & SONENBERG, N. 2001. The requirement for eukaryotic initiation factor 4A (eIF4A) in translation is in direct proportion to the degree of mRNA 5' secondary structure. RNA, 7, 382-94.

TABANCAY, A. P., JR., GAU, C. L., MACHADO, I. M., UHLMANN, E. J., GUTMANN, D. H., GUO, L. & TAMANOI, F. 2003. Identification of dominant negative mutants of Rheb GTPase and their use to implicate the involvement of human Rheb in the activation of p70S6K. J Biol Chem, 278, 39921-30.

TANG, H., HORNSTEIN, E., STOLOVICH, M., LEVY, G., LIVINGSTONE, M., TEMPLETON, D., AVRUCH, J. & MEYUHAS, O. 2001. Amino acid-induced translation of TOP mRNAs is fully dependent on phosphatidylinositol 3-kinase-mediated signaling, is partially inhibited by rapamycin, and is independent of S6K1 and rpS6 phosphorylation. Mol Cell Biol, 21, 8671-83.

TAPON, N., ITO, N., DICKSON, B. J., TREISMAN, J. E. & HARIHARAN, I. K. 2001. The Drosophila tuberous sclerosis complex gene homologs restrict cell growth and cell proliferation. Cell, 105, 345-55.

TEE, A. R., MANNING, B. D., ROUX, P. P., CANTLEY, L. C. & BLENIS, J. 2003. Tuberous sclerosis complex gene products, Tuberin and Hamartin, control mTOR signaling by acting as a GTPase-activating protein complex toward Rheb. Curr Biol, 13, 1259-68.

TEE, A. R. & PROUD, C. G. 2002. Caspase cleavage of initiation factor 4E-binding protein 1 yields a dominant inhibitor of cap-dependent translation and reveals a novel regulatory motif. Mol Cell Biol, 22, 1674-83.

TERADA, N., PATEL, H. R., TAKASE, K., KOHNO, K., NAIRN, A. C. & GELFAND, E. W. 1994. Rapamycin selectively inhibits translation of mRNAs encoding elongation factors and ribosomal proteins. Proc Natl Acad Sci U S A, 91, 11477-81.

THEDIECK, K., POLAK, P., KIM, M. L., MOLLE, K. D., COHEN, A., JENO, P., ARRIEUMERLOU, C. & HALL, M. N. 2007. PRAS40 and PRR5-like protein are new mTOR interactors that regulate apoptosis. PLoS One, 2, e1217.

THOMAS, G. & LUTHER, H. 1981. Transcriptional and translational control of cytoplasmic proteins after serum stimulation of quiescent Swiss 3T3 cells. Proc Natl Acad Sci U S A, 78, 5712-6.

THOREEN, C. C., KANG, S. A., CHANG, J. W., LIU, Q., ZHANG, J., GAO, Y., REICHLING, L. J., SIM, T., SABATINI, D. M. & GRAY, N. S. 2009. An ATP-competitive mammalian target of rapamycin inhibitor reveals rapamycin-resistant functions of mTORC1. J Biol Chem, 284, 8023-32.

TICHY, A., VAVROVA, J., PEJCHAL, J. & REZACOVA, M. 2010. Ataxia-telangiectasia mutated kinase (ATM) as a central regulator of radiation-induced DNA damage response. Acta Medica (Hradec Kralove), 53, 13-7.

TINTON, S. A. & BUC-CALDERON, P. M. 1999. Hypoxia increases the association of 4E-binding protein 1 with the initiation factor 4E in isolated rat hepatocytes. FEBS Lett, 446, 55-9.

TORRES, J. & PULIDO, R. 2001. The tumor suppressor PTEN is phosphorylated by the protein kinase CK2 at its C terminus. Implications for PTEN stability to proteasome-mediated degradation. J Biol Chem, 276, 993-8.

TRAN, H., BRUNET, A., GRIFFITH, E. C. & GREENBERG, M. E. 2003. The many forks in FOXO's road. Sci STKE, 2003, RE5.

TSCHOPP, C., KNAUF, U., BRAUCHLE, M., ZURINI, M., RAMAGE, P., GLUECK, D., NEW, L., HAN, J. & GRAM, H. 2000. Phosphorylation of eIF-4E on Ser 209 in response to mitogenic and inflammatory stimuli is faithfully detected by specific antibodies. Mol Cell Biol Res Commun, 3, 205-211.

UEDA, T., SASAKI, M., ELIA, A. J., CHIO, II, HAMADA, K., FUKUNAGA, R. & MAK, T. W. 2010. Combined deficiency for MAP kinase-interacting kinase 1 and 2 (Mnk1 and Mnk2) delays tumor development. Proc Natl Acad Sci U S A, 107, 13984-90.

UEDA, T., WATANABE-FUKUNAGA, R., FUKUYAMA, H., NAGATA, S. & FUKUNAGA, R. 2004. Mnk2 and Mnk1 are essential for constitutive and inducible phosphorylation of eukaryotic initiation factor 4E but not for cell growth or development. Mol Cell Biol, 24, 6539-49.

UETSUKI, T., NAITO, A., NAGATA, S. & KAZIRO, Y. 1989. Isolation and characterization of the human chromosomal gene for polypeptide chain elongation factor-1 alpha. J Biol Chem, 264, 5791-8.

UM, S. H., FRIGERIO, F., WATANABE, M., PICARD, F., JOAQUIN, M., STICKER, M., FUMAGALLI, S., ALLEGRINI, P. R., KOZMA, S. C., AUWERX, J. & THOMAS, G. 2004. Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity. Nature, 431, 200-5.

VANDER HAAR, E., LEE, S. I., BANDHAKAVI, S., GRIFFIN, T. J. & KIM, D. H. 2007. Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat Cell Biol, 9, 316-23.

VEZINA, C., KUDELSKI, A. & SEHGAL, S. N. 1975. Rapamycin (AY-22,989), a new antifungal antibiotic. I. Taxonomy of the producing streptomycete and isolation of the active principle. J Antibiot (Tokyo), 28, 721-6.

VIVANCO, I. & SAWYERS, C. L. 2002. The phosphatidylinositol 3-Kinase AKT pathway in human cancer. Nat Rev Cancer, 2, 489-501.

WAN, X. & HELMAN, L. J. 2007. The biology behind mTOR inhibition in sarcoma. Oncologist, 12, 1007-18.

WANG, G. L. & SEMENZA, G. L. 1993. General involvement of hypoxia-inducible factor 1 in transcriptional response to hypoxia. Proc Natl Acad Sci U S A, 90, 4304-8.

WANG, G. L. & SEMENZA, G. L. 1995. Purification and characterization of hypoxia-inducible factor 1. J Biol Chem, 270, 1230-7.

WANG, L., HARRIS, T. E., ROTH, R. A. & LAWRENCE, J. C., JR. 2007a. PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. J Biol Chem, 282, 20036-44.

WANG, X., BEUGNET, A., MURAKAMI, M., YAMANAKA, S. & PROUD, C. G. 2005. Distinct signaling events downstream of mTOR cooperate to mediate the effects of amino acids and insulin on initiation factor 4E-binding proteins. Mol Cell Biol, 25, 2558-72.

WANG, X., FLYNN, A., WASKIEWICZ, A. J., WEBB, B. L., VRIES, R. G., BAINES, I. A., COOPER, J. A. & PROUD, C. G. 1998. The phosphorylation of eukaryotic initiation factor eIF4E in response to phorbol esters, cell stresses, and cytokines is mediated by distinct MAP kinase pathways. J Biol Chem, 273, 9373-7.

WANG, X., LI, W., PARRA, J. L., BEUGNET, A. & PROUD, C. G. 2003. The C terminus of initiation factor 4E-binding protein 1 contains multiple regulatory features that influence its function and phosphorylation. Mol Cell Biol, 23, 1546-57.

WANG, X., LI, W., WILLIAMS, M., TERADA, N., ALESSI, D. R. & PROUD, C. G. 2001. Regulation of elongation factor 2 kinase by p90(RSK1) and p70 S6 kinase. EMBO J, 20, 4370-9.

WANG, X., YUE, P., CHAN, C. B., YE, K., UEDA, T., WATANABE-FUKUNAGA, R., FUKUNAGA, R., FU, H., KHURI, F. R. & SUN, S. Y. 2007b. Inhibition of mammalian target of rapamycin induces phosphatidylinositol 3-kinase-dependent and Mnk-mediated eukaryotic translation initiation factor 4E phosphorylation. Mol Cell Biol, 27, 7405-13.

WASKIEWICZ, A. J., FLYNN, A., PROUD, C. G. & COOPER, J. A. 1997. Mitogen-activated protein kinases activate the serine/threonine kinases Mnk1 and Mnk2. EMBO J, 16, 1909-20.

WASKIEWICZ, A. J., JOHNSON, J. C., PENN, B., MAHALINGAM, M., KIMBALL, S. R. & COOPER, J. A. 1999. Phosphorylation of the cap-binding protein eukaryotic translation initiation factor 4E by protein kinase Mnk1 in vivo. Mol Cell Biol, 19, 1871-80.

WEBB, D. W., THOMAS, R. D. & OSBORNE, J. P. 1993. Cardiac rhabdomyomas and their association with tuberous sclerosis. Arch Dis Child, 68, 367-70.

WELLS, S. E., HILLNER, P. E., VALE, R. D. & SACHS, A. B. 1998. Circularization of mRNA by eukaryotic translation initiation factors. Mol Cell, 2, 135-40.

WENDEL, H. G., DE STANCHINA, E., FRIDMAN, J. S., MALINA, A., RAY, S., KOGAN, S., CORDON-CARDO, C., PELLETIER, J. & LOWE, S. W. 2004. Survival signalling by Akt and eIF4E in oncogenesis and cancer therapy. Nature, 428, 332-7.

WENDEL, H. G., SILVA, R. L., MALINA, A., MILLS, J. R., ZHU, H., UEDA, T., WATANABE-FUKUNAGA, R., FUKUNAGA, R., TERUYA-FELDSTEIN, J., PELLETIER, J. & LOWE, S. W. 2007. Dissecting eIF4E action in tumorigenesis. Genes Dev, 21, 3232-7.

WHEATER, M. J., JOHNSON, P. W. & BLAYDES, J. P. 2010. The role of MNK proteins and eIF4E phosphorylation in breast cancer cell proliferation and survival. Cancer Biol Ther, 10, 728-35.

WHITE, M. F. 1998. The IRS-signalling system: a network of docking proteins that mediate insulin action. Mol Cell Biochem, 182, 3-11.

WHITE, M. F., MARON, R. & KAHN, C. R. 1985. Insulin rapidly stimulates tyrosine phosphorylation of a Mr-185,000 protein in intact cells. Nature, 318, 183-6.

WHITE, R. J. 2005. RNA polymerases I and III, growth control and cancer. Nat Rev Mol Cell Biol, 6, 69-78.

WIEDMANN, B., SAKAI, H., DAVIS, T. A. & WIEDMANN, M. 1994. A protein complex required for signal-sequence-specific sorting and translocation. Nature, 370, 434-40.

WILKINSON, K. A. & HENLEY, J. M. 2010. Mechanisms, regulation and consequences of protein SUMOylation. Biochem J, 428, 133-45.

WITHERS, D. J., BURKS, D. J., TOWERY, H. H., ALTAMURO, S. L., FLINT, C. L. & WHITE, M. F. 1999. Irs-2 coordinates Igf-1 receptor-mediated beta-cell development and peripheral insulin signalling. Nat Genet, 23, 32-40.

WITHERS, D. J., GUTIERREZ, J. S., TOWERY, H., BURKS, D. J., REN, J. M., PREVIS, S., ZHANG, Y., BERNAL, D., PONS, S., SHULMAN, G. I., BONNER-WEIR, S. & WHITE, M. F. 1998. Disruption of IRS-2 causes type 2 diabetes in mice. Nature, 391, 900-4.

WOO, S. Y., KIM, D. H., JUN, C. B., KIM, Y. M., HAAR, E. V., LEE, S. I., HEGG, J. W., BANDHAKAVI, S. & GRIFFIN, T. J. 2007. PRR5, a novel component of mTOR complex 2, regulates platelet-derived growth factor receptor beta expression and signaling. J Biol Chem, 282, 25604-12.

XU, X., VATSYAYAN, J., GAO, C., BAKKENIST, C. J. & HU, J. 2010. Sumoylation of eIF4E activates mRNA translation. EMBO Rep, 11, 299-304.

YAN, J., KUROYANAGI, H., TOMEMORI, T., OKAZAKI, N., ASATO, K., MATSUDA, Y., SUZUKI, Y., OHSHIMA, Y., MITANI, S., MASUHO, Y., SHIRASAWA, T. & MURAMATSU, M. 1999. Mouse ULK2, a novel member of the UNC-51-like protein kinases: unique features of functional domains. Oncogene, 18, 5850-9.

YAN, L., MIEULET, V., BURGESS, D., FINDLAY, G. M., SULLY, K., PROCTER, J., GORIS, J., JANSSENS, V., MORRICE, N. A. & LAMB, R. F. 2010. PP2A T61 epsilon is an inhibitor of MAP4K3 in nutrient signaling to mTOR. Mol Cell, 37, 633-42.

YANG, H. S., JANSEN, A. P., KOMAR, A. A., ZHENG, X., MERRICK, W. C., COSTES, S., LOCKETT, S. J., SONENBERG, N. & COLBURN, N. H. 2003. The transformation suppressor Pdcd4 is a novel eukaryotic translation initiation factor 4A binding protein that inhibits translation. Mol Cell Biol, 23, 26-37.

YANG, Q. & GUAN, K. L. 2007. Expanding mTOR signaling. Cell Res, 17, 666-81.

YANG, Q., INOKI, K., IKENOUE, T. & GUAN, K. L. 2006. Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev, 20, 2820-32.

YORIMITSU, T., HE, C., WANG, K. & KLIONSKY, D. J. 2009. Tap42-associated protein phosphatase type 2A negatively regulates induction of autophagy. Autophagy, 5, 616-24.

YUAN, R., KAY, A., BERG, W. J. & LEBWOHL, D. 2009. Targeting tumorigenesis: development and use of mTOR inhibitors in cancer therapy. J Hematol Oncol, 2, 45.

YUAN, X., ZHAO, J., ZENTGRAF, H., HOFFMANN-ROHRER, U. & GRUMMT, I. 2002. Multiple interactions between RNA polymerase I, TIF-IA and TAF(I) subunits regulate

preinitiation complex assembly at the ribosomal gene promoter. EMBO Rep, 3, 1082-7.

ZASK, A., VERHEIJEN, J. C. & RICHARD, D. J. 2011. Recent advances in the discovery of small-molecule ATP competitive mTOR inhibitors: a patent review. Expert Opin Ther Pat, 21, 1109-27.

ZATSEPINA, O. V., ROUSSELET, A., CHAN, P. K., OLSON, M. O., JORDAN, E. G. & BORNENS, M. 1999. The nucleolar phosphoprotein B23 redistributes in part to the spindle poles during mitosis. J Cell Sci, 112 (Pt 4), 455-66.

ZEQIRAJ, E., FILIPPI, B. M., DEAK, M., ALESSI, D. R. & VAN AALTEN, D. M. 2009. Structure of the LKB1-STRAD-MO25 complex reveals an allosteric mechanism of kinase activation. Science, 326, 1707-11.

ZHANG, H., CICCHETTI, G., ONDA, H., KOON, H. B., ASRICAN, K., BAJRASZEWSKI, N., VAZQUEZ, F., CARPENTER, C. L. & KWIATKOWSKI, D. J. 2003a. Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR. J Clin Invest, 112, 1223-33.

ZHANG, Y., GAO, X., SAUCEDO, L. J., RU, B., EDGAR, B. A. & PAN, D. 2003b. Rheb is a direct target of the tuberous sclerosis tumour suppressor proteins. Nat Cell Biol, 5, 578-81.

ZHOU, B. P., LIAO, Y., XIA, W., ZOU, Y., SPOHN, B. & HUNG, M. C. 2001. HER-2/neu induces p53 ubiquitination via Akt-mediated MDM2 phosphorylation. Nat Cell Biol, 3, 973-82.

ZINZALLA, V., STRACKA, D., OPPLIGER, W. & HALL, M. N. 2011. Activation of mTORC2 by association with the ribosome. Cell, 144, 757-68.

ZLATANOVA, J., SEEBART, C. & TOMSCHIK, M. 2007. Nap1: taking a closer look at a juggler protein of extraordinary skills. FASEB J, 21, 1294-310.

ZONG, Q., SCHUMMER, M., HOOD, L. & MORRIS, D. R. 1999. Messenger RNA translation state: the second dimension of high-throughput expression screening. Proc Natl Acad Sci U S A, 96, 10632-6.

ZUBEREK, J., WYSLOUCH-CIESZYNSKA, A., NIEDZWIECKA, A., DADLEZ, M., STEPINSKI, J., AUGUSTYNIAK, W., GINGRAS, A. C., ZHANG, Z., BURLEY, S. K., SONENBERG, N., STOLARSKI, R. & DARZYNKIEWICZ, E. 2003. Phosphorylation of eIF4E attenuates its interaction with mRNA 5' cap analogs by electrostatic repulsion: intein-mediated protein ligation strategy to obtain phosphorylated protein. RNA, 9, 52-61.