IDENTIFYING AND CHARACTERISING DOWNSTREAM SUBSTRATES OF mTORC1

Kayleigh Dodd

2011

Thesis submitted to Cardiff University in fulfilment of the requirements for the degree of Doctor of Philosophy.

ProQuest LLC
789 East Eisenhower Parkway
P.O. Box 1346
Ann Arbor, MI 48106-1346

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

CONTENTS

FIGURES & TABLES

FIGURES & TABLES

FIGURES & TABLES

ACKNOWLEDGEMENTS

I would like to thanks the following people:

My supervisors; Dr Andy Tee and Prof. Julian Sampson for their guidance and support and for providing me with this opportunity.

Prof. John Blenis, Prof. Chris Proud, Prof. Cheryl Walker and Dr. Mark Nellist's laboratories for providing me with various reagents and valuable discussions.

The Tee lab; thank you to Dr Elaine Dunlop for all the help with kinase assays and cloning, David Hunt, Rachael Preston, Lyndsey Seymour, Tijs Claessens, Katy Harper, Rienk Doetjes, Ellie Rad, Sara Seifan and everyone else who has been a part of the Tee lab, thank you for all of your help and support over the years.

The gene park staff for allowing us to take over your lab, I hope we weren't too much of a pain! Thank you to Vikki Humphries, Pete Davies, Julie Maynard, James Colley and also thank you to Shelley Idziaszczyk for being so helpful and welcoming.

I also like to thank all the people who have helped me to feel at home in Cardiff, in particular Mark Aldred, Becky Harris, Chris Smith, Hannah West, JP Hothi, Laura Thomas and everyone else that has been part of the PhD office or nearby; Maria Kalogerou, Duncan Azzopardi, Cleo Bonnet and Natalie Jones. It has been an absolute pleasure.

Finally I would like to thank my family and friends in Bristol, particularly the residents of Weavers Mill Close, for their continuing support and for always being available for a pint when needed, it is greatly appreciated.

SUMMARY

mTORC1 functions as a nutrient sensor within the cell. It integrates signalling inputs from growth factors, nutrients and amino acids to regulate key cellular processes involving growth, proliferation and development. Perturbed mTORC1 signalling is a feature of a variety of diseases including the hamartoma syndromes, various cancer types as well as both autoimmune and neurological diseases; yet many downstream signalling effects remain uncharacterised.

The central aim of this study was to characterise mTORC1 as a regulator of transcription. This has been carried out focusing upon the regulation of two transcription factors which are fundamental to the pathophysiology of the hamartoma disorders and many types of cancer; HIF-1α and STAT3.

I have shown evidence that mTORC1 is able to regulate HIF-1α on a translational level through phosphorylation of 4E-BP1. I have also shown that mTORC1 is able to regulate the synthesis of HIF-1α mRNA to further augment its activity.

I present evidence that HIF-1α is subject to mTORC1 independent regulation from the upstream regulator TSC2. Significantly, mTORC1 inhibitors were able to normalise HIF-1α elevation through TSC1/2 loss in the absence of TSC1 but not TSC2 in a cell line model for the disease Tuberous Sclerosis.

Furthermore I have shown that mTORC1 can directly phosphorylate STAT3 at Ser727, promoting its transcriptional activity. I have also shown direct functional link between STAT3 and HIF-1α.

This study includes an analysis of the current knowledge regarding these two transcription factors and highlights the possibilities for targeting this signalling pathway in the treatment of disease.

ABBREVIATIONS

17-AAG	17-allylaminogeldanamycin
4E-BPs	eIF4E-binding proteins
5'TOP	5' tract of pyrimidine
5'UTR	5' untranslated region
AA	Amino acids
AGC	cAMP-dependent, cGMP-dependent and protein kinase C
ALPS	Autoimmune lymphoproliferative syndrome
AML	Angiomyolipomas
AMP	Adenosine Monophosphate
AMPK	AMP-activated protein kinase
AP-1/2	Activator-protein 1/2
APE	Apyrimidinic endonuclease
ASD	Autism spectrum disorder
ATGL	Adipose triglyceride lipase
ATM	Ataxia telangiectasia mutated-dependent kinase
Aβ	Amyloid β-peptide
BAD	BCL2-associated agonist of cell death
B-CLL	B-cell chronic lymphocytic leukemia
BNIP3	Bcl-2/adenovirus E1B 19-kDa interacting protein 3
BRRS	Bannayan-Riley-Ruvalcaba syndrome
BSA	Bovine serum albumin
CaM	Calmodulin
CamKII	Ca2+/calmodulin-dependent kinase-II
cAMP	Cyclic-AMP
CBC	Cap-binding-complex
CBP80	Nuclear cap binding protein subunit 1 of 80kDa
Cdc42	Cell division cycle 42 (GTP binding protein of 25kDa)
CdKs	Cyclin dependent kinases
cDNA	Complementary deoxyribonucleic acid
CHiP	Chromatin immunprecipitation
CMA	Chaperone mediated autophagy
CNTF	Ciliary neurotrophic factor
CREMτ	cAMP-response modulator
DEP	Dishevelled, egl-10, pleckstrin domain
Deptor	DEP domain containing mTOR-interacting protein
DKO	Double knockout
DMEM	Dulbecco's modified eagle medium
DMOG	Dimethyloxallyl glycine
DNA	Deoxyribonucleic acid
D-PBS	Dulbecco's phosphate-buffered saline

ABBREVIATIONS

dsDNA	Double stranded-deoxyribonucleic acid
ECL	Enhanced chemiluminescence
eEFs	Eukaryotic elongation factors
Egr-1	Early growth response-1,
eIFs	Eukaryotic initiation factors
EJC	Exon junction complex
EMSA	Electrophoretic mobility shift assay
ER	Endoplasmic reticulum
ERK1/2	Extracellular signal regulated kinase
ERR-α	Oestrogen related receptor-alpha
ERα	Oestrogen receptor-alpha
FAT(C)	FRAP, ATM, and TRRAP (COOH-terminal) domain
FIP200	Focal adhesion kinase family interacting protein of 200 kD
FKBP12	FK506-binding protein of 12/36-kDa
FOXO	Forkhead box A to S
FRB	FK506-binding protein of 12-kDa-rapamycin binding domain
GAP	GTPase activating protein
GDP	Guanine diphosphate
GEF	Guanine exchange factor
GLUT1-4	Glucose transporters 1-4
GSK3β	Glycogen synthase kinase-3-beta
GST	Glutothione S-transferase
GTFs	General transcription factors
GTP	Guanine triphosphate
GTPase	Guanine triphosphate-hydrolase
HD	Huntingtons disease
HEAT	Huntingtin, elongation factor 3, a subunit of protein phosphatase 2A, and TOR1 domain
HEK293s	Human embryonic kidney cells
HIFs	Hypoxia inducible factors
HIV	Human immunodeficiency virus
HRE	HIF response elements
HSL	Hormone sensitive lipase
Hsp90	Heat shock protein-90
ID	Inhibitory domain
IFN-γ	Interferon-gamma
IGF	Insulin-like growth factor
IL-6	Interleukin-6
IRES	Internal ribosomal entry site

ABBREVIATIONS

IRF-1	Interferon response factor-1
IRS1-4	Insulin receptor substrate 1-4
ITAF	Internal ribosomal entry site - transactivation factors
JAKs	Janus kinases
JNK	c-Jun N-terminal kinases
LAM	Lymphangioleiomyomatosis
LIF	Leukaemia inhibtory factor
LKB1	Also known as Ser/Thr kinase 11
LPA	Lysophosphatidic acid
MAD-1	MAX dimerization protein-1
MAP4K3	Mitogen-activated protein kinase kinase kinase kinase 3
MCM5	Minichromosome maintenance complex component 5
MDM	Monocyte derived macrophages
MEFs	Mouse embryonic fibroblasts
MEK	MAPK/ERK kinase
MEKK1	MEK kinase-1
MEM	Minimum essential media
mLST8	mTOR associated protein, LST8 homolog (S. cerevisiae)
mRNA	messenger ribonucleic acid
mTOR	mechanistic/mammalian target of rapamycin
mTORC1/2	mechanistic target of rapamycin complex 1/2
NBFL	Human neuroblastoma cell line
NF-1	Neurofibromin-1
NPR2/3	Nitrogen permease regulators 2/3
NRFs	Nuclear respiratory factors
NS	Not signficant
ODDD	Oxygen dependent degradation domain
OSM	Oncostatin-M
p90-RSK1	90 kDa ribosomal protein S6 kinase-1
PA	Phosphatidic acid
PABP	Poly-A binding proteins
PAS-A	Period-ARNT-Sim conserved domain-A
Pdcd4	Programmed cell death 4
PDGFR	Platelet-derived growth factor receptor-alpha
PDK1	Pyruvate dehydrogenase kinase, isozyme-1
PDZ	PSD-95/discs-large/ZO-1 domains
PEXs	Peroxisomal import receptors
PGC-1α	Peroxisome proliferator-activated receptor gamma, coactivator 1-alpha
PI3K	Phosphoinositide kinase
PIK3CA	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
PIKK	Phosphoinositide kinase-related kinase

ABBREVIATIONS

PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKB/C/δ/ε	Protein kinase A/B/C δ/ε
PKD	Polycystic kidney disease
PLD2	Phospholipase D2
PML	Promyelocytic leukaemia tumour suppressor
Pol I/II/III	RNA Polymerases
POLYPHEN	Polymorphism phenotyping
PP2A	Protein phosphatase type 2A
PPARγ	Peroxisome proliferator-activated receptor gamma
PPIase	Peptidylprolylisomerase
PRAS40	Proline enriched Akt substrate of 40-kDa
PROTOR1/2	Protein observed with rictor-1
PtdIns	Phosphoinositides
PtdIns3P	Phosphatidylinositol 3-phosphate
PTEN	Phosphatase and tensin homolog
PTS1/2	Peroxisome targeting signal type-1
PVDF	Polyvinylidene fluoride
Q-PCR	Quantitative-polymerase chain reaction
RalA/B	V-ral simian leukemia viral oncogene homolog A/B (ras related)
Raptor	Regulatory associated protein of mTORC1
Rb	Retinoblastoma gene product
REDD1/2	Also known as DNA-damage-inducible transcript 4 (DDIT4)
REPO	Recruitment of polycomb domain
Rheb	Ras homolog enriched in brain
Rictor	Raptor independent companion of mTORC2
RNC	Raptor N-terminal conserved domain
ROS	Reactive oxygen species
rpS6	Ribsomal protein S6
RTKs	Receptor tyrosine kinases
S1p	Sphingosine-1-phosphate
S6Ks	rpS6 kinases
SCAP	Sterol regulatory element-binding proteins-cleavage-activating protein
$SCF^{\beta TRCP}$	Skp1, Cdc53, and the F-box protein Cdc4 - β-transducin repeat-containing protein
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SH2	Src-homology-2 domain
shRNA	Small hairpin ribonucleic acid
SIFT	Sorting intolerant from tolerant
Sin-1	Stress-activated protein kinase-interacting protein-1
siRNA	Small interfering ribonucleic acid

ABBREVIATIONS

SKAR	S6K1 Aly/REF-like substrate
SLE	Systemic lupus erythematosus
SOCS	Suppressor of cytokine signalling
SREBP1	Sterol regulatory element-binding protein-1
STATs	Signal transducer and activator of transcription transcription factors
TAD	Transcriptional activation domain
TBS(T)	Tris buffered saline (plus tween)
TCTP	Translationally controlled tumour protein
TIP41	TAP42-interacting protein of 41 kDa
TM	Transmembrane domain
Tor1/2	Target of rapamycin (yeast homolog)
TOS	mTOR signalling motif
TPR	Tetratricopeptide repeat
tRNA	Transfer ribonucleic acid
TSC(1/2)	Tuberous sclerosis complex (1/2 - also known as tuberin and hamartin)
UBR1/2	Ubiquitin protein ligase E3 component n-recognin-1/2
ULK-1/2/3/4	UNC-51 like kinase-1/2/3/4
VEGF-A	Vascular endothelial growth factor-A
VHL	Von hippel-lindau tumour suppressor
Vps34/15	Vacuolar protein sorting 34/15
YY1	Yin-yang-1

CODON TABLE

	Second position of codon									
		T		C		A		G		
First position	T	TTT	F	TCT	S	TAT	Y	TGT	C	T
		TTC	F	TCC	S	TAC	Y	TGC	C	C
		TTA	L	TCA	S	TAA	Stop	TGA	Stop	A
		TTG	L	TCG	S	TAG	Stop	TGG	W	G
	C	CTT	L	CCT	P	CAT	H	CGT	R	T
		CTC	L	CCC	P	CAC	H	CGC	R	C
		CTA	L	CCA	P	CAA	Q	CGA	R	A
		CTG	L	CCG	P	CAG	Q	CGG	R	G
	A	ATT	I	ACT	T	AAT	N	AGT	S	T
		ATC	I	ACC	T	AAC	N	AGC	S	C
		ATA	I	ACA	T	AAA	K	AGA	R	A
		ATG	M	ACG	T	AAG	K	AGG	R	G
	G	GTT	V	GCT	A	GAT	D	GGT	G	T
		GTC	V	GCC	A	GAC	D	GGC	G	C
		GTA	V	GCA	A	GAA	E	GGA	G	A
		GTC	V	GCG	A	GAG	E	GGG	G	G

Third position

Key:

G – Glycine (Gly) W – Tryptophan (Trp)
P – Proline (Pro) H – Histidine (His)
A – Alanine (Ala) K – Lysine (Lys)
V – Valine (Val) R – Arginine (Arg)
L – Leucine (Leu) Q – Glutamine (Gln)
I – Isoleucine (Ile) N – Asparagine (Asn)
M – Methionine (Met) E – Glutamic Acid (Glu)
C – Cysteine (Cys) D – Aspartic Acid (Asp)
F – Phenylalanine (Phe) S – Serine (Ser)
Y – Tyrosine (Tyr) T – Threonine (Thr)

CHAPTER 1: INTRODUCTION

1.1 OXYGEN

Throughout evolution, the earth's atmospheric oxygen levels have fluctuated. The arrival of photosynthetic organisms initiated a gradual rise in atmospheric oxygen, which increased exponentially as plant life flourished [1, 2]. This rise facilitated the evolution of eukaryotic organisms with cellular mitochondria. Complex organisms are dependent upon the mitochondrial process of oxidative phosphorylation, which produces significant levels of ATP from molecular glucose; this process is critically dependent upon oxygen. It is this process which facilitated the evolution of complex, multicellular organisms, including the evolution of man. These variations in atmospheric oxygen levels have therefore been one of most significant driving forces behind the evolution of our species.

A vast network of blood vessels distributes nutrients and oxygen around the human body. Oxygen diffuses through the tissue and into the cell where it reaches the mitochondria. In the mitochondria, an electrochemical gradient is formed as electrons are passed from electron donors such as NADH (nicotinamide adenine dinucleotide).. Oxygen is the final electron acceptor, and is ultimately reduced to H_2O [1, 3]. The electrochemical gradient produced from these reactions drives the synthesis of ATP by ATPases, producing significant levels of cellular energy [4].

This process is not without its drawbacks, molecular oxygen can be released before it is fully reduced to H_20, one-electron reduction of O_2 results in the formation of reactive oxygen species (ROS) [2, 4]. This process, referred to as oxidative stress, has been linked to the aging process [5], as well as the pathology of a number of neurodegenerative diseases [6]. For these reasons, the maintenance of oxygen homeostasis is a critical process.

The human body is therefore highly sensitive to fluctuations in oxygen on both a systemic and a cellular level, oxygen levels must be maintained within strict parameters. Critical components in cellular oxygen sensing are the evolutionary conserved HIF proteins (hypoxia inducible factors). HIFs are transcription factors which, as indicated by the name, become activated under conditions of hypoxia. HIFs can alter gene expression in order to promote glucose uptake, increase anaerobic respiration and promote new blood vessel formation, increasing blood flow to the hypoxic tissue. This promotes cells survival during hypoxia and also facilitates

growth [7]. The involvement of HIFs in these processes makes them an interesting potential therapeutic target in the pathology of cancer. One unifying feature of all cancers is uncontrolled cellular growth and proliferation. As a tumour expands, the core becomes hypoxic; it therefore relies upon the activation of HIFs to facilitate new blood vessel formation, promoting survival and expansion of the tumour. Understanding how HIFs are regulated on a cellular level is therefore paramount to developing potential therapeutics in this field.

HIFs are continuously synthesised within the cell however the protein itself is highly unstable in the presence of oxygen (discussed in detail later) so is therefore only active when oxygen levels are low. Interestingly HIFs can be transactivated by various cellular signalling pathways. The molecular mechanisms governing this regulation are only just becoming apparent. This project is primarily focused upon elucidating links between an evolutionary conserved master of protein synthesis, referred to as mechanistic target of rapamycin complex-1 (mTORC1) and the cellular response to hypoxia. mTORC1 is regulated by nutrients, growth factors, hormones, mechanical stimulus, stress and hypoxia. It responds to fluctuations in the cellular environment to control a myriad of cellular processes. It is best characterised as a key mediator of protein synthesis, this process and the role mTORC1 plays in the cell are described in detail in the next section.

1.2 PROTEIN SYNTHESIS

Protein synthesis is a pivotal cell process whereby our genetic code is translated into physical characteristics. The physical characteristics of proteins are determined by the 20 different amino acids which make them. Cells translate a diverse array of proteins with different properties to fulfil specific cellular tasks. In order to efficiently translate genetic code, regulation of protein synthesis is subject to a massively diverse array of inputs and feedback mechanisms. Synthesis begins with the process of 'transcription' which is described below.

1.2.1 Transcription

Before protein synthesis, a copy of the DNA has to be made (or transcribed) this is messenger RNA (mRNA) and is synthesised using a single strand of the DNA as a template.

Transcription involves the copying of the nucleotide sequence from the gene into a single stranded polymer of nucleotides. The initiation of transcription is a major point of control for the regulation of gene expression and is primarily controlled by RNA polymerases. One of these polymerases in particular, RNA polymerase II (Pol II) regulates the transcription of all protein encoding genes. Pol II is subject to strict modulation from transcription factors, which have a substantial role to play in the regulation of gene expression, recent advances in biochemical and molecular techniques have identified thousands of factors which regulate transcription [8], thus furthering our understanding of this highly conserved process.

The rate of transcription can be also determined by gene-specific transcription factors, these bind to specific response elements contained within promoter or enhancer regions of the DNA to accelerate the rate of transcription. There are a large number of different transcription factors and the number and position of binding response elements differs between genes.

Differing combinations of transcription factors are produced depending upon cell type; this is thought to be how particular cell types are able to control specific gene expression [9]. The transcription cycle is outlined in figure 1.1 below.

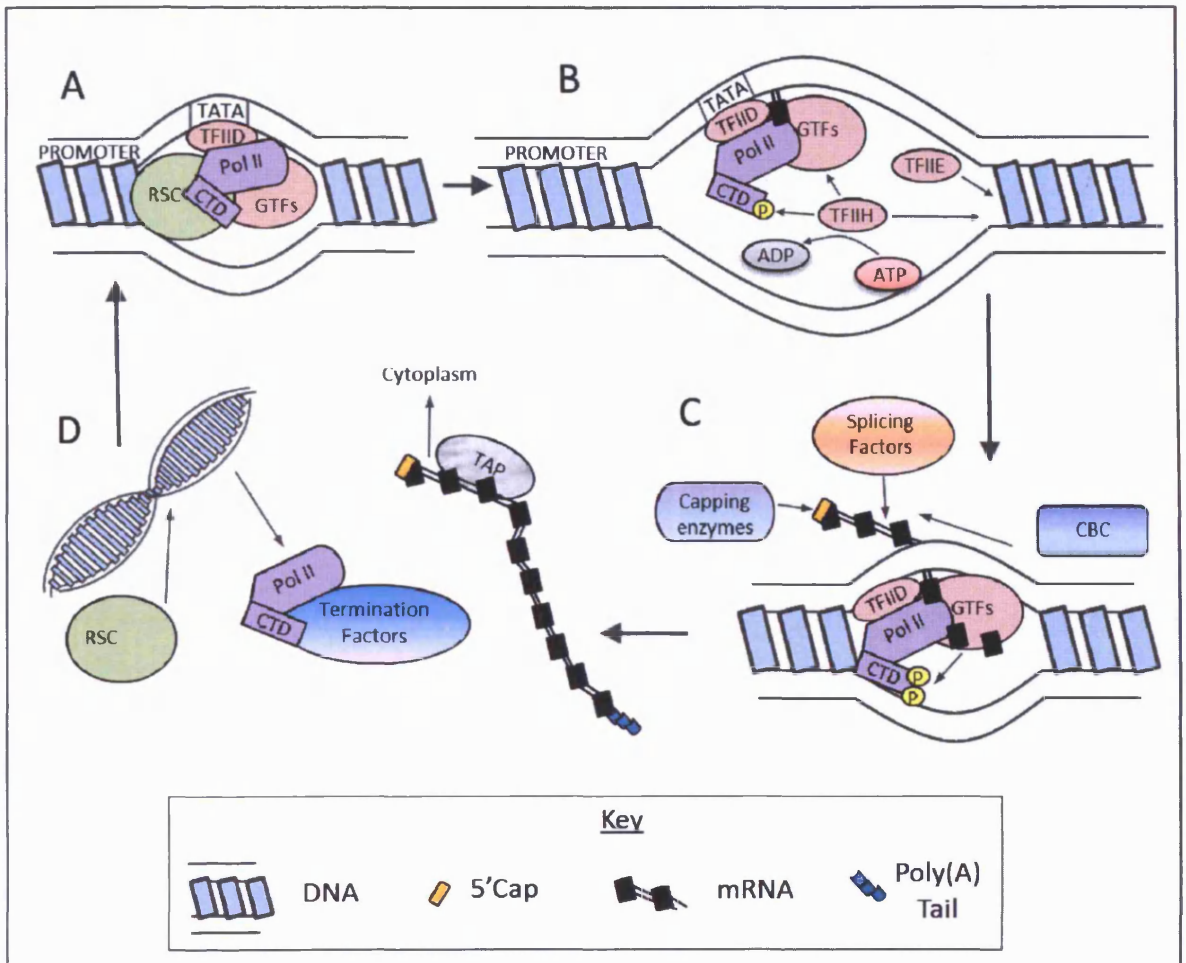


Figure 1.1: The Transcription cycle: **A:** Once transcription has been activated, RSC functions to remodel the nucleosome; facilitating chromatin removal in order to expose the DNA sequence[10]. The TATA-binding protein contained within the TFIID binds directly to the TATA-box motif within the promoter region, this forms a platform for the assembly of the initiation complex containing the regulatory polymerase; Pol II in association with general transcription factors [11, 12]. **B:** TFIIH and TFIIE function to unzip the DNA, the CTD of Pol II is phosphorylated by CdK7 contained within TFIIH, and TFIIH is also required to re-organise the GTFs in order to form the elongation complex [13-15]. Pol II in association with GTFs facilitates the addition of the first corresponding NTP, this continues until the promoter is cleared when the complex becomes more stable [15]. **C:** GTFs mediate further phosphorylation of the CTD of Pol II enabling it to activate mRNA processing by activation of splicing factors and capping enzymes [14]. The cap binding complex recognises the cap structure and facilitates mRNA splicing and poly(A) tail synthesis. Pol II facilitates the addition of NTPs and translocation of the elongation complex along the DNA until a stop codon is reached [16]. **D:** Pol II facilitates the endonucleolytic cleavage followed by synthesis of the poly-(A) tail at the 3'end, this recruits termination factors and the transcript is cleaved downstream of the poly (A) tail. The mRNA transcript is released and TAP proteins facilitate it's transport out of the nucleus to the ribosomes, Pol II dissociates from the DNA for recycling and the RSC restructures the DNA into chromatin [10].

1.2.2 Translation

Once the mRNA has been transcribed, it can be translated into protein. The majority of eukaryotic translation (cap-dependent) occurs when the 5'-cap end of the mRNA transcript is recognised and bound by eIF4E. Like the transcriptional process, translation can be divided into three phases, initiation, elongation and termination. These are described below.

1.2.2.1 Initiation of cap-dependent translation

The m^7-5'-cap structure at the 5'end of the mRNA transcript is essential for translation. The cap is recognised by eIF4E which is part of a heterotrimeric eIF4F initiation complex. The eIF4F complex consists of an adaptor protein eIF4G, this forms the backbone between eIF4E (associated with the m^7GpppN cap structure) and eIF4A, an RNA helicase [17-20]. The eIF4Gs then associate with eIF3, which is complexed with the 40S ribosomal subunit.

Association of eIF4F to the 40S forms a tertiary complex (referred to as the 43S initiation complex) which also comprises of GTP-bound eIF2 and initiator methionine transfer RNA (tRNA). The assembly of the 43S initiation complex allows the unwinding of the mRNA's 5' terminal secondary structure, mediated by eIF4A, however eIF4A alone has limited helicase activity and requires accessory proteins, eIF4G and eIF4H (or it's homolog eIF4B) to stimulate it's intrinsic helicase activity [21].

The 43S complex then migrates along the 5'-UTR of the mRNA transcript in the 5'-3' direction until an initiation codon is located (AUG) [22]. After recognition of the AUG start codon, eIF5 and eIF5B facilitate the addition of the 60S subunit [22]. eIF4Gs also interact with poly-A binding proteins (PABP) bridging the 5'- to the 3'-end causing mRNA circularisation, this aids recycling of the 40S ribosomal subunit [19], causes synergistic enhancement of translation [23] and may play a role in translational silencing of various genes [24].

The initiation step is characterised by the bringing together of the ribosome, mRNA and initiator tRNA, this comprises the elongation-competent ribosome and allows progression to the elongation phase of translation. This initiation step is a rate-limiting step in translation, primarily due to the activity of eIF4E inhibitory proteins. These inhibitory binding proteins often associate with eIF4E to inhibit the

translational complex, or they may also be found tethered to specific mRNA subsets for more specific and targeted inhibition [19]. (See A & B of figure 1.2).

1.2.2.2 Elongation

Elongation is the process of assembling the polypeptide and therefore has high metabolic energy requirements. Initially, the ribosome is in association with the newly transcribed mRNA as well as initiator tRNA (see above), this allows incorporation of the first amino acid into the ribosome. Amino acids are complexed with tRNA and GTP-bound eIF2. When the corresponding amino acid finds a matching codon, eIF5 promotes GTP hydrolysis of GTP-eIF2 and subsequently GDP-eIF2 dissociates from the ribosome, this is referred to as decoding[25, 26]. The dissociation of eIF2 allows the 3'arm of tRNA to move into the peptidyl transfer centre of the large ribosomal subunit which facilitates the formation of peptide bonds.

After peptide bond formation, the mRNA translocates through the ribosome by an exact distance of one codon, as catalysed by the GTPase eEF2, the ribosome also undergoes a conformational change to facilitate the addition of the next amino acid. This is a cyclic process which continues until the entire mRNA transcript has been read [25, 27, 28] (See 'C' of figure 1.2).

1.2.2.3 Termination

The third and final stage is known as 'termination' and is triggered by the recognition of the stop codon within the mRNA transcript. Stop codon recognition stimulates the release of the protein by catalysing the hydrolysis of the peptidyl-tRNA ester bond between the complete peptide chain and the tRNA. This process is modulated via various release factors and signifies the end of protein synthesis [28] (See 'D' of figure 1.2).

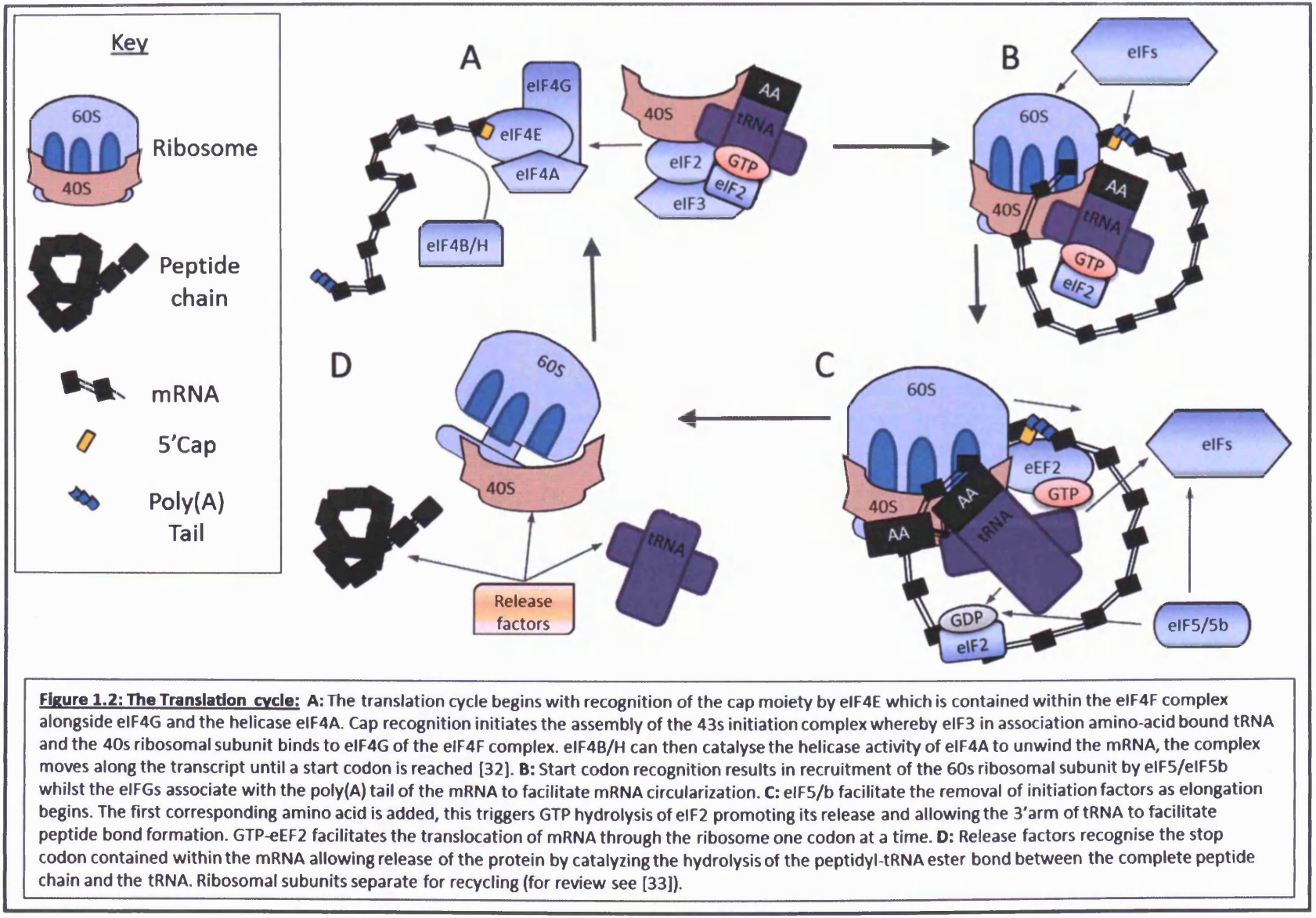


Figure 1.2: The Translation cycle: **A:** The translation cycle begins with recognition of the cap moiety by eIF4E which is contained within the eIF4F complex alongside eIF4G and the helicase eIF4A. Cap recognition initiates the assembly of the 43s initiation complex whereby eIF3 in association amino-acid bound tRNA and the 40s ribosomal subunit binds to eIF4G of the eIF4F complex. eIF4B/H can then catalyse the helicase activity of eIF4A to unwind the mRNA, the complex moves along the transcript until a start codon is reached [32]. **B:** Start codon recognition results in recruitment of the 60s ribosomal subunit by eIF5/eIF5b whilst the eIFGs associate with the poly(A) tail of the mRNA to facilitate mRNA circularization. **C:** eIF5/b facilitate the removal of initiation factors as elongation begins. The first corresponding amino acid is added, this triggers GTP hydrolysis of eIF2 promoting its release and allowing the 3'arm of tRNA to facilitate peptide bond formation. GTP-eEF2 facilitates the translocation of mRNA through the ribosome one codon at a time. **D:** Release factors recognise the stop codon contained within the mRNA allowing release of the protein by catalyzing the hydrolysis of the peptidyl-tRNA ester bond between the complete peptide chain and the tRNA. Ribosomal subunits separate for recycling (for review see [33]).

1.2.3 Alternatives to cap-dependent translation

Approximately 3-5% of mRNAs are transcribed independently of cap-dependent translation, however the mechanism behind this has not been fully elucidated. It is thought that this occurs through internal ribosome entry sites (IRES) found within the 5'UTRs of a subset of mRNAs encoding proteins associated with cell growth, proliferation or survival [29]. These IRES-containing mRNAs carry a translational advantage under conditions of cellular stress, with increased stress coupled with a repression of cap-dependent but not IRES mediated translation. IRESs are typically found in the 5'-UTR of the mRNA transcript and are usually several hundred nucleotides long, so consequently have a substantial secondary structure and increased GC content [29].

IRESs recruit the 40S ribosomal subunit directly to the 5'-UTR and thus bi-pass assembly of the initiation complex. This allows translation to occur independently of the 5'cap and is facilitated by a set of factors referred to as ITAFs (IRES trans-acting factors) which may be generally acting or specific to the mRNA which is being translated [29, 30].

1.2.4 Significance

This section has provided a general overview of the mechanisms which regulate the translation of the genetic code into protein, this is a fundamental cellular process which explains how genes and traits are translated into physical characteristics. The central facet of this study is to identify and characterise downstream substrates of mTOR, mTOR is a key mediator of protein synthesis and exerts many of its cellular effects through regulation of this process. The mTOR signalling pathway, including downstream substrates, cellular inputs and negative feedback mechanisms are described in detail in the next section.

1.3 mTOR STRUCTURE AND RELATED COMPLEXES

Mechanistic or mammalian target of rapamycin (mTOR) is a serine/threonine kinase that was first identified after the discovery of the immunosuppressant drug, rapamycin. Rapamycin is a product of the bacterium *Streptomyces hygroscopicus* which was found in soil samples taken from Easter Island (Rapa Nui). Rapamycin is a macrolide which exerts immunosuppressive effects by limiting the growth cycle of T-lymphocytes and is currently approved for treatment of transplant patients to

prevent graft rejection. Yet perhaps more importantly, rapamycin lead to the discovery of its drug target, mTOR. Rapamycin exerts its effects by binding within the cell to FKBP12, this complex inhibits the functions of mTORC1. mTOR functions as two distinct protein kinase complexes, mTORC1 consisting of mTOR/Raptor and mLST8 and mTORC2 consisting of mTOR bound to Rictor, mLST8, Sin-1 and Protor-1/2 [31-33] see figure 1.3. The two distinct complexes have different downstream targets and regulate distinct pathways, this study aims to identify and characterise downstream substrates of mTORC1.

mTORC1 acts as an energy sensor within the cell and co-ordinates a wide range of cellular processes in response to growth factors and nutrient availability [34, 35]. mTORC1 is a key modulator of cell growth and proliferation, it also regulates metabolic processes via transcriptional regulation including ribosomal biogenesis, autophagy, glucose transport and angiogenesis (discussed later) [36]. mTORC1 is subject to tight regulation from feedback loops, upstream components and nutrient availability and integrates a number of upstream signalling inputs. Instances where mTORC1 signalling is improperly regulated invariably result in pathogenesis, consequently, mTOR inhibitors are currently being utilised in a number of clinical trials.

1.3.1 mTORC2

As stated earlier, mTOR can also form a rapamycin insensitive complex, mTORC2. mTORC2 consists of mTOR, Rictor, mLST8 and Sin 1 [37-39] and, like mTORC1 is negatively regulated by DEPTOR (see section 1.3.7). A new component of the mTORC2 complex was recently identified, Protor1/2, protein observed with rictor-1, which as the name indicates, is a Rictor binding protein. It was demonstrated that both Protor 1 and 2 isoforms bind directly to Rictor and that knockdown of Rictor could reduce the expression levels of Protor1/2 and Sin-1 [39]. The role of Protor1/2 within mTORC2 has yet to be determined (for comparison of mTORC1 and mTORC2 complexes see figure 1.3).

Little is known regarding the regulation of mTORC2, it was previously shown that insulin stimulation could induce Ser473 phosphorylation of Akt however it wasn't until 2005 that mTORC2 was identified as the kinase responsible for this [40, 41]. Akt is involved in regulation of the cell-cycle, cell survival, glucose uptake, gluconeogenesis regulation and maintaining neuronal synapse activity through

phosphorylation of ion-channels (see review [42]). Importantly it also plays a role in the activation of mTORC1.

Akt activation occurs in response to insulin or growth factor stimulation through phosphorylation at two sites, Ser473 within the hydrophobic motif is mediated by mTORC2 and appears to act as a priming site for phosphoinositide-dependent kinase 1 (PDK1) to bind and phosphorylate Thr308 causing full Akt activation [40, 41].

Studies utilising Rictor knockout mice have indicated that Ser473 phosphorylation of Akt is essential for embryonic development [43] whilst other studies have demonstrated that mTORC2 plays a role in maintaining the actin cytoskeleton of the cell [44].

Our knowledge of mTORC2 mediated substrate inhibition is behind that of mTORC1, primarily due to the fact that rapamycin can be utilised to investigate mTORC1 but not mTORC2 signalling. There are multiple studies indicating that the rapamycin/FKBP12 complex cannot inhibit mTORC2 [40, 44, 45]. More recently however, it has been demonstrated that prolonged rapamycin treatment does infact perturb mTORC2 signalling by binding to 'free mTOR' thus limiting mTORC2 complex assembly. Prolonged (24 hour) rapamycin treatment results in saturation of newly synthesised mTOR with the rapamycin/FKBP12 complex, causing a suppression of Akt signalling. This effect appears to be variable between cell types with some being more sensitive to inhibition of mTORC2 assembly with rapamycin than others [46]. This could suggest that there are multiple mechanisms of mTORC2 complex assembly.

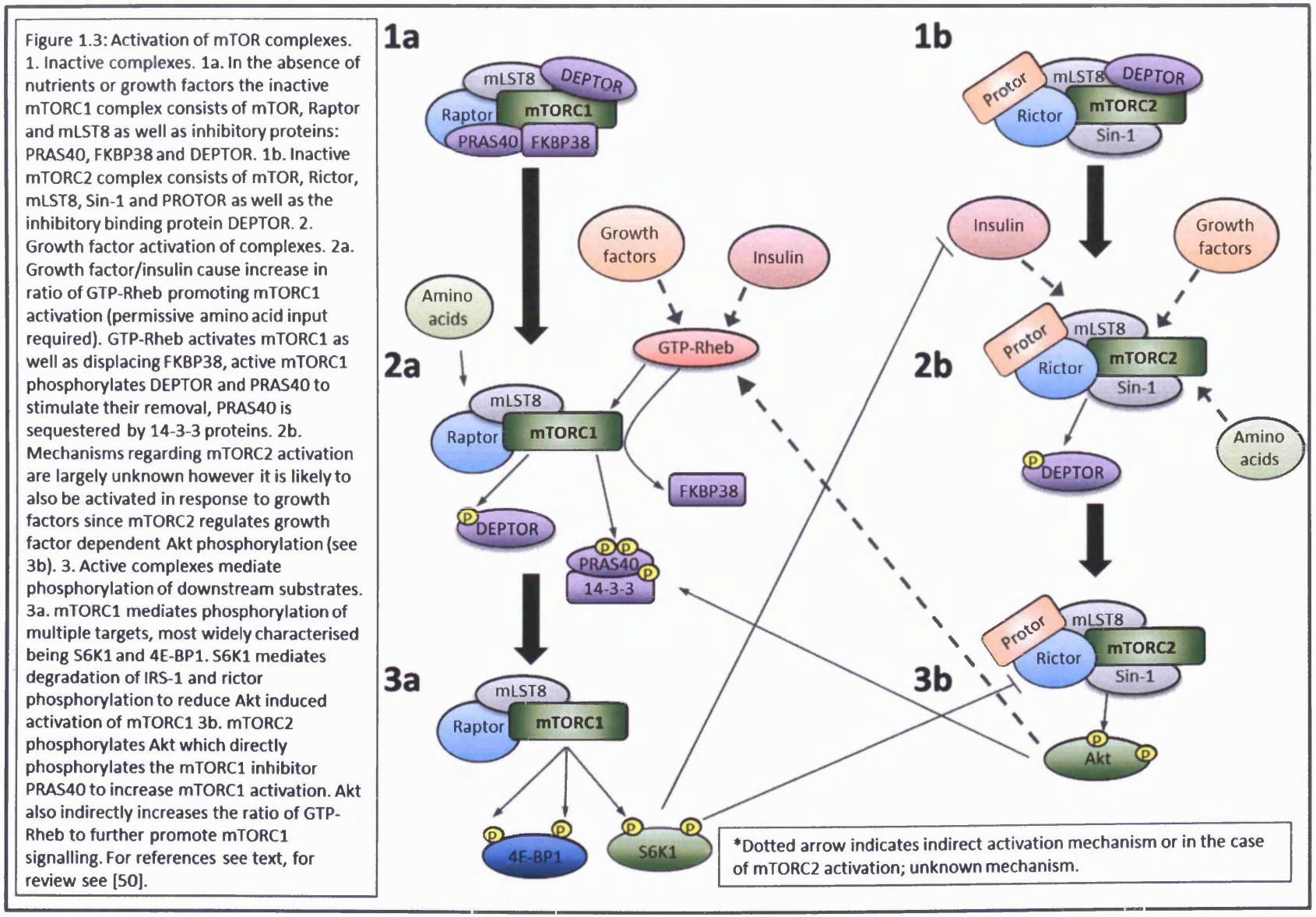


Figure 1.3: Activation of mTOR complexes. 1. Inactive complexes. 1a. In the absence of nutrients or growth factors the inactive mTORC1 complex consists of mTOR, Raptor and mLST8 as well as inhibitory proteins: PRAS40, FKBP38 and DEPTOR. 1b. Inactive mTORC2 complex consists of mTOR, Rictor, mLST8, Sin-1 and PROTOR as well as the inhibitory binding protein DEPTOR. 2. Growth factor activation of complexes. 2a. Growth factor/insulin cause increase in ratio of GTP-Rheb promoting mTORC1 activation (permissive amino acid input required). GTP-Rheb activates mTORC1 as well as displacing FKBP38, active mTORC1 phosphorylates DEPTOR and PRAS40 to stimulate their removal, PRAS40 is sequestered by 14-3-3 proteins. 2b. Mechanisms regarding mTORC2 activation are largely unknown however it is likely to also be activated in response to growth factors since mTORC2 regulates growth factor dependent Akt phosphorylation (see 3b). 3. Active complexes mediate phosphorylation of downstream substrates. 3a. mTORC1 mediates phosphorylation of multiple targets, most widely characterised being S6K1 and 4E-BP1. S6K1 mediates degradation of IRS-1 and rictor phosphorylation to reduce Akt induced activation of mTORC1 3b. mTORC2 phosphorylates Akt which directly phosphorylates the mTORC1 inhibitor PRAS40 to increase mTORC1 activation. Akt also indirectly increases the ratio of GTP-Rheb to further promote mTORC1 signalling. For references see text, for review see [50].

The aim of this study is to characterise signalling downstream of mTORC1, however crosstalk between the signalling pathways must be considered. The next section goes on to describe the structure and function of mTORC1 signalling components.

1.3.2 Rheb

Loss of heterozygosity of either the *TSC1* or *TSC2* genes leads to the disease Tuberous Sclerosis Complex (TSC). TSC is characterised by the development of benign hamartomas in multiple organ systems and is a result of upregulation of the mTORC1 signalling pathway. It wasn't until 2003 that the link between *TSC1*, *TSC2* and the mTOR signalling pathway was established.

The pivotal point in this research was the discovery of the small G-protein, Rheb as an activator of mTORC1. Ras-homologue enriched in brain is a novel member of the Ras family of small GTP-binding proteins. As the name suggests, high levels are found in the brain and its sequence is highly homologous to Ras. As with all of the Ras family of proteins, Rheb activity is determined by its nucleotide bound status. This is determined by the activity of their corresponding GAPs and GEFs (GTPase Activating Proteins and Guanine Exchange Factors). With GAPs function to negatively regulate small G-proteins, GEFs perform the opposing function and increase GTP-loading to positively regulate them.

It was discovered in 2003 that Rheb was an activator of mTOR and that the TSC1 and TSC2 proteins suppressed mTORC1 signalling by acting as a GAP towards Rheb when complexed together [47-51]. The TS complex binds to GTP-Rheb stimulating GTP-hydrolysis.

There is some speculation regarding the GEF which regulates Rheb. It was recently demonstrated that the translationally controlled tumour protein (TCTP) could act as a GEF towards Rheb in *Drosophila* [52]. However, several subsequent studies have found that this is unlikely to be the case is mammalian cells [53, 54].

There is evidence of cross-talk between sphingosine-1-phosphate (S1P) signalling and the mTORC1 pathway. Similarly to mTOR, S1P regulates cellular growth, mitogenesis and apoptosis. It has been demonstrated that S1P can activate mTORC1 in certain cell types [55, 56], a mechanism has been proposed whereby E3 ubiquitin ligase Protein Associated with Myc (PAM) (downstream of S1P) acts as a GEF to directly activate Rheb, however this has yet to be substantiated [57].

The precise mechanism by which Rheb functions to activate mTORC1 is yet to be fully elucidated. mTORC1 cannot be activated by other G-proteins, KRas, RalA/B or Cdc42 but is activated by Rheb1 and Rheb-Like-1 (also known as Rheb 2). It has been demonstrated that Rheb can activate mTORC1 but not mTORC2 [38, 58, 59] suggesting that Rheb is a highly specific activator of mTORC1. Although Huang *et al.* recently reported that the TS complex interacts directly with mTORC2 and was required for mTORC2 kinase activity but this occurs independently of Rheb [38].

Expression of Rheb induces mTORC1 activity under conditions of nutrient withdrawal [47] suggesting that Rheb is proximal to mTOR in the signalling pathway. Its association with mTOR appears to be fragile and may involve a direct interaction with the catalytic domain of mTOR that is independent from Rhebs association with TSC2 [60]. It does not however appear to be dependent upon the nucleotide bound status of Rheb, as truncated mutants of Rheb which are nucleotide deficient are still able to bind to mTORC1 but do not confer kinase activity [60]. A mechanism of Rheb activation of mTORC1 involving FKBP38 has been proposed (described below in section 1.3.8), however current thinking suggests that this is not the primary mechanism for Rheb activation of mTORC1. Recent studies have suggested that Rheb increases the substrate binding capability of mTORC1 causing more efficient phospho-transfer, rather than just enhancing the kinase activity directly [59]. However other studies have seen increases in phosphorylation of mTORC1 substrates by addition of Rheb and hypothesised an increase in kinase activity [60].

Rheb contains a C-terminal CaaX motif whereby the 'a's represent aliphatic amino acids and X represents the C-terminal amino acid, usually serine, alanine, glutamine, cysteine or methionine. The CaaX motif becomes post-translationally farnesylated and many studies have indicated that this is required for Rheb activation of mTORC1. This is supported by use of farnesyltransferase inhibitors which downregulate mTORC1 signalling [47, 61]. It has also been shown that unfarnesylated Rheb can activate mTORC1 *in vitro* [59] but not *in vivo* [47]. It is therefore likely that the farnesylation of Rheb is required for its localisation to allow its interaction with mTOR at the membrane. Further research investigating the structure of Rheb by Tee *et al.* revealed that the Thr38 and Asp41 residues contained within the switch 1 region of Rheb are required for maximal interaction with mTOR [62].

Whilst comparisons between Ras and Rheb revealed that the effector region is highly homologous, Rheb contains an arginine at position 12 rather than a glycine residue. This confers intrinsically low GTPase activity as it allows for a conformational change in the switch I region during GTP/GDP cycling. This causes displacement of the Gln64 residue making it incapable of participating in GTP hydrolysis [63]. Further research is required to determine how these functional domains interact with mTORC1 and the exact mechanism behind mTORC1 activation.

1.3.3 mTOR

mTOR belongs to a family of phosphoinositide kinase-related kinase (PIKK) protein kinases. mTOR contains a serine/threonine kinase domain in the C-terminal which is structurally similar to the catalytic domain found in the lipid kinase, phosphoinositide kinase (PI3K) (see figure 1.4). However, mTOR only functions as a ser/thr kinase [35, 64]. Structurally, the N-terminal half is made up of 20 tandem HEAT repeats (see figure 1.4 'A' for schematic) consisting of two α-helices of approximately 40 amino acids arranged in terms of their hydrophilic and hydrophobic residues [65]. HEAT repeats are thought to coordinate protein to protein interactions so it is likely that this region of mTOR provides docking sites for other protein interactions [66]. Adjacent to the HEAT repeats is the FRB (FKBP12/rapamycin binding) domain. This is the region that the FKBP12/rapamycin heterodimer binds to [67]. The FRB domain is essential for mTOR kinase activity since mTOR mutants lacking the FRB domain are unable to promote cell cycle progression into the G_1 phase [68]. Within the FRB domain is a Ser2035 residue which appears to be necessary for interactions with FKBP12/rapamycin. Ser2035 substitution to any amino acid structurally larger than an alanine reduces the binding affinity of FKBP12/rapamycin and also affects mTOR kinase activity indicating that Ser2035 may be a regulatory site [24].

Oshiro *et al.* demonstrated that rapamycin treatment disrupted mTOR binding to Raptor independently of mTOR kinase activity [69] however, it is still thought that there is a functional relationship between the FRB domain and the kinase domain [68]. In addition to the HEAT repeats and FRB domain, there are two conserved FAT domains (see figure 1.4), one being substantially smaller and situated at the C-terminus, termed FATC. This is also seen in PIKK's and is adjacent to the kinase domain. The FATC domain is crucial for mTOR kinase activity, where single point

amino acid substitutions cannot be tolerated *in vivo* or *in vitro* [70]. FATC contains a structural motif entailing an α-helix and a di-sulphide bonded loop situated between two cysteines. Reduction of the di-sulphide bond results in a conformational change increasing flexibility of the carboxyl-terminal loop region, potentially revealing hydrophobic regions of the domain. This may impact binding partners and interactions with other domains of mTOR [71].

Altering the redox status of mTOR by introducing mutations to this FATC domain causes in a reduction in mTOR protein levels, suggesting that the redox status of the FATC domain is involved in protein stability [71]. The larger 550 amino acid length FAT domain is composed of α-helices structures, similarly to the HEAT repeats suggesting that the FAT domain also plays a role in protein-protein interactions [72]. Since FAT domains are usually found in conjunction with FATC domains [73], it is likely that these domains interact with one another upon mTOR activation, causing a conformational change and exposing the protein kinase domain to induce activity [65, 70].

The actions of mTOR are determined by what proteins it is complexed with. The rapamycin sensitive mTORC1 complex is responsive to growth factors, hormones and nutrients. The mTORC2 complex is less well described and is known to regulate the actin cytoskeleton and moderate Akt phosphorylation and therefore may also play a role in mTORC1 regulation. The mTORC1 complex consists of mTOR, Raptor, mLST8, PRAS40, Deptor, FKBP38 and Protor-1/2 (as shown in figure 1.3), and it is directly activated by GTP-Rheb. The structure and function of each of the proteins within the mTORC1 complex are described below.

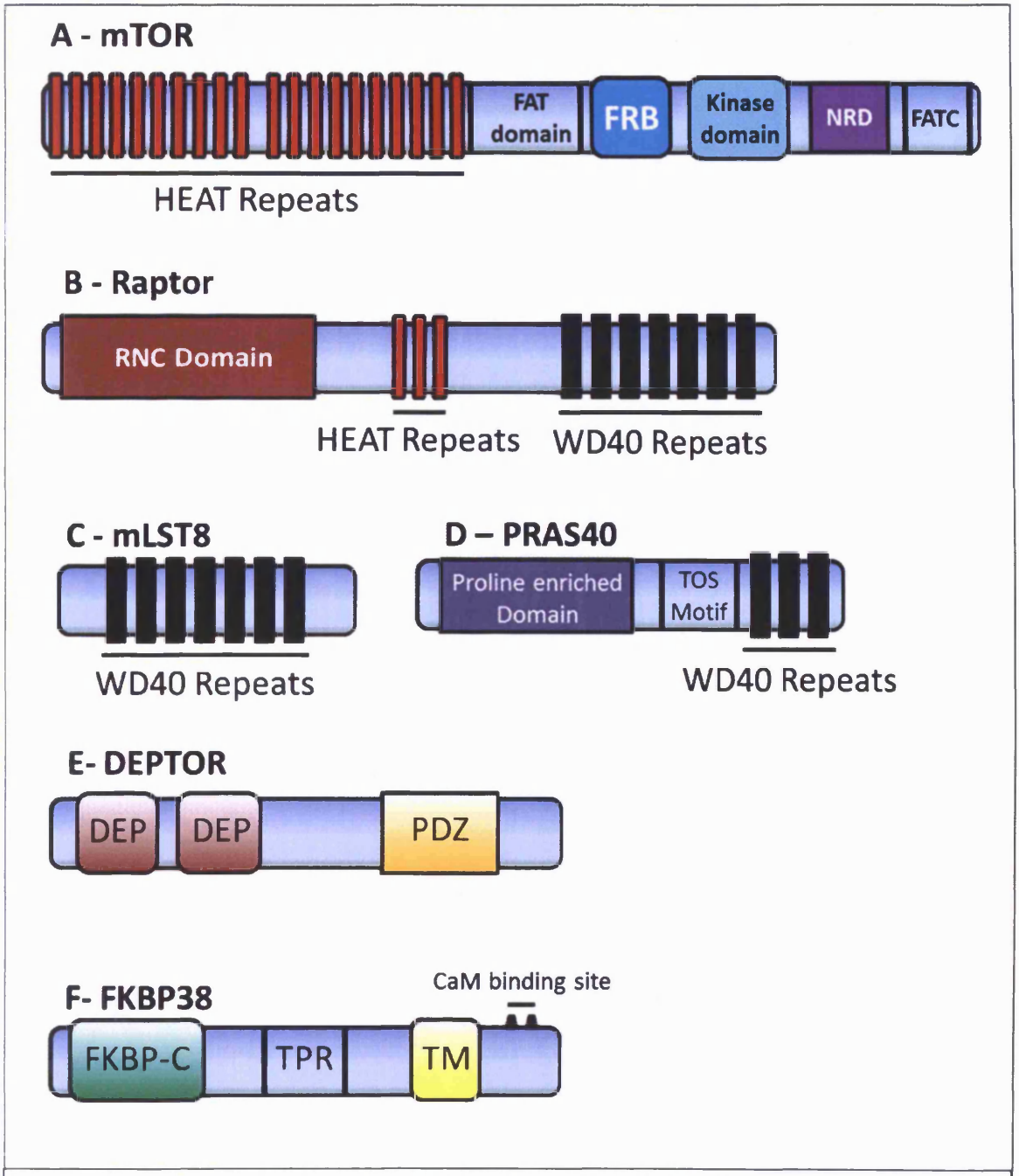


Figure 1.4 Schematic of mTOR complex components showing conserved domains. FRB domain: FKBP12/rapamycin binding domain. NRD domain: Negative regulatory domain. RNC domain: Raptor N-terminal conserved. TOS motif: mTORC1 signalling motif required for mTOR directed phosphorylation. DEP domain: dishevelled, egl-10, pleckstrin. PDZ domain: postsynaptic density 95, discs large, zonula occludens-1. TPR: tetratricopeptide repeat. TM: Trans membrane domain. CaM:Calmodulin binding site. Diagram adapted from [77].

1.3.4 Raptor

Raptor was first identified in 2002 by Hara e*t al.* and Kim e*t al.* Both groups identified Raptor as a 150kDa mTOR binding protein which was also able to interact with 4E-BP1 and S6K1. [74, 75]. It is thought that Raptor is the substrate recognition component of the complex and is therefore indispensible in mTORC1 signalling.

Raptor is an evolutionary conserved protein containing a highly conserved N-terminal domain named the RNC (Raptor N-terminal conserved) domain followed by three HEAT repeats and then seven WD40 repeats (see figure 1.4 'B'). The RNC domain is made up of three blocks with around 67-79% sequence homology [74]. This region has a tendency to form α-helices and work by Dunlop *et al.* identified that this region is also required for mTOR/Raptor interaction with substrates [76]. Thus identifying that Raptor mutant 4, containing a mutation to the RNC domain, was able to interact with mTOR but not facilitate substrate phosphorylation. Raptor mutant 4 therefore acts as a dominant inhibitor of mTORC1 signalling and has been utilised within these studies.

mTORC1 substrate phosphorylation has been found to be dependent upon a conserved regulatory motif which is found in the N-terminal of all known S6K's and the C-terminal of the 4E-BPs, which is referred to as the mTOR signalling (TOS) motif [77-79]. The TOS motif acts as a docking site for mTORC1 interaction and Raptor acts as the recognition component to facilitate this. A fully intact TOS motif is required for both interaction with Raptor and mTORC1 mediated substrate phosphorylation. It has therefore been proposed that the TOS motif mediates substrate binding to Raptor, recruiting mTOR to the substrate/Raptor complex so that phosphorylation can occur [78]. More recently, putative TOS motifs have been found in a range of potential or candidate mTORC1 substrates, described in table 1.1. The TOS motif consists of a five amino acid sequence with a phenylalanine at position one which is indispensible for Raptor binding. It has been suggested that an aliphatic uncharged residue is also preferred at position three [80] however eIF3F has more recently been demonstrated to contain a potential TOS motif with a Threonine residue at the third position, suggesting that this is not necessary [81]. It has also been suggested that the remaining amino acids consist of alternating acidic and hydrophobic residues [82]. However, there are some discrepancies with this notion, for instance the TOS motif found in HIF-1α is made up entirely of hydrophobic

residues (see table 1.1) and the Threonine residue in eIF3F is polar and hence hydrophilic.

More recently it has been shown that Raptor is phosphorylated by mTOR at Ser863 in response to insulin and other mTOR stimuli. This appears to trigger phosphorylation of Raptor at five other sites: Ser696/Thr706/Ser855/Ser859/Ser877.

Mutation of the mTOR directed phosphorylation site (Ser863) renders mTOR unresponsive to Rheb induced activation. This indicates that Raptor not only functions in the recognition of substrates but also plays a role in modulating mTOR kinase activity [83, 84].

Table 1.1: Substrates containing potential TOS motifs

Protein (Homo Sapien)	Motif	Amino acid position
S6K1	FDIDL	5-9
S6K2	FDLDL	5-9
4EBP1	FEMDI	114-118
4EBP2	FEMDI	116-120
4EBP3	FEMDI	86-90
HIF-1α	FVMVL	99-103
PLD2	FEVQV	264-269
PRAS40	FVMDE	129-133
PKCδ	FVMEF	425-429
PKCε	FVMEY	484-488
STAT3	FPMEL/FDMDL	26-30/756-760
eIF3f	FETML	323-327
GTF3C	FEVDR	90-94

*Table adapted from [83].

1.3.5 mLST8

mLST8 is a widely expressed 36 kDa protein which consists mainly of seven WD40 repeats (see figure 1.4 'C'). It was first identified in 2003 and was thought to bind directly to the kinase domain of mTOR increasing the stability of mTOR/Raptor interactions and promoting mTORC1 kinase activity. The interaction between mTOR and mLST8 was reported to be stable, independent of Raptor and unaffected by nutrient status [85]. It was later found to also be a component of the mTORC2 complex [45]. More recent work suggests that although it does form part of the mTORC1 complex, it is more contributory to mTORC2 mediated signalling. As demonstrated by studies of mLST8-/- MEFs which exhibit impaired mTORC2 signalling whilst mTORC1 signalling remains intact [86]. Additionally, knockout of mLST8 disturbs mTOR/Rictor association but not mTOR/Raptor association, producing a phenotype similar to Rictor knockouts [86, 87]. mLST8 is therefore indispensible in mTORC2 complex formation but not mTORC1.

1.3.6 PRAS40

PRAS40 is a 40 kDa proline enriched Akt substrate. It was identified in 2007 by several research groups. PRAS40 was demonstrated to interact with mTORC1 but not mTORC2 via Raptor recognition of a TOS motif identified in the C-terminal (see schematic, figure 1.4 'D').

PRAS40 acts as a negative regulator of mTORC1 activity under conditions of insulin-deprivation. It is thought to bind to the C-terminal kinase domain of mTOR [88] inhibiting its activation by Rheb [82, 88-91]. Insulin stimulation causes Akt and mTORC1 to directly phosphorylate PRAS40 causing it's dissociation from the complex. This promotes mTORC1 interaction with substrates via their TOS motifs. Phosphorylated PRAS40 then binds to 14-3-3 chaperone proteins, fully alleviating the suppression of mTORC1 [92].

1.3.7 DEPTOR

Research into inhibitory components of the mTOR complexes revealed a 48kDa protein referred to as DEPTOR. Unlike PRAS40 it is present in both mTORC1 and mTORC2 complexes and is only conserved among invertebrates. It has been shown to bind directly to mTOR regardless of mLST8 expression. Its interaction is modulated by the PDZ domain located in the C-terminus of DEPTOR (see figure 1.4

'E'). It binds to a region of mTOR in the C-terminus adjacent to the kinase domain. DEPTOR depleted cells exhibit increased kinase activity towards mTORC1 substrates 4E-BP1 and S6K1 as well as the mTORC2 substrate Akt. Serum-starvation causes a rise in DEPTOR expression and re-stimulation with serum causes a reduction in its expression. mTORC1 and mTORC2 both negatively regulate the expression of DEPTOR when activated by phosphorylating it at one of the thirteen potential serine/threonine phosphorylation sites located between the C-terminal DEP domain and the PDZ domain. This appears to induce its removal from the complex in a manner similar to that of PRAS40 [93].

1.3.8 FKBP38

FKBP38 belongs to the peptidyl prolyl cis/trans isomerase (PPIase) family of FK506-binding proteins (FKBP). Also included in this family is the rapamycin binding partner FKBP12. This PPIases act as chaperones to modulate protein folding, biogenesis, assembly and trafficking [94]. They are characterised by a PPIase or PPIase-like domain referred to as the FKBP-C domain (see figure 1.4 'F'). The PPIase family catalyse the *cis/trans* isomerisation between native-state prolyl bond isomers of different biological activity via FKBP-C domains [94].

The FKBP-C domain of FKBP38 is situated in the N-terminus (see figure 1.4 'F'). It also contains a tetratricopeptide repeat (TPR domain) involved in protein-protein interactions, a binding site for calcium induced calmodulin, and finally a TM (transmembrane) domain. The TM domain allows FKBP38 to anchor itself to mitochondrial membranes and is unique to FKBP38 [95]. FKBP38's PPIase domain becomes active in response to calcium influx and can then bind to Bcl-2 to regulate apoptosis [94].

There are several reports indicating that FKBP38 may form part of the mTORC1 complex functioning as an endogenous inhibitor [76, 96, 97]. Studies have also demonstrated that like rapamycin, FKBP38 binds to the FRB region of mTOR, suggesting a similar mechanism of inhibition as with rapamycin [95]. A mechanism has been suggested whereby GTP-Rheb interacts with the inactive mTORC1 complex in order to displace FKBP38. Consistent with this theory, mTORC1 has been demonstrated to localise to the mitochondrial membrane [98, 99].

Work by Ma *et al.* has implicated the switch I region of Rheb as necessary for displacing FKBP38 interactions [96]. Tee *et al.* had also previously demonstrated

that mutations to this switch 1 region prevent Rheb induced activation of mTOR [62]. This is consistent with research on other small G-proteins such as Ras, which in its GTP bound state can interact with its effectors via its switch I region [96]. Both Rheb and mTORC1 have been demonstrated to localise to the mitochondria where FKBP38 is tethered [96]. This is consistent with the role of mTORC1 as an energy and oxygen sensor. Studies have shown that Rheb interaction with FKBP38 is dependent upon the presence of amino acids and growth factors [97], indicating that interactions are determined by its guanine nucleotide status. Increases in Rheb binding to FKBP38 appear to be coupled with decreases in the amount of FKBP38 bound to mTOR, supporting the notion of Rheb modulated displacement [97]. It has been suggested that this may be the mechanism by which Rheb activates mTOR, however this remains controversial. Some studies have been unable to demonstrate any inhibition of mTORC1 through FKBP38 expression but show direct interaction [54, 59, 100]. Dunlop *et al.* demonstrated that FKBP38 induced a modest inhibition of mTORC1 kinase activity *in vivo* and *in vitro* that was not as potent as PRAS40 [76]. This may explain why some groups did not see inhibition. It also makes it unlikely that the sole mechanism for Rheb induced activation of mTORC1 is via removal of FKBP38 as Rheb is a potent activator of mTORC1. It is likely that Rheb modulates mTORC1 through FKBP38 dependent and independent mechanisms.

1.4 ACTIVATION OF mTORC1

mTORC1 is activated by a number of different mechanisms, this is unsurprising given the diverse and complex nature of the cellular processes moderated by mTORC1. mTORC1 is regulated by nutrient status, growth factors, insulin and other mitogens. The known mechanisms resulting in mTORC1 activation are described in detail below and summarised in figure 1.5.

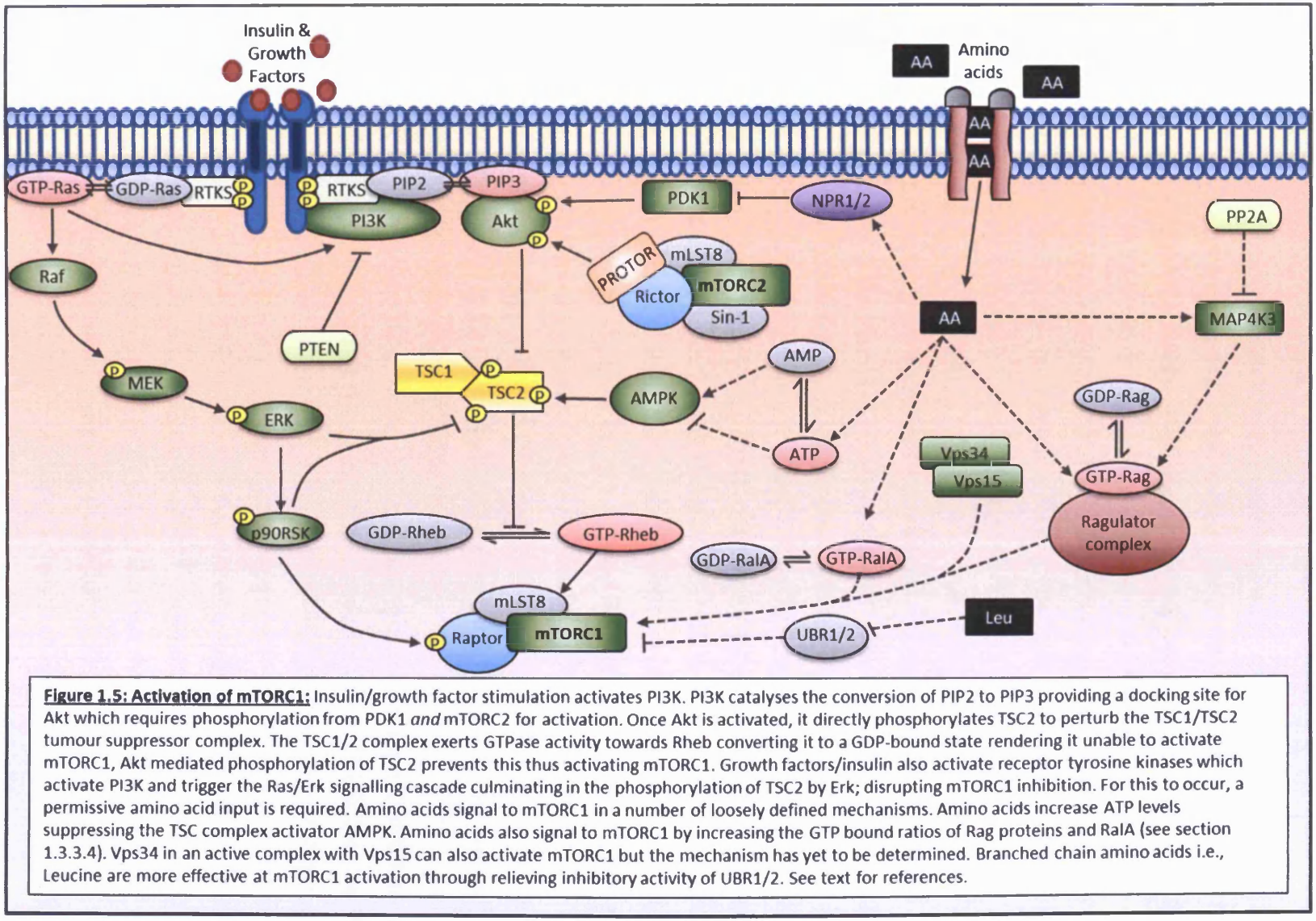


Figure 1.5: Activation of mTORC1: Insulin/growth factor stimulation activates PI3K. PI3K catalyses the conversion of PIP2 to PIP3 providing a docking site for Akt which requires phosphorylation from PDK1 *and* mTORC2 for activation. Once Akt is activated, it directly phosphorylates TSC2 to perturb the TSC1/TSC2 tumour suppressor complex. The TSC1/2 complex exerts GTPase activity towards Rheb converting it to a GDP-bound state rendering it unable to activate mTORC1, Akt mediated phosphorylation of TSC2 prevents this thus activating mTORC1. Growth factors/insulin also activate receptor tyrosine kinases which activate PI3K and trigger the Ras/Erk signalling cascade culminating in the phosphorylation of TSC2 by Erk; disrupting mTORC1 inhibition. For this to occur, a permissive amino acid input is required. Amino acids signal to mTORC1 in a number of loosely defined mechanisms. Amino acids increase ATP levels suppressing the TSC complex activator AMPK. Amino acids also signal to mTORC1 by increasing the GTP bound ratios of Rag proteins and RalA (see section 1.3.3.4). Vps34 in an active complex with Vps15 can also activate mTORC1 but the mechanism has yet to be determined. Branched chain amino acids i.e., Leucine are more effective at mTORC1 activation through relieving inhibitory activity of UBR1/2. See text for references.

1.4.1 Insulin and Growth Factors

PI3Ks are a family of lipid kinases which play regulatory roles within the cell. They are classified by their structure and their ability to recognise substrates. Of particular interest in this project is the Class 1a PI3Ks which are heterodimers consisting of a p85 regulatory subunit coupled to a p110 catalytic subunit. The 1a class of PI3Ks are activated via cell surface receptors and are switched on with insulin and growth factor stimulation. The insulin receptor substrates (IRS1-4) are potent activators of PI3Ks when phosphorylated. PI3Ks also demonstrate an affinity for the phosphorylated tyrosine residues found in activated growth factor RTKS. Phosphorylation of these tyrosine residues facilitates the recruitment of PI3Ks to the membrane where the p85 regulatory subunit directly binds to the receptors (see figure 1.5). The p110 catalytic subunit then converts phosphatidylinositol-4,5-bisphosphate (PIP2) to phosphatidylinositol-3,4,5-trisphosphate (PIP3) [101]. It is significant to note that PI3K is negatively regulated by phosphatase and tensin homolog (PTEN), which reverses the conversion of PIP2 to PIP3 and thus functions as a tumour suppressor [102]. Importantly, the conversion of PIP2 to PIP3 provides a docking site for Akt (also known as Protein Kinase B (PKB)). This 'docking' of Akt, causes a conformational change exposing two phosphorylation sites within Akt. The first, Ser473 is phosphorylated by mTORC2, this allows PDK1 to phosphorylate the second Thr308 site. Akt/PKB also up-regulates mTORC1 signalling by phosphorylation of PRAS40 and TSC2 [101].

Phosphorylation of PRAS40 by Akt triggers its removal from mTORC1 by 14-3-3 chaperone proteins (see section 1.3.6) while phosphorylation of TSC2 at Ser939 and Thr1462 by Akt causes disruption of the TSC1/TSC2 heterodimer. Tuberin and Hamartin (TSC1 and 2) form a heterodimer in response to mitogenic withdrawal. When complexed together, TSC1 and TSC2 are able to confer GAP activity towards the small G-protein Rheb to suppress mTORC1. Activation of the TSC complex, therefore, results in the conversion of Rheb to the inactive GDP-bound state and suppression of mTORC1 signalling (as shown in figure 1.5).

Other mitogenic signalling pathways activate mTORC1 signalling through disruption of the TSC1/2 complex. Growth factor binding to membrane receptors activates Ras, which in turn activates PI3K, inducing Akt mediated phosphorylation of TSC2 (as described above). It also activates a Raf/Mek1 signalling cascade which stimulates the phosphorylation of TSC2 at Ser664 (and possibly to a lesser extent

Ser540) by ERK1/2, disrupting the TSC tumour suppressor complex [103]. ERK1/2 is also able to phosphorylate and subsequently activate p90-RSK1 [104] which phosphorylates TSC2 at Ser1798 [105], p90-RSK1 also phosphorylates Raptor to increase mTORC1 kinase activity directly [106]. TSC2 is therefore the axis at which Raf/Mek and PI3K signalling cascades meet, convergence of the two pathways to mediate mTORC1 signal transduction indicates the significance of mTOR in the regulation of cellular growth and metabolism.

1.4.2 Phospholipase-D

Phospholipase-D (PLD) is a widely expressed enzyme which is activated in response to a wealth of hormones, growth factors, cytokines and neurotransmitters (see review [107]). PLD1 hydrolyses phosphatidycholine, producing phosphatidic acid (PA) and choline [107]. Phosphatidic acid (PA) is a positive regulator of mTORC1. It was demonstrated *in vitro* that PA bound directly to the FRB domain within mTOR (FKBP12/rapamycin binding domain – see figure 1.4 'A') and as expected was displaced by the rapamycin/FKBP12 complex. Elevation of intracellular PA renders cells less sensitive to rapamycin treatment indicating a competitive relationship between PA and rapamycin, with PA binding causing activation and rapamycin binding causing inhibition [108].

Interestingly, not all production of PA results in mTORC1 activation, for instance RhoA activates PLD1 but not mTORC1 [109]. This suggests that the cellular localisation of PA production is significant in the regulation of mTORC1. In addition to the direct binding of PA to mTORC1, there is evidence that PLD1 itself is a direct effector of Rheb [110]. Sun *et al.* demonstrated that Rheb knockdown impaired serum-induced activation of PLD1 whilst over-expression of Rheb resulted in PLD1 activation in the absence of mitogenic stimulation. They were able to demonstrate that Rheb bound directly to PLD1 in a GTP dependent manner *in vitro* and have therefore proposed a mechanism by which Rheb signals to PLD1 in order to stimulate PA production, resulting in mTORC1 activation [110]. A more recent study however provided evidence disputing the direct effect of PA upon mTORC1, suggesting that PA must first be metabolised to LPA (lysophosphatidic acid) to permit activation of mTORC1, furthermore they suggested that neither PA nor LPA bound directly to mTORC1 and that activation is a result of upregulation of the ERK

pathway [111]. The reason for these discrepancies is unclear within the literature and further studies are required to determine the correct mechanism.

1.4.3 AMPK

A mechanism has been described whereby AMPK activation results in the phosphorylation of TSC2 (see figure 1.5). AMP levels are inversely proportionate to ATP levels, therefore when ATP becomes depleted, AMP levels rise and AMPK is activated. When ATP levels are depleted, homeostatic mechanisms are induced to switch off energy consuming cellular processes such as protein synthesis and cell growth. Therefore AMPK acts to switch off mTORC1 signalling via phosphorylation of TSC2 [112]. Although phosphorylation of TSC2 by RSK, Akt and ERK results in disruption of TSC1/TSC2 causing its inhibition, phosphorylation at a different site, Ser1345 by AMPK results in TSC2 activation and subsequent inhibition of mTORC1 [112].

It was later established that GSK3β also phosphorylates TSC2 at Ser1341 as well as Ser1337 to mediate its activation and subsequent inhibition of mTORC1 [113]. AMPK mediated Ser1345 phosphorylation of TSC2 is required for this to occur so it is likely that AMPK and GSK3β act in synergy to promote the GAP activity of TSC towards Rheb. Thus indicating crosstalk between the Wnt signalling pathway which negatively regulates GSK3β and mTORC1 [114].

Studies have also identified a TSC2 independent mechanism of AMPK mediated inhibition of mTORC1 whereby AMPK directly phosphorylates Raptor. This results in the recruitment of inhibitory 14-3-3 proteins to the mTORC1 complex and subsequent inhibition of signal transduction [115].

1.4.4 Nutrient Regulation

As mTORC1 regulates a significant range of energy costly processes, it is paramount that mTORC1 activity is appropriate for the nutrient status of the cell. It is not fully understood how mTORC1 is regulated by nutrients, with amino acid regulation being the best characterised. In addition to this, several other mediators have also been identified which contribute to nutrient mediation of mTORC1 and are described below.

1.4.4.1 Amino Acid Regulation

A permissive amino acid input is required for Rheb to activate mTORC1 in response to growth factors however it is unknown how amino acids signal to mTORC1. Recently, details of this mechanism have been uncovered, implicating a series of proteins referred to as the Rag GTPases. A model has been described whereby amino acids stimulate the GTP loading of Rag proteins. Once this occurs, they are able to bind to the Raptor component within mTORC1 and trigger re-localisation of the complex to membrane surfaces where farnesylated Rheb is also situated. This translocation occurs independently of mTORC1 kinase activity and is therefore insensitive to rapamycin inhibition. It is thought that for the above described activation of mTORC1, this amino acid induced localisation of mTORC1 must first occur [116, 117]. More recent work by Sancak *et al.* identified that amino acids induce the translocation of mTORC1 to the lysosomal membrane where it interacts with the aptly named 'Ragulator complex' consisting of MP1, p14 and p18. The Ragulator complex functions to recruit Rag GTPases to the lysosomal membranes where they function to activate mTORC1. It has been demonstrated that constitutive targeting of mTOR to the lysosomal surface is sufficient to render it unresponsive to amino acids [118].

1.4.4.2 Vps34

Vacuolar protein sorting 34 (Vps34) was recently identified as a positive regulator of mTORC1 signalling. Vps34 is involved in vesicular trafficking processes and autophagy [119]. It was shown that over expression of Vps34 causes upregulation of mTORC1 signalling to its downstream substrates. In addition to this, Vps34 was inhibited by amino acid/glucose withdrawal but not rapamycin, indicating that it lies upstream of mTORC1 [120, 121].

Vps34 binds to Vps15 to form an active complex, then uses PtdIns (phosphoinositides) as a substrate to produce PtdIns(3)P_1 [122], this results in the recruitment of various proteins to the early endosome where it may provide a platform for mTORC1 signalling [120, 121].

It was later established that amino acid stimulation causes an influx of calcium, Vps34 contains a calmodulin binding domain and has been postulated that binding of calcium/calmodulin to Vps34 increases lipid kinase activity and increases mTORC1 signalling, suggesting in fact that Vps34 senses calcium rather than amino

acids [123]. However Yan e*t al.* in 2009 indicated that Vps34 activity was unaffected by calcium chelators or calmodulin inhibitors disputing this argument [124]. In addition, a recent study implementing *Drosophila* containing loss-of-function mutations to the Vps34 ortholog demonstrated that TOR signalling was unaffected while autophagy and endocytosis were disrupted [119]. This may indicate that Vps34 signalling is only conserved among invertebrates. Alternatively it may be possible that Vps34 activates mTORC1 when amino acids are present in order to repress autophagy (see figure 1.6) since it has a well characterised role as an activator of autophagy under appropriate nutrient conditions.

Further research is required to determine whether amino acids signal to Vps34 within the cell.

1.4.4.3 MAP4K3

MAP4K3 is an Ste20 related kinase which was identified in 2007 as a regulator of mTORC1 in response to amino acids [125]. Findlay *et al.* established that over-expression of MAP4K3 could propagate mTORC1 signalling as determined by increased phosphorylation of S6K1 and 4E-BP1 (see section 1.5). This was subject to inhibition by rapamycin but not the PI3K inhibitor wortmannin. MAP4K3 activity was shown to be regulated by amino acids but not insulin or rapamycin. Furthermore, knockdown of MAP4K3 prevented amino acid induced S6K1 phosphorylation. This suggests that MAP4K3 lies upstream of mTORC1 and may function to regulate mTORC1 in response to amino acids [125]. Later work in 2010 utilised *Drosophila* MAP4K3 mutants to investigate this further. Interestingly, the mutant flies were viable indicating that TOR signalling could still occur. The mutants however displayed reduced TOR signalling which may suggest that MAP4K3 functions to modulate mTOR but is not required for its activation. Interestingly, differences between the mutant flies and wild-type could be diminished if the flies were raised under low-nutrient conditions. This suggests that MAP4K3 plays an important role in modulation of TOR signalling when nutrients are plentiful. Yan *et al.* observed that suppression of the Rag proteins reduced the ability of MAP4K3 to propagate mTORC1 signalling but concluded that it was unlikely that RagGTPases were directly modulated by MAP4K3. Further work is required to determine how the two pathways are related.

Yan *et al.* also used mass spectrometry phosphopeptide analysis to identify a phosphorylation site, Ser170 within the kinase activation domain of MAP4K3. Phosphorylation at this site is thought to be required for amino acid modulation of MAP4K3 activity and for MAP4K3 activation of mTORC1. Phosphorylation at Ser170 was eliminated by amino acid withdrawal but unaffected by insulin treatment. Interestingly, they showed that amino acid withdrawal caused an acute drop in Ser170 phosphorylation within 5 minutes and postulated that this rapid dephosphorylation was likely to be the action of a phosphatase. Further studies revealed that MAP4K dephosphorylation could be inhibited by incubation with the specific PP2A inhibitor okadaic acid [126]. PP2A is a multiprotein serine/threonine phosphatase which functions to reverse the action of many kinases in many major signalling pathways. Consisting of a structural A-subunit, a regulatory B-subunit and a catalytic C-subunit, the regulatory B-subunit is thought to determine substrate specificity [127]. Yan *et al.* revealed that MAP4K3 could bind to the B-subunit of PP2A, PR61ε. It was demonstrated that ectopic expression of PR61ε could abolish Ser170 phosphorylation of MAP4K and hence mTORC1 signalling even in the presence of amino acids. Furthermore, amino acid deprivation lead to an increase in the binding of PP2A to MAP4K3 [126]. This may suggest a model of competitive inhibition between PP2AT61ε (PP2A in complex with the B-subunit) and amino acids (more likely a factor regulated by both) but further work is required to fully elucidate how amino acids regulate MAP4K3 (see figure 1.5).

1.4.4.4 RalA

RalA is a member of the Ras superfamily of GTPases involved in modulation of protein transcription, cellular membranes and cell migration. It has also been implicated in cellular proliferation and participates in Ras-induced oncogenic transformation of cells. More recently however it has been implicated in nutrient regulation of mTORC1. Maehema *et al.* demonstrated that amino acid and glucose induced S6K1 phosphorylation could be inhibited by knockdown of RalA or its activator Ral-GDS. It was reported that amino acids increased the levels of GTP-bound RalA but not RalB, concluding that amino acids were able to regulate RalA to activate mTORC1. Furthermore, RalA knockdown was sufficient to suppress mTORC1 signalling in cells overexpressing a hyperactive mutant of Rheb and that RalA knockdown did not affect Rheb's nucleotide bound status, thus placing RalA

downstream of Rheb. However they were not able to show direct interaction of RalA with mTORC1 or with FKBP38 indicating that RalA is not able to activate mTORC1 alone. From this, they hypothesised that nutrients may be able to activate mTORC1 independently or downstream from Rheb [100], however the mechanism is yet to be uncovered.

1.4.4.5 UBR1 and UBR2

The branched chain amino acids, in particular leucine, elicit a much stronger impact upon mTORC1 signalling than other amino acids [128, 129]. Leucine withdrawal alone is as effective as complete amino acid starvation at suppressing mTORC1 signalling and stimulation with leucine is sufficient to promote mTORC1 signal transduction [128]. It has been speculated that leucine may be more frequently utilised in protein synthesis and therefore mTORC1 is more responsive to their depletion [128].

A recent study has however shed light on how leucine in particular is able to modulate mTORC1 by identifying UBR1 and UBR2 as both leucine binding proteins and negative regulators of mTORC1. UBR1 and UBR2 are E3 ubiquitin ligases which specifically recognise 'N-degrons' which are destabilising N-terminal basic or bulky hydrophobic residues of protein substrates. UBR1 and 2 function as 'N-recognins' which identify N-degrons and target these specific proteins for ubiquitination and subsequent degradation at the 26S proteasome (N-end rule pathway) [130]. A study by Kume *et al.* demonstrated that over-expression of UBR1 and UBR2 causes a suppression of mTORC1 signal transduction which can be rescued by stimulating with high concentrations of leucine. They demonstrated that leucine directly binds to the substrate recognition domain of UBR2 preventing degradation via the N-end rule pathway, this promotes signalling via mTORC1 [131]. It is unclear how they function to inhibit mTORC1 signalling and whether this is related to their roles as ubiquitin ligases, leucine may also be able to exert a more direct effect but this has yet to be discovered.

1.4.4.6 NPR2 and NPR3

Within yeast, conditions of rapamycin treatment or amino acid withdrawal result in the nuclear translocation of transcription factors Gln3 and Gat1 causing expression of Dal80 and suppression of Tor. When nutrients are plentiful, Gln3 and Gat1 remain

cytoplasmic and hence inactive. Neklesa *et al.* developed a flow-cytometry based genetic screen to discover regulators of TOR1 in yeast. They identified a highly conserved complex consisting of NPR2 and NPR3 which responds specifically to amino acid deprivation in yeast to inactivate TOR1 [132]. Unfortunately they were unable to identify the mechanistic action of NPR2 and NPR3, nor whether this activity was conserved among higher eukaryotes.

NPR2 and 3 are nitrogen permease regulators, the human analogues of which are NPRL2 and NPRL3 which are now known to function as tumour suppressors [133] so it may be likely that the human analogues of NPRL2/3 perform similar functions in mammalian cells. A recent study by Kuruta *et al.* also demonstrated that NPRL2 interacts with and inhibits PDK1 [134]. PDK1 phosphorylates Akt to promote activation of mTORC1 (see section 1.4.1). It was demonstrated that active NPRL2 functions to inhibit PDK1 and subsequently decrease phosphorylation of the inhibitory mTORC1 regulators PRAS40 and TSC2, suppressing mTORC1. Unfortunately Kurata *et al.* did not investigate NPRL2 activity under the context of nutrient deprivation. Since a similar mechanism occurs in eukaryotes as yeast and NPRL2 can inhibit mTORC1, it is possible that NPRL2 may also be subject to amino acid regulation and may present one of the mechanisms by which amino acids signal to mTORC1.

1.4.5 Hypoxic Regulation

The cell is continuously orchestrating a fine balance between ensuring sufficient oxygen concentration for metabolic processes without allowing it to reach toxic levels. During O_2 deprivation, the cell rapidly adapts to compensate for the lack of O_2, this is regulated in part by increased production of HIFs (see section 1.1 Oxygen),but also by suppression of energy demanding process such as protein translation. This is modulated in part by a hypoxia induced suppression of mTORC1 signalling. Hypoxia induces suppression of mTORC1 by several distinct mechanisms, all of which are dispensable. HIF-1α has been identified as a potential target of mTORC1 signalling and is a focus of this study [135]. There are multiple mechanisms of hypoxia mediated mTORC1 suppression, both HIF-1α dependent and independent mechanisms are described. See figure 1.6 for overview.

1.4.5.1 AMPK

As described above, induction of AMPK as a result of energy stress results in mTORC1 suppression. AMPK phosphorylates TSC2 at Ser1345 to induce formation of the inhibitory TSC [112] (see figure 1.6). In addition to this, AMPK can also directly phosphorylate Raptor to reduce mTORC1 activity [115]. Both these events play a role in hypoxia induced mTORC1 suppression.

1.4.5.2 REDD1 and REDD2

REDD1/2 expression is upregulated in response to hypoxia, cell stress and DNA damage and it is thought to be a direct target for the transcription factor HIF-1α [136]. When mTORC1 is active, inhibitory 14-3-3 proteins bind to TSC2 to disrupt TSC-mediated inhibition of mTOR [137-140]. Under hypoxia, REDD1/2 binds to and sequesters 14-3-3 proteins, thus releasing TSC2 and thereby rescuing TSC Rheb-GAP activity to inhibit mTORC1 [35, 141, 142].

1.4.5.3 PML (Promyelocytic leukaemia tumour suppressor)

PML induces growth arrest, cellular senescence and apoptosis. In 2006, Bernardi *et al.* identified PML as a key regulator of angiogenesis, demonstrating that PML could be induced by hypoxia and that it led to a down-regulation of HIF-1α. They identified a novel mechanism by which PML functions to sequester mTOR to the nucleus, away from its activator Rheb. This results in suppression of mTORC1 signalling and therefore down-regulation of HIF-1α [143].

1.4.5.4 BNIP3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3)

BNIP3 is a member of the of the Bcl-2 family of apoptosis regulating proteins [144]. It is also a target for HIF-1α and it is thought to play a role in hypoxic-induced cell death [144, 145]. More recently it has been identified as one of the mediators of hypoxia-induced mTOR suppression. BNIP3 has been shown to bind directly to Rheb where it appears to reduce the GTP-level, thus preventing its activation of mTORC1 [146].

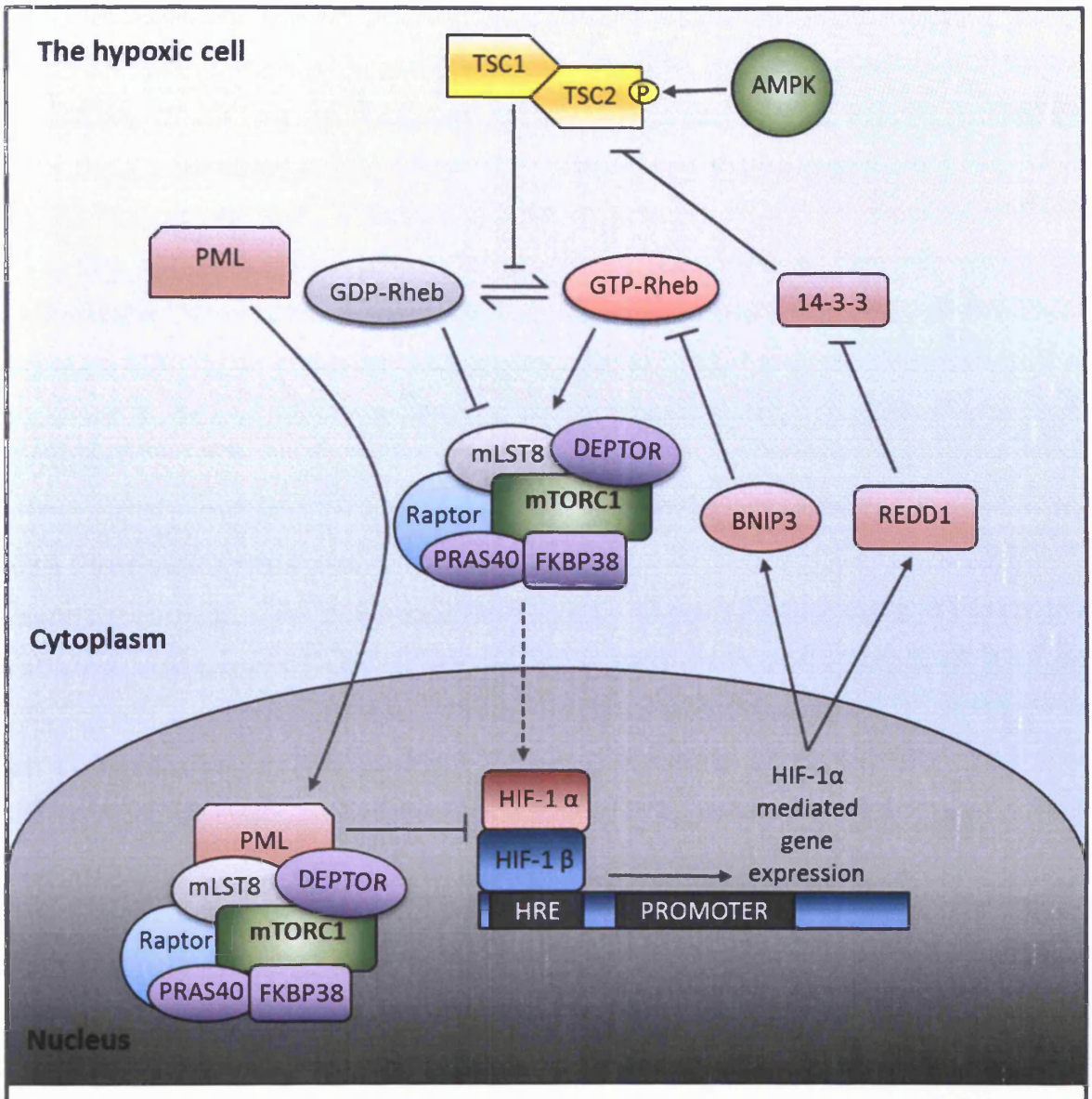


Figure 1.6: Hypoxia induced mTORC1 suppression: mTORC1 increases activity of HIF-1α, HIF-1α increases the transcription of BNIP3 which binds directly to Rheb causing an increase in the ratio of GDP-Rheb: GTP-Rheb, suppressing mTORC1 activity. HIF-1α also increases expression of REDD1 which sequesters inhibitory 14-3-3 proteins which normally interfere with TSC1/2 complex formation. This allows TSC1/2 to exert GAP activity towards Rheb, also functioning to decrease it's GTP-bound state. Hypoxia also increases the cellular stress response kinase AMPK, AMPK directly phosphorylates TSC2 at Ser1345 which promotes the formation of the TSC1/2 complex. Finally, hypoxia also induces PML activity, PML interacts directly with mTORC1, sequestering it to the nucleus away from the activator Rheb.

1.4.5.5 Hypoxia and mTORC1

The relationship between O_2 homeostasis is complex, mTORC1 appears to regulate the activity of HIF-1α which is an essential mediator of the hypoxic response, paradoxically however mTORC1 itself is suppressed during hypoxia but HIF-1α is not. This suggests that mTORC1 is able to upregulate HIF-1α specifically whilst mediating suppression of other cellular processes; however this has yet to be demonstrated. One of the primary aims of this study is to characterise how mTORC1 regulates HIF-1α. In order to understand how mTORC1 can regulate HIF-1α it is important to dissect what we already know about mTORC1 and its downstream substrates.

1.4.6 Feedback Loops

There are many positive and negative feedback loops in place which fine tune signal transduction through mTOR. Several mechanisms have been described involving S6K1 which is a direct substrate for mTORC1. For instance, S6K1 phosphorylates Ser/Thr residues in IRS proteins inhibiting tyrosine phosphorylation and thus preventing PI3Kinase activation, this renders cells resistant to insulin [147, 148]. Active S6K1 also reduces the expression of both α and β isoforms of the platelet derived growth factor receptor (PDGFR), causing insensitivity to serum and platelet derived growth factor when mTORC1 is active. TSC1/2 deficient cells lines thus exhibit reduced expression of PDGFR isoforms in comparison to their wild-types [149]. Finally, S6K1 is also reported to phosphorylate Rictor in order to downregulate mTORC2 mediated phosphorylation of Akt [150, 151].

Recent reports suggest inhibition of mTORC1 by rapamycin causes upregulation of ERK1/2 signalling to TSC2, disrupting the TSC1/2 tumour suppressor complex (see section 1.4.1). It is thought that active S6K1 functions to suppress activation of the Ras/Raf signalling cascade, therefore mTORC1 inhibition with rapamycin alleviates the S6K1 mediated repression of Ras/Raf signalling [152]. This may lead to activation of other pathways downstream of Raf and Ras which may be inappropriately upregulated. Feedback mechanisms have direct implications for the therapeutic use of mTORC1 inhibitors since they too become dysregulated when signalling pathways are manipulated. Identifying these feedback loops is paramount to understanding how diseases manifest through perturbed signalling, and also for successful therapeutic intervention.

1.5 DOWNSTREAM SUBSTRATES OF mTORC1

mTORC1 regulates multiple processes, including cell growth and proliferation, cell cycle progression, angiogenesis, erythropoiesis, glucose transport and mitochondrial biogenesis (see figure 1.7). It exerts many effects through regulation of protein synthesis at a translational and transcriptional level, mediating phosphorylation of multiple downstream substrates. Our knowledge base is rapidly expanding in the field with identification of several new mTORC1 substrates in recent years and many still yet to be uncovered.

Below is a review of the current knowledge of signalling downstream of mTORC1. See figure 1.7 for an overview of mTORC1 downstream signalling effects.

1.5.1 The ribosomal protein S6-kinases

The S6-kinases are a subset of the AGC family of protein kinases, several other AGC kinases are also involved in mTOR signal transduction including Akt and p90-RSK. S6K1 and S6K2, like mTOR, are serine/threonine kinases and are known regulators of protein synthesis. In addition to this role, they have been implicated in the regulation of mRNA processing, cell growth and survival as well as glucose homeostasis (see review [153]). The S6-kinases exert many of their effects through phosphorylation of ribosomal protein S6 (rpS6) however a full list of the known S6 kinase substrates is shown in table 1.2.

The family of AGC protein kinases share an analogous mechanism for activation, for maximal induction they require phosphorylation at two conserved Ser/Thr residues. One of which is situated in the T-loop domain (or activation-loop) and one is contained within a hydrophobic motif found in the C-terminal catalytic domain. PDK1 is thought to be the major T-loop kinase for the S6 kinases and many other AGC kinases downstream of PI3K. It is thought that phosphorylation of the Thr389 site (Thr388 in S6K2) contained within the hydrophobic motif, creates a docking site for PDK1 which promotes its phosphorylation at the T-loop domain and hence its full activation [154].

It was demonstrated in 2002 that mTORC1 specifically interacts with S6K1 via an mTORC1 signalling motif to mediate its phosphorylation and subsequent activation in a rapamycin sensitive manner. The TOS motif is found in the N-terminus of both S6K1 and S6K2, mutation to this motif was found to mimic the effects of rapamycin [77]. Rapamycin treatment is thought to repress S6K1 activation not only

through downregulation of mTORC1, but also through increased activity of the phosphatase PP2A towards S6K1 [155].

Studies utilising *Drosophila* have revealed the importance of the S6K's in modulating cell growth and development. Disruption to the singular S6K gene found in *Drosophila* (dS6K1) resulted in lethality for the majority of flies at the larval stage. Those that did survive presented with a significantly smaller phenotype than the wild-type as a result of a reduction in cell size not proliferation [156].

The S6Ks exert the majority of their effects through phosphorylation of rpS6, rpS6 becomes activated after sequential phosphorylation at 5 different Serine sites within its C-terminus: Ser236 > Ser235 > Ser240 > Ser244 > Ser247, the S6Ks are able to mediate phosphorylation of Ser240/244 [157]. Mice studies demonstrated that rpS6-/- mice share a similar phenotype to S6K1-/- mice and both exhibit defects in cell growth. Interestingly, S6K1-/- mice show minimal changes to rpS6 phosphorylation whereas S6K2-/- mice show significantly reduced levels of phosphorylated rpS6 but normal growth [158]. This would indicate that S6K2 is the primary kinase for rpS6 but does not explain the discrepancies in the cell growth phenotype. It may indicate that rpS6 is only able to effect cell growth when phosphorylated by S6K1, and could be explained by differential localisation of S6K1 and S6K2. S6K2 contains an additional C-terminal nuclear localisation sequence whilst S6K1 contains an additional PDZ domain associated with recruitment to the cytoskeleton [153] in support of this.

S6K1 also has multiple roles in protein translation (see figure 1.8). Introns within a gene promote splicing which enhances gene expression via recruitment of the exon junction complex (EJC) [159, 160]. In 2004, Richardson *et al.* identified SKAR to be a specific target of S6K1 [161]. Ma *et al.* later demonstrated that S6K1 was recruited to the EJC via binding to SKAR where it functions to enhance translational efficiency mediated via increased splicing [162].

Furthermore, a recent a study by Yamnik *et al.* demonstrated that S6K1 directly phosphorylates the oestrogen receptor (ERα) at Ser167 promoting its transcriptional activity in a rapamycin sensitive manner [163]. Increased transcriptional activity of ERα promotes cell proliferation and is thought to confer enhanced proliferation in breast cancer cell lines, implicating mTORC1 dysregulation in the manifestation of breast cancer. See figure 1.8 for an overview of the effects of S6 kinases upon protein synthesis.

Table 1.2: Substrates of the S6 kinases

Substrate	Function
rpS6	Component of the ribosomal 40s subunit, required for translation.
eIF4b	Assists eIF4A in unwinding of mRNA for translation.
eEF2k	Inhibits eEF2 required for elongation (protein translation), also activates autophagy. Inactivated by S6 kinase mediated phosphorylation.
MAD1	Also inactivated by the S6 kinases, MAD1 is an inhibitor of myc-directed transcription which upregulates genes involved in cell proliferation and apoptosis, it is also associated with oncogenic transformation. MAD1 has also been shown to regulate UBF which is a transcription factor required for ribosomal biogenesis.
CBP80	80 kDa subunit of the nuclear cap binding complex. Activated by S6 kinases and facilitates pre-mRNA splicing and synthesis of poly (A) tail (see figure 1.1 'The Transcription Cycle').
Pdcd4	Pdcd4 is a tumour suppressor which inhibits protein translation by blocking eIF4A. S6 kinases mediate its degradation to enhance protein translation.
SKAR	Activated by S6K1 but not S6K2. Interacts with the DNA polymerase delta p50 subunit and appears to play a role in cell growth regulation.
IRS-1	S6 kinases phosphorylate IRS-1 to facilitate its degradation preventing further insulin induced S6 Kinase activity as a means of negative feedback.
mTOR	S6 kinase mediate phosphorylation of mTOR directly at Thr-2446/Ser-2448, the function of this is unclear but may be a positive feedback loop.
BAD	BAD is a pro-apoptotic protein which can dimerise with BCL-2 to promote cell death. Phosphorylation of BAD by S6 kinases prevents BCL-2 binding.
MDM2	MDM2 functions as an E3 ligase to p53 and is regulated by S6 kinases. p53 is involved in a plethora of cellular processes including cell cycle arrest, apoptosis and senescence. S6 kinase represses these processes via MDM2.
CREM	cAMP-responsive modulator (CREMτ) regulates the transcription of cAMP-responsive genes and plays a role in spermatogenesis.

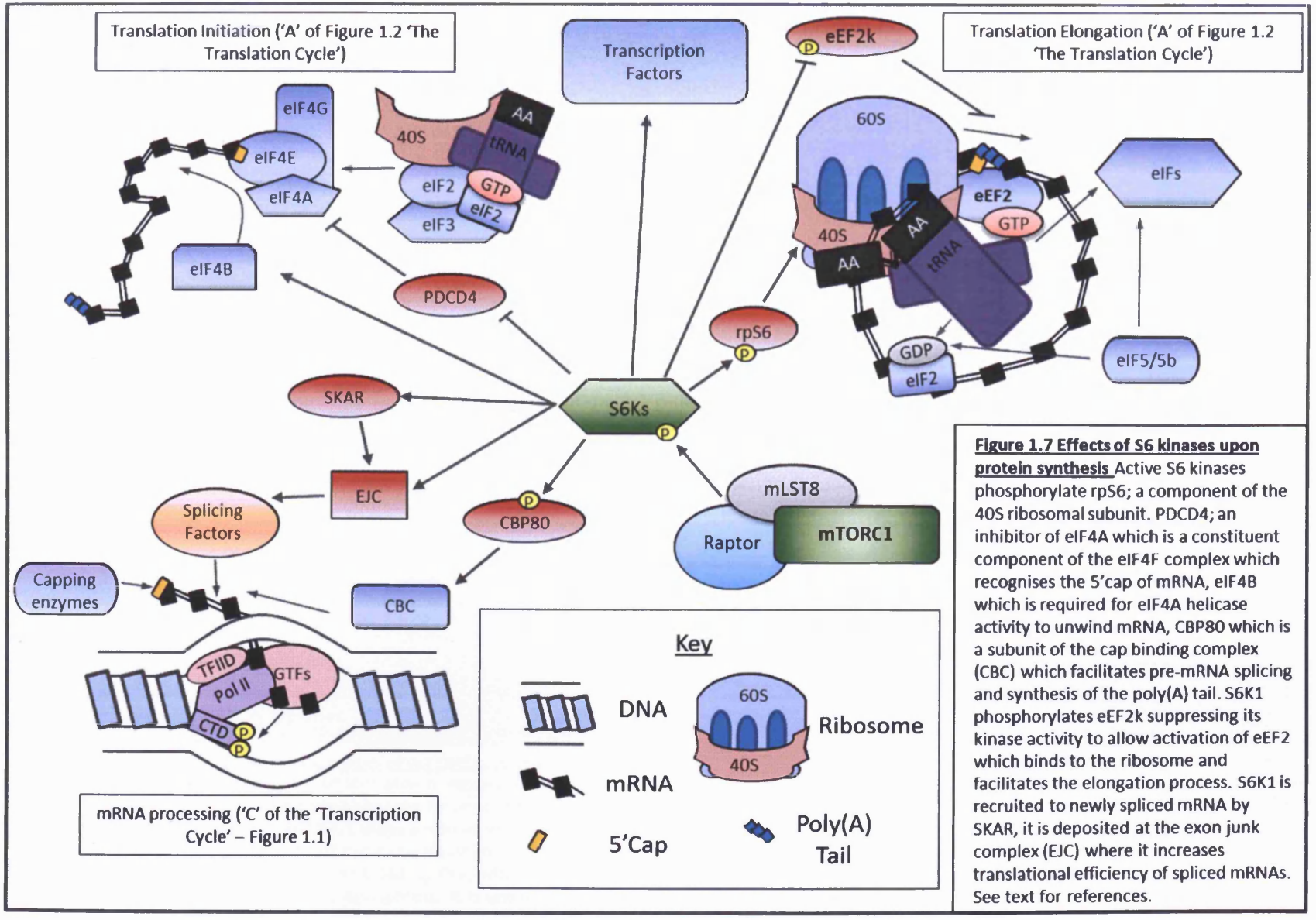


Figure 1.7 Effects of S6 kinases upon protein synthesis Active S6 kinases phosphorylate rpS6; a component of the 40S ribosomal subunit. PDCD4; an inhibitor of eIF4A which is a constituent component of the eIF4F complex which recognises the 5'cap of mRNA, eIF4B which is required for eIF4A helicase activity to unwind mRNA, CBP80 which is a subunit of the cap binding complex (CBC) which facilitates pre-mRNA splicing and synthesis of the poly(A) tail. S6K1 phosphorylates eEF2k suppressing its kinase activity to allow activation of eEF2 which binds to the ribosome and facilitates the elongation process. S6K1 is recruited to newly spliced mRNA by SKAR, it is deposited at the exon junk complex (EJC) where it increases translational efficiency of spliced mRNAs. See text for references.

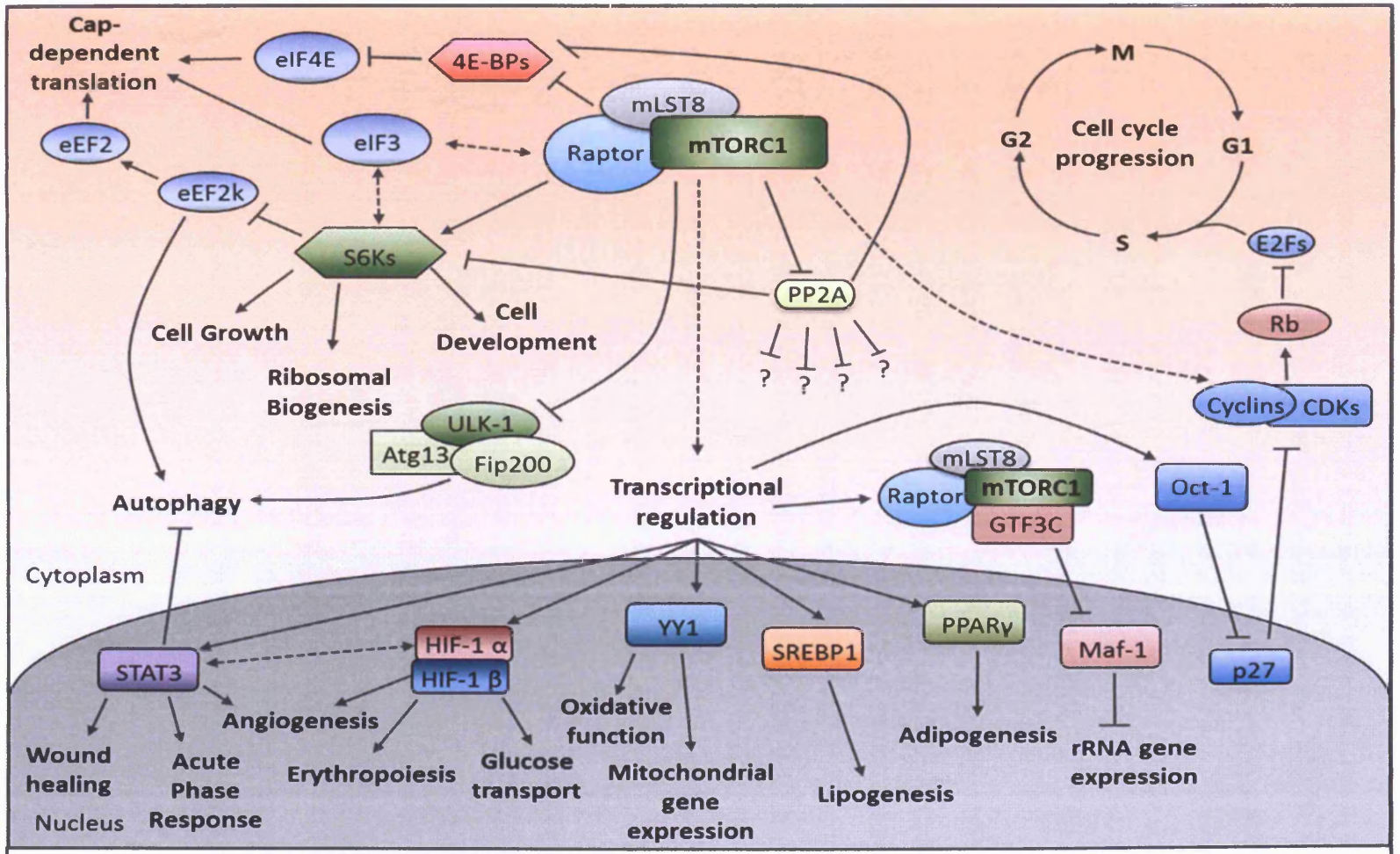


Figure 1.8 Signalling downstream of mTORC1: Active mTORC1 regulates cap-dependent translation via phosphorylation of 4E-BPs and S6 kinases. Active S6 kinases are also able to regulate cellular growth, ribosomal biogenesis and development. It is thought that active mTORC1 may repress the phosphatase PP2A to prevent dephosphorylation of substrates, however the full repertoire of PP2A targets has yet to be determined. mTORC1 plays a role in cell cycle progression by affecting the formation of active cyclin:CDK complexes (see section 1.5.1) which regulate the cell cycle repressor Rb. mTORC1s role as a transcriptional regulator has yet to be fully elucidated, mTORC1 has been reported to regulate STAT3, HIF-1, YY1, SREBP1, PPARγ, Maf-1 and p27 (indirectly) which in turn regulate gene expression relating to a diverse range of functions (highlighted). It is unknown if this regulation is direct or mediated by an mTORC1 effectors such as S6K1.

1.5.2 4E-BPs

As described in section 1.2.2.1, eIF4G interacts directly with the cap-bound protein eIF4E for the initiation of cap-dependent translation. The 4E-BPs regulate protein translation at this rate-limiting stage by disrupting this interaction. Hypo-phosphorylated 4E-BPs compete for and bind reversibly to the binding site on eIF4E also targeted by eIF4G, thus preventing its binding and suppressing cap-dependent translation [164, 165]. Phosphorylation from mTORC1 reduces the affinity of the 4E-BPs towards eIF4E, triggering removal from the cap-bound eIF4E so cap-dependent translation can then commence [166].

There are three 4E-BP isoforms, they belong to a poorly understood group of largely unstructured proteins which are thought to regulate a diverse range of cellular functions. 4E-BP1, 2 and 3 all share the eIF4E binding region found in eIF4G and hence are regulated in the same manner [167]. Binding of the 4E-BPs to eIF4E causes a change in conformation with the 4E-BPs adopting a more structured state consisting of around 50% α-helices, this may aid their inhibitive action [168].

4E-BPs tend to differ primarily in terms of their expression with 4E-BP1 being the most widely expressed and best characterised. Phosphorylation of 4E-BP1 is thought to be a two-step mechanism. There are six identified mTORC1 phosphorylation sites contained within the 4E-BPs. mTORC1 phosphorylates Thr37 and Thr46 and this acts as a priming event for phosphorylation at other sites. These sites are more readily phosphorylated whilst 4E-BP1 is in complex with eIF4E, and do not appear to influence the affinity of 4E-BP1 for eIF4E. However 4E-BP1 must be initially phosphorylated at these residues to allow subsequent phosphorylation of Ser65, Thr70, Ser83 and Ser112 (by mTORC1/Akt/PI3Ks) [166]. Phosphorylation at Ser65 has the greatest impact upon eIF4E binding affinity which is perhaps expected as it is the site situated closest to the eIF4E binding domain (see figure 1.9 below).

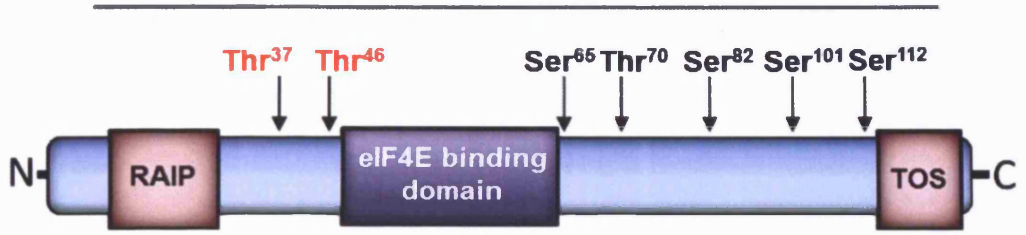


Figure 1.9 Schematic of 4E-BP1: Conserved domains indicated; RAIP motif (named after its constituent amino acids) required for maximal phosphorylation. TOS motif – mTORC1 signalling motif required for Raptor recognition of substrates. mTORC1 directed phosphorylation sites are highlighted in red, phosphorylation of which results promotes phosphorylation at other site to facilitate removal from eIF4E [80].

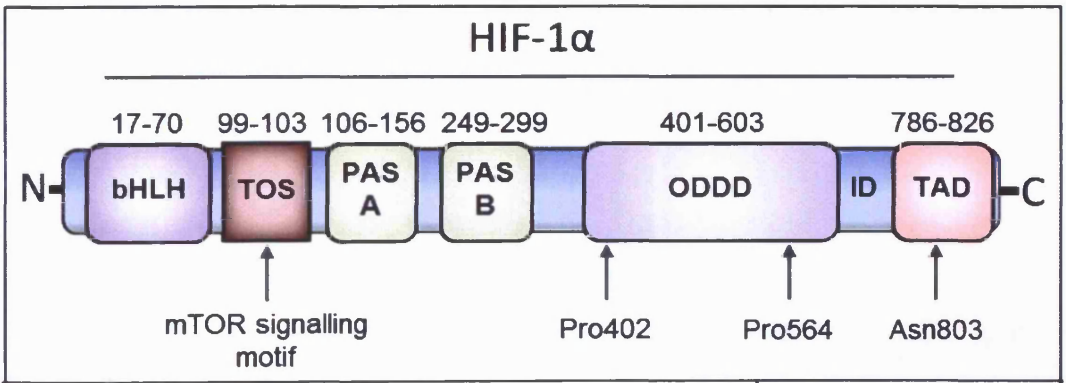


Figure 1.10 HIF-1α Schematic: Diagram indicating conserved domains within HIF-1α subunit, numbers above indicate amino acid positions. TOS motif is required for mTORC1 directed regulation. Proline residues within ODDD become hydroxylated in the presence of O2 to allow ubiquitination by the Von Hippel Lindau tumour suppressor. Asn803 is hydroxylated to prevent cofactors p300 and CBP from binding [172].

Key
bHLH: β-hoop looped helix domain.
ODDD: Oxygen dependent degradation domain.
ID: Inhibitory domain
TAD: Transcriptional activation domain.

mTORC1 interaction with 4E-BP1 is modulated by two distinct regulatory motifs. The mTOR signalling motif or TOS motif and an additional RAIP motif, named after its amino acid sequence (see figure 1.9). As with other mTORC1 substrates, mutations to the TOS motif of 4E-BP1 prevent interaction of mTORC1. The TOS mutant of 4E-BP1 therefore acts as a dominant inhibitor of cap-dependent translation [169], still able to bind to and inhibit eIF4E but not phosphorylated by mTORC1 to initiate its removal. The RAIP motif was later identified by Tee *et al.* [169] and appears to facilitate maximal phosphorylation of 4E-BP1 although there are discrepancies within the literature regarding whether it is involved in Raptor binding [170, 171], further characterisation of this motif is carried out as part of these studies.

1.5.3 HIF-1α

HIF-1α is a ubiquitously expressed HIF protein. HIF-1α is primarily responsible for the hypoxic response. It can modulate over 100 different target genes and regulates processes involved in angiogenesis, erythropoiesis, energy metabolism as well as cell cycle progression [135, 172]. It functions as a heterodimer which is comprised of a constitutively expressed HIF-β subunit and an inducible α sub-unit. The α-subunit is present in 3 isoforms, HIF-1α, HIF-2α and HIF-3α. HIF-1α and HIF-2α are similar in their structure and function whereas HIF-3α appears to play more of an opposing role, functioning to inhibit the expression of hypoxia induced genes [173]. HIF-1α and HIF-2α both appear to be activated and regulated in the same oxygen dependent manner however HIF-1α is the only form which is expressed ubiquitously. HIF-2α expression is restricted to the endothelium of blood vessels and distinct cells of the kidney, brain, heart, lung, liver, pancreas, and intestine [174, 175]. It is the ubiquitously expressed HIF-1α isoform which is the focus of this study due to its widespread expression and the presence of an mTOR signalling motif within the N-terminus which is not present in the HIF-2α isoform. Land and Tee (2007) discovered the mTOR signalling motif at the 3'-end of the period-ARNT-Sim conserved domain-A (PAS-A) of HIF-1α. In their study, the TOS motif, FVMVL, was mutated at the first crucial phenylalanine to an inactivating alanine residue (see figure 1.10). This produced a dominant negative mutant of HIF-1α and indicated a regulatory input from the mTORC1 signalling pathway [135].

Many diseases linked with mTOR feature the development of tumours. Whether they are benign hamartomas like those seen in TSC patients, or highly

metastatic cancers like those seen in renal cell carcinoma, activation of HIF-1α is required to induce the formation of new blood vessels. The diffusion of O_2 through tissues is very limited therefore tumours absolutely require activation of HIFs to increase O_2 and nutrient delivery to all areas of the tissue. The effects of inappropriate HIF-1α activation can be seen best in sufferers of Von-Hippel Lindau (VHL) syndrome. VHL results from mutations to the Von Hippel-Lindau tumour suppressor protein. The disease is characterised by the development of highly vascularised, both benign and malignant tumours with increased risk of hemangioblastoma and clear-cell renal carcinoma [176, 177]. VHL modulates the oxygen dependent degradation of the HIF-1α subunit (see figure 1.11 for an overview of this degradation). HIF-1α contains an oxygen dependent degradation domain, within this domain are two proline residues of significance, Pro564 and Pro402 (see figure 1.10). These proline sites become hydroxylated by any of three 2-oxoglutarate and Fe(II) dependent prolyl hydroxylases (PHDs) in the presence of O_2. This reaction causes a significant increase in the binding of VHL to HIF-1α, where VHL is the recognition component of the E3 ligase for HIF-1α, ubiquitinating it for degradation at the proteasome [178, 179]. OS-9 (osteosarcoma amplified 9) has also been demonstrated to influence the stability of HIF-1α. OS-9 appears to stabilise the interaction between HIF-1α and PHD2/3 to promote HIF-1α hydroxylation [180].

In addition to this, there is further hydroxylation of an asparaginyl residue C-terminal transcriptional activational domain of HIF-1α, which acts to inhibit interactions with the transcriptional co-activator of HIF-1α, p300 [179]. This is mediated by factor inhibiting HIF (FIH) [181] Oxygen is required for the hydroxylation of the proline and asparagine sites, therefore under hypoxic conditions this does not occur. Therefore under hypoxia, HIF-1α remains stable enough to form a heterodimer with the β-subunit, it then translocates into the nucleus. This heterodimer then binds to HIF response elements located upstream of the promoters on target genes (see figure 1.11.)

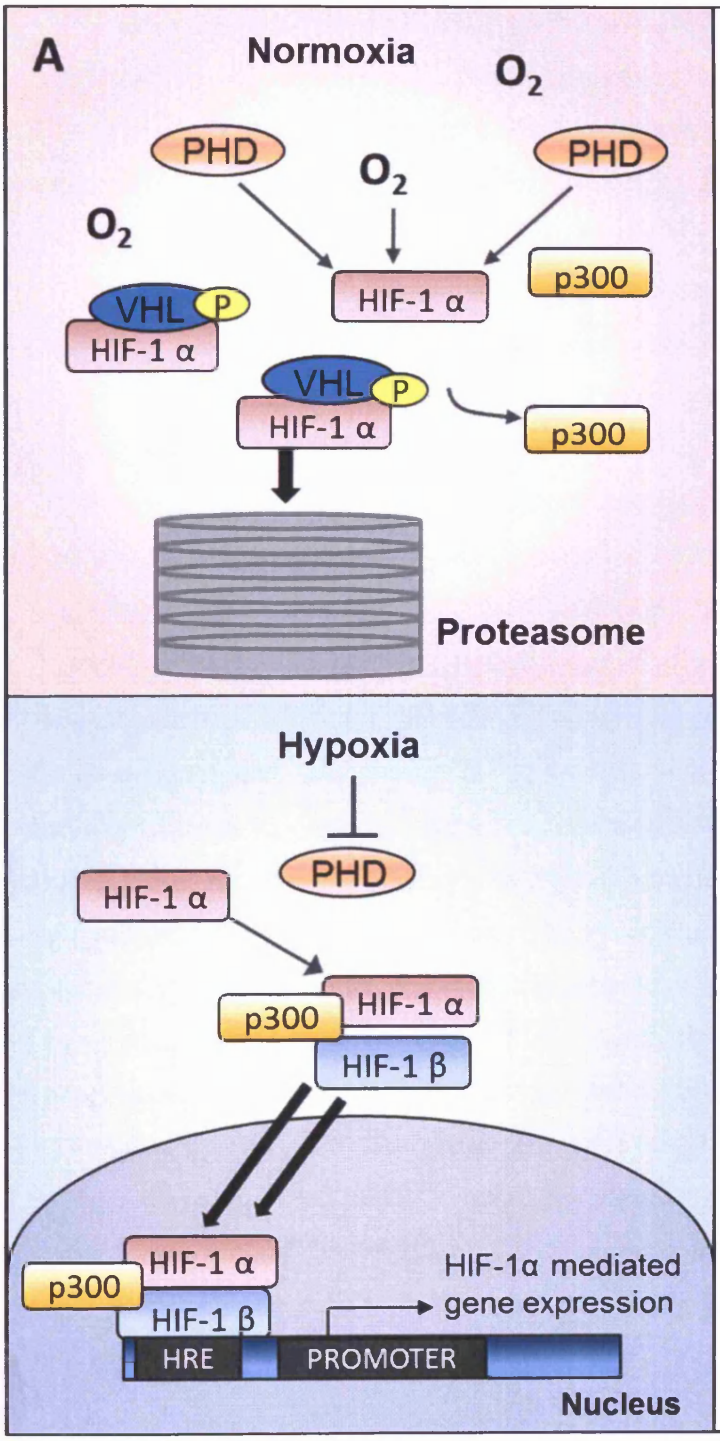


Figure 1.11 Oxygen dependent degradation of HIF-1α.

A: During normoxia; PHDs hydroxylate Pro402, Pro 564 and Asn803 In an O_2 dependent fashion. Hydroxylation of proline residues allows phosphorylated VHL to bind to the alpha subunit facilitating ubiquitination and thus tagging the alpha subunit for degradation at the proteasome. Hydroxylation of Asn803 prevents interactions between the alpha subunit and the transcriptional cofactor: p300. **B:** Under hypoxia, PHD enzymes are inhibited preventing hydroxylation events and subsequent degradation. This allows binding to the constitutively expressed HIF-β subunit as well as p300. This stabilises the transcriptional complex so that it can translocate to the nucleus where it binds directly to HIF response elements located upstream of the promoters on target genes. For review see [185].

Key

PHDs: Prolyl hydroxylases
VHL: Von Hippel Lindau tumour suppressor protein
HIF: Hypoxia inducible factor
HRE: HIF response elements

HIF-1α is also subject to other post-translational modications. For instance, it is acetylated by an acetyltransferase referred to as ARrest Defective-1 protein (ARD1). This interaction promotes VHL interaction with HIF-1α and therefore promotes HIF-1α proteasomal degradation. ARD1 is thought to be suppressed by hypoxic conditions to promote HIF-1α stability [182].

Conversely, HIF-1α stability can also be increased through post-translational modifications. Recent studies show evidence that HIF-1α is sumoylated by SUMO-1 (small ubiquitin-related modifier-1) at Lys391 and Lys477 resulting in an upregulation of its transcriptional activity and stability [183]

Nitric oxide has also recently been demonstrated to upregulate HIF-1α, it is thought that all 15 thiol groups found in human HIF-1α are subjected to S-nitrosation which promotes HIF-1α stabilisation and activation during hypoxia [184].

HIF-1α transcriptional activity appears to be dependent upon its interaction with co-activators p300 and CBP (CREB binding protein). p300/CBP bind to HIF-1α via the C-terminal and N-terminal TAD domains. It is thought that p300/CBP facilitate histone acetylation to promote chromatin remodelling, this enhances HIF-1α DNA-binding to HRE on target gene promoters. Interaction with p300 is thought to be a critical aspect of HIF-1α activation. Other acetyl-transferases such as steroid-receptor co-activator-1 (SRC-1) and transcription intermediary factor 2 (TIF2) are also thought to act in synergy with p300 to activate HIF-1α and this appears to be regulated by the redox regulated protein, Ref-1 [185]. Furthermore s-nitrosation on cys800 appears to promote it's interaction with p300

As indicated above, mTOR has been shown to regulate HIF-1α and diseases associated with mTORC1, such as TSC, exhibit highly vascularised hamartomas, however the mechanism behind this regulation has yet to be determined. One of the primary aims of this project is to characterise mTORC1 regulation of HIF-1α.

1.5.4 STAT3

1.5.4.1 The STAT family

STAT3 is a member of the STAT protein family. The STATs are a group of latent transcription factors which become activated in response to cytokine or growth factor interactions with cell membrane receptors [155-157]. There are currently seven recognised STAT proteins which are subject to similar mechanisms of regulation and activation, STAT-1, 2, 3, 4, 5a, 5b and 6.

Cytokine/growth factor stimulation causes recruitment of STAT proteins to the appropriate receptor as mediated by a highly conserved Src-homology-2 (SH2) domain found within the STAT family of proteins [186]. In resting cells, STAT proteins reside primarily as homodimers in the cytoplasm [187]. Upon ligand receptor binding, associated Janus-activated kinase family kinases (JAK kinases) are activated to promote phosphorylation of specific tyrosine residues within the STAT protein. The phospho-tyrosine residue then interacts directly with the SH2 domains within inactive STAT proteins to create protein dimers. The inactive STAT proteins are also phosphorylated at their tyrosine residue promoting the release and nuclear translocation of the activated dimers to upregulate cytokine mediated gene expression [186-188].

1.5.4.2 STAT3 Structure and function

STAT3 is ubiquitously expressed and activated by a number of cytokines, growth factors and other stimuli [188]. It is reportedly involved in cellular processes including cellular differentiation and survival, wound healing and the acute phase immune response. It also facilitates neuronal development in the brain in response to Ciliary Neurotrophic Factor (CNTF) [188-190] [191]. More recently, it has been demonstrated that STAT3 plays a role in carcinogenesis and tumour formation, in part due to the protective effect activated STAT3 can have from apoptosis [192-194]. A number of STAT3 target genes are reported to be upregulated during tumour formation including Bcl-X_L survivin, Hsp70, cyclin-D1 and c-myc which are likely to contribute to the pathogenesis [190]. Many genes have been proposed to be regulated in a STAT3 dependent manner, microarray analysis has indicated hundreds of potential target genes for STAT3 however not all these have been shown to be direct targets. A more recent study by Snyder *et al.* used chromatin immunoprecipitation (ChIP) to specifically look at STAT3 DNA binding and cross referenced interactors with those previously identified by microarray giving an extensive list (see below figure 1.12 for overview of probable STAT3 targets) [195].

Structurally, STAT3 is similar to the other members of the STAT family. It is activated by ligand binding to the gp-130 receptor, this receptor is activated by IL-6, CNTF, LIF (Leukaemia Inhibitory Factor), IL-11, oncostatin-M and cardiotrophin-1, causing dimerisation of the gp-130 receptor subunit. This recruits JAK kinases to

mediate tyrosine phosphorylation, firstly of the gp-130 receptor, providing a docking site for STAT3, then of STAT3 itself for activation [196-198]. STAT3 contains two characterised phosphorylation sites, Tyr705 and Ser727 both situated within the transcriptional activation domain (TAD) - see figure 1.11. It was initially thought that STAT proteins were modulated entirely by phosphorylation at the tyrosine residue (this is still thought to be the case for STAT2). However, further studies identified a secondary serine phosphorylation site within the TAD of STAT proteins which regulate the transcriptional activity. Phosphorylation at Ser727 on STAT1 is required for its maximal activation and facilitates the recruitment of its transcriptional cofactors, MCM5 and CamKII [199, 200], leading to enhanced activation. In the case of STAT3, there is substantial evidence for a similar positive role of Ser727 in STAT3 activation. STAT3 also contains two putative TOS motifs, FDMDL at amino acids 26-30 and FDMDL at amino acids 756-760 [80] (see figure 1.12) although research has not been carried out to determine whether they are functional mTORC1 signalling motifs.

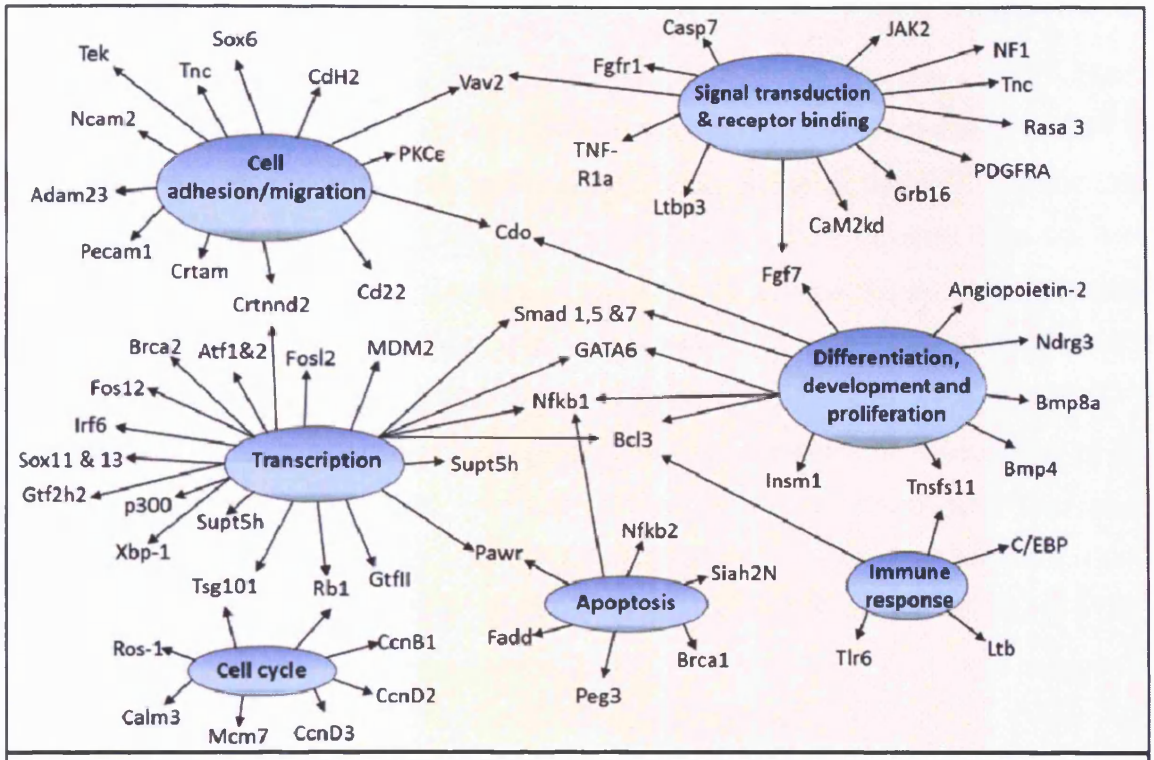


Figure 1.12 Gene targets of STAT3 Processes regulated by STAT3; figure shows potential gene targets of STAT3 as classified by function. Data adapted from CHiP of STAT3 DNA binding elements carried out by Snyder *et al.* [177].

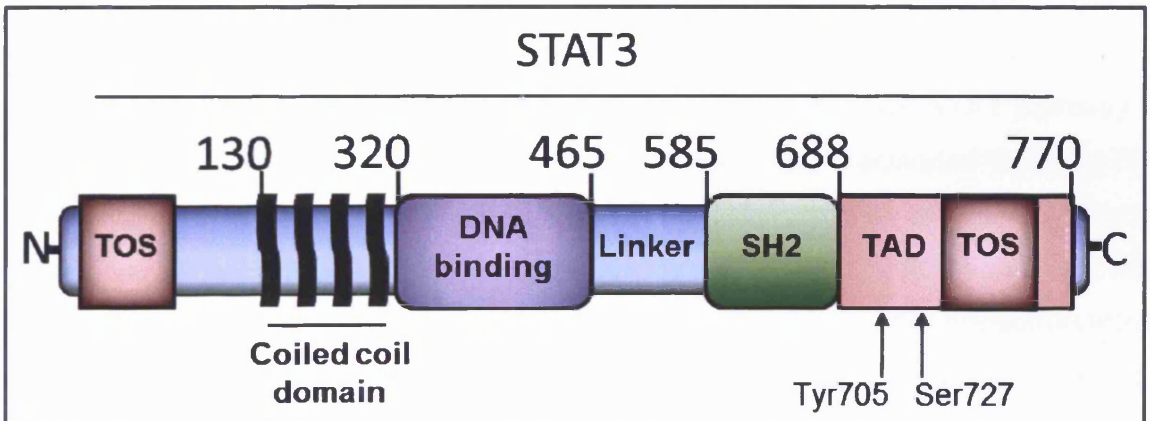


Figure 1.13: Schematic of STAT3: Demonstrating two potential mTOR signalling motifs (TOS), one of which is located within the carboxyl terminal transcriptional activation domain (TAD). SH2 domain allows interaction with gp-130 receptor and between other STAT3 molecules (via the Tyr705 residue) to allow dimerisation and subsequent nuclear localisation. Tyr705 phosphorylation is mediated by JAK kinases, Ser727 phosphorylation appears to regulate the transcriptional activity, several kinases have been implicated with Ser727 phosphorylation [204].

1.5.4.3 Serine phosphorylation of STAT3

Mice engineered to express a mutant form of STAT3 whereby the Ser727 site is substituted with an alanine (S727A) revealed that serine phosphorylation of STAT3 is required for embryonic growth and development. Many of the S727A mice died shortly after birth, those that didn't were approximately 50% smaller than the wild-type at one week old and exhibited altered IGF-1 serum levels and increased apoptosis [191]. This indicates that the supplementary signalling via growth factors to Ser727 may serve to regulate alternative cellular processes of STAT3. STAT3 is often referred to as the acute phase response gene due to its modulation of this process, however, S727A mice exhibited a normal STAT3 dependent liver acute phase response. This indicates that the acute phase response is modulated mainly via Tyr705 phosphorylation [191]. Shen *et al* suggested a model whereby STAT3 effects are determined by its phosphorylation status at both sites and suggest a more prominent role for Ser727 of STAT3 than was first thought [191].

However, other studies have indicated that Ser727 phosphorylation functions to negatively regulate Tyr705 phosphorylation, with cells expressing the S727A mutant exhibiting higher levels of tyrosine phosphorylation [201]. This could suggest that Ser727 phosphorylation may play a role in the negative feedback loops for STAT3. A study by Yokogami *et al.* demonstrated that in the case of neuroblastoma cell lines, CNTF (a neuropoeitic cytokine which stimulates the JAK STAT pathway in addition to ERK1/2, PI3K and mTOR signalling) stimulation activated both Tyr705 phosphorylation and Ser727 phosphorylation of STAT3, and phosphorylation of both sites was required for maximal transcriptional activation. Furthermore they showed evidence that mTORC1 may be the kinase which modulates Ser727 phosphorylation and demonstrated mTORC1 directed phosphorylation of a C-terminal STAT3 peptide *in vitro* [202].

A more recent study indicated that STAT3 could be activated at Ser727 but not Tyr705 phosphorylation by amino acid stimulation in a rapamycin sensitive manner [203] adding support for the hypothesis that mTORC1 is a regulator of STAT3. However, aside from Yokogami *et al.* studies providing evidence for mTORC1 mediated regulation of STAT3 are based upon inhibition seen with rapamycin treatment. Therefore it is unclear from these whether STAT3 is a direct substrate for mTORC1 or whether it occurs further downstream. In addition to this, more recently it was reported that the phosphatase PP2A could bind to a

dephosphorylate the Ser727 residue of STAT3 [204], since PP2A is activated by rapamycin treatment [205] it is possible that the rapamycin sensitivity demonstrated in many studies is a result of PP2A activation as opposed to mTORC1 inhibition. This study aims to determine whether STAT3 is indeed a direct substrate for mTORC1.

1.5.5 STAT3 and HIF-1α

The first evidence for a link between STAT3 and HIF-1α was published in 2002. As described earlier, VEGF is a gene target of HIF-1α. Analysis of the promoter region of VEGF revealed a putative STAT3 binding site, indicating that VEGF may be activated by STAT3 as well as HIF-1α [206] (it should be noted that there are also binding sites within the VEGF promoter for other transcription factors including Sp-1, AP-1, AP-2 and Egr-1 etc. [207]).

Nui *et al.* described how STAT3 and VEGF activity correlated and saw upregulation of VEGF with expression of a constitutively active STAT3 mutant [206]. This was confirmed by Wei *et al.* who demonstrated that VEGF was upregulated by STAT3 directly in pancreatic cancer cell lines [208].

In 2005, Gray *et al.* demonstrated that STAT3 and HIF-1α could bind to the VEGF promoter simultaneously in complex with the transcriptional co-activators p300 and APE. They postulated that both STAT3 and HIF-1α were required for maximal activation of the VEGF gene [209]. A second group later reported that hypoxia could induce STAT3 phosphorylation, promoting recruitment of HIF-1α and p300 as well as inducing histone H3 acetylation to cause transactivation of the VEGF promoter [210]. These studies provide clear evidence that STAT3 and HIF-1α function in synergy. The latter study suggests that STAT3 may in fact be a regulator of HIF-1α, this was confirmed shortly afterwards when Xu *et al.* showed that knockdown of STAT3 prevented both basal and growth-factor induced HIF-1α protein expression [211].

In 2008 it was reported that STAT3 is able to interact directly with the C-terminal domain of HIF-1α whereby it functions to directly compete with VHL for binding. Jung *et al.* demonstrated that over expression of constitutively active STAT3 inhibited the binding of VHL to HIF-1α in a dose dependent manner. (See figure 1.11 for VHL mechanistic action) [212]. This therefore represents at least one of the

mechanisms by which STAT3 is able to modulate HIF-1α. This study aims to investigate this relationship further.

1.5.6 YY1

It was demonstrated in 2006 that rapamycin treatment caused a reduction in mitochondrial membrane potential, oxygen consumption and ATP synthesis. Similar results were achieved through knockdown of TSC2/Raptor indicating that this is not a result of rapamycin induced phosphatase activity [213]. A later publication revealed a potential mechanism behind this showing evidence that Yin-Yang-1 may be a direct substrate for mTORC1 [214]. They established this via studies of the transcriptional co-activator, peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α). PGC-1α is a regulator of mitochondrial function, it modulates the expression of genes related to mitochondrial oxidative function, including the oestrogen-related receptor α (ERR-α) and nuclear respiratory factors (NRFs) [215]. Rapamycin treatment inhibits PGC-1α, ERR-α and NRF-1 mRNA levels and genomic analysis revealed that YY1-binding motifs were highly enriched within these rapamycin sensitive genes [214]. Cunningham *et al.* used shRNA interference to knockdown YY1 causing a dramatic decrease in mitochondrial activity. Knockdown of YY1 also rendered the cells insensitive to rapamycin inhibition of mitochondrial gene expression suggesting that YY1 is the intermediary component linking mTORC1 to mitochondrial function. They revealed that YY1 and PGC-1α formed a transcriptional complex and that this interaction could be disrupted by rapamycin treatment, resulting in a reduction in mitochondrial gene expression. YY1 is a zinc-finger transcription factor of the Polycomb group protein family. It is a 44kDa protein which is 414 amino acids in length. Eight different isoforms of YY1 are generated from alternative splicing of the YY1 gene, however the functional significance of this remains unknown [216]. It contains several conserved domains relating to its function, the N-terminus region appears to function as an activating domain, whereas the C-terminus appears to be involved in its repression. The four zinc fingered motifs are involved in its repression as well as its interaction with PGC-1α [214, 216]. Cunningham *et al.* demonstrated that mTORC1 could bind to the conserved 'REPO' domain of YY1 (so called because it is thought to mediate recruitment of polycomb proteins to their appropriate target genes [217]), however analysis of the sequence reveals only one phenylalanine within this domain. Therefore the only potential TOS

motif within this domain is VTMWS which would be unconventional. Although there are several reports implicating mTORC1 with mitochondrial biogenesis [213, 218, 219], little is known about the mechanisms governing this regulation. Further research is required to confirm whether YY1 is indeed a direct substrate for mTORC1.

1.5.7 GTF3C and Maf-1

Ribosomal synthesis is an expensive and complex process which requires all three classes of the RNA polymerases (Pol I, II and III). A proliferating HeLa cell is reported to produce approximately 7,500 ribosomes per minute, this requires transcription of around 150–200 rRNA genes and synthesis of around 300,000 ribosomal proteins [220]

It is well established that mTORC1 is a key regulator in this process. Studies examining the effects of rapamycin upon mammalian cells and yeast have demonstrated that mTOR plays a role in modulating transcription, pre-rRNA processing, expression of ribosomal proteins and synthesis of 5*S* rRNA [221-224]. However the mechanisms behind this regulation are not fully understood.

Recent evidence has demonstrated a direct link between mTORC1 and the regulation of RNA polymerase III (pol III), identifying GTF3C (general transcription factor IIIC or TFIIIC) as a key modulator of this process. Pol III is an RNA polymerase required for the production of 5S rRNA and tRNA which is negatively regulated by Maf-1. In yeast, Tor interacts with and phosphorylates Maf-1 *in vivo.* Wei *et al.* demonstrated how Tor translocates to the nucleus, to facilitate the release and cytoplasmic export of Maf-1, allowing Pol III mediated transcription to occur [225]. More recently it was demonstrated that mTOR is also involved in Maf-1 phosphorylation in mammalian cells [226].

Kantidakis *et al.* described a mechanism by which mTOR binds directly to GTF3C via an mTOR signalling motif (see table 1.1). GTF3C recognises the promoters of the tRNA and 5s rRNA genes and functions to relocate mTOR to the target genes (see figure 1.7). Mammalian Maf-1 appears to be is then phosphorylated by mTORC1, alleviating its repressive activity towards Pol III. However, Maf-1 remains at the Pol III site rather than translocating to the cytoplasm as is seen in yeast [227].

This case is particularly interesting as the TOS motif has been identified in the co-factor not the substrate. It will be of interest to see if the research group takes this work further to establish whether GTF3C is also phosphorylated by mTOR or whether this is a newly described mechanism.

1.5.8 PRAS40

See section 1.3.6 for details.

1.5.9 PKCδ and ε isoforms

Table 1.1 indicates that mTOR signalling motifs have been identified in the the δ and ε isoforms of PKC. It was reported in 1999 that these isoforms could in fact be regulated and phosphorylated in a rapamycin sensitive manner [228]. However, there has been no further evidence to support this. A more recent study investigating PKC δ and ε in the context of cardiac hypertrophy has shown evidence that PKCδ and ε isoforms are actually located upstream of mTORC1 and required for auto-phosphorylation of mTOR at Ser2448, as well as Thr389 phosphorylation of S6K1 during hypertrophy. This may explain the correlation in activity. [229]. Further research is required however to determine whether mTORC1 can in fact phosphorylate PKCδ and ε isoforms.

1.6 Cellular processes regulated downstream of mTORC1

1.6.1 Cell cycle progression

The cell cycle is made up of four distinct growth phases, G1, S, G2 and M. Whereby 'S' represents the DNA synthesis phase and 'M' represents the mitotic or dividing phase. Cells in a quiescent non-dividing state are said to be in G_0. For review see [230]. Progression through each phase of the cycle is subject to tight regulation from various inputs. The first indication that mTORC1 may be involved in cell cycle progression came from studies of the drug rapamycin. Analysis into the effects of the drug revealed its immunosuppressant qualities, with rapamycin treatment inducing growth arrest in T-lymhocytes, preventing progression into the S-phase of the cycle.

Although we are yet to discover exactly how mTORC1 regulates the cell cycle, there have been several recent advancements which are outlined below.

1.6.1.1 p27

p27 is a cyclin-dependent-kinase inhibitor (CDKI) which as the name indicates has inhibitory activity towards cyclin-dependent-kinases (CDKs) [231]. For a cell to progress from the G1 phase to the S-phase of the growth cycle, active cyclin complexes are formed between cyclins and CDKs. These active complexes phosphorylate the retinoblastoma gene product (Rb).

Rb is nuclear protein which is active whilst cells are in the G1 phase of the cell cycle, it inhibits the progression into S-phase, primarily through inhibition of the E2F family of transcription factors. Once Rb is phosphorylated by the active cyclin complexes, its inhibitory activity towards the E2F family is relieved and progression to S-phase commences [232].

Whilst the cell is in G1 phase, CDKs are repressed by p27 [231]. Active mTORC1 reduces expression of p27 to allow active cyclin complexes to form, promoting Rb phosphorylation and subsequent activation of E2F target genes. This allows progression to the s-phase of the cell cycle [233, 234] (see figure 1.7). When p27 is phosphorylated, its nuclear translocation is prevented, and its inhibitory activity is lost [235]. A recent publication also indicated a potential involvement of Oct1 in the expression of p27. Initial findings indicate that Oct1 mediates the transcription of p27 downstream of mTORC1. Further work is required to confirm this and also to confirm whether Oct1 is a direct downstream substrate of mTORC1 or one of its effectors [236].

1.6.1.2 Cyclin-D1

As outlined above, cyclins are important for the progression from G1 to the S-phase. There is growing evidence to suggest that mTORC1 may be able to regulate the expression of cyclin-D1, this may provide an alternative mechanism of cell cycle control. Cyclin-D1 forms an active cyclin complex with Cdk4, this stimulates activation of cyclin-E/CdK2 complexes by altering the binding activity of the inhibitory p27 [237]. Furthermore the active cyclin-D1 complex mediates phosphorylation of Rb on Ser795 [238], hyper-phosphorylation of Rb results in cell cycle progression to the S-phase.

The first evidence of mTORC1 directed regulation of cyclin-D1 appeared in 1993, a study by Rosenwald *et al.* showed that overexpression of eIF4E caused an increase in the expression of cyclin-D1 protein levels [239]. It was later demonstrated

that elevation of cyclin-D1 mRNA did not always lead to increased protein levels [240]. This indicates that cyclin-D1 is likely to be subject to post-transcriptional modulation and therefore makes eIF4E a likely regulator. In 1998 it was reported that rapamycin treatment of serum stimulated NIH 3T3 cells suppresses cyclin-D1 mRNA accumulation whilst reducing the stability of the transcript, rapamycin was also reported to accelerate its degradation at the proteasome [241].

However in 2004, Koziczak and Hynes demonstrated that siRNA mediated depletion of S6K1 caused a 20-30% reduction in cyclin-D1 expression which could be rescued by expression of an active S6K1 mutant construct in MDA-MB-453 cells. They also reported that rapamycin treatment reduced the association of cyclin-D1 mRNA with the polysome, suggesting that rapamycin can reduce cyclin-D1 levels via inhibition of S6K1 and subsequent downregulation of translational efficiency [242] rather than targeting the transcription and stability of the mRNA transcript or protein as was suggested by Hashemolhosseini *et al.* The most recently published study in this area however shows evidence that knockdown of 4E-BP1 abolishes rapamycin induced suppression of cyclin-D1 expression. It was shown that
4E-BP1 controls the association between cyclin-D1 mRNA and the polysomes, this may represent a mechanism by which mTORC1 regulates cyclin-D1 expression. It is likely that mTORC1 regulation of cyclin-D1 can occur by multiple mechanisms dependent upon the cell type and stimulus.

It is also notable that cyclin-D1 is a downstream gene target of STAT3 [243] which has shown evidence of mTORC1 dependent regulation [202]. This will add to the suppressive effect of rapamycin upon cyclin-D1 expression and adds to the complexity in dissecting the specific mechanisms of cyclin-D1 regulation downstream of mTORC1.

1.6.1.3 Other cyclins

It has been demonstrated that rapamycin treatment of T-lymphocytes diminishes the formation of active complexes between cyclin-D3 and Cdk4 or Cdk6, repressing the phosphorylation of Rb. Furthermore, over-expression of cyclin-D3 can alleviate the anti-proliferative effects of rapamycin treatment at low doses [244]. Decker *et al.* also reported that rapamycin treatment prevented upregulation of cyclins E and A, resulting in a decrease in CdK2 activity [245].

It appears from this evidence that cyclins, Cdks and CdK inhibitors are the key modulators of the cell cycle across most if not all cell types, many of which are subject to regulation from mTORC1. However difficulty arises given that the expression of specific cyclins and CdK inhibitors appears to be cell type and stimulus specific. The cell cycle is an appealing potential pharmacological target in the development of anti-cancer therapeutics and mTORC1 inhibitors may be a viable therapeutic tool.

1.6.2 Autophagy

So far this study has highlighted how mTORC1 acts as a nutrient sensor within eukaryotic cells, fine tuning the intricate balance between the rate of anabolic processes and the availability of nutrients. As logic dictates, mTORC1 is therefore also able to regulate catabolic processes within the cell to counterbalance this regulation. It has been known for some time that mTORC1 is a regulator of autophagy, however the mechanisms behind this have yet to be fully elucidated (see review [246]). Autophagy is a homeostatic mechanism induced by serum starvation, stress or reduced availability of growth factors. Low nutrient availability leads to inhibition of growth and autophagy induction as a mechanism of increasing intracellular nutrient levels [247, 248]. Autophagy consists of the breakdown of cytoplasmic proteins and organelles and is suppressed by mTORC1 signalling when nutrients are plentiful [248] [246]. As stated earlier, the mechanism governing mTORC1 mediated suppression of autophagy is unclear in the case of mammalian cells, however studies in yeast have increased our understanding (outlined below). Autophagy can be classified into three distinct pathways, microautophagy, macroautophagy and chaperone mediated autophagy. Chaperone mediated autophagy (CMA), as the name suggests, is a process whereby chaperones modulate the translocation of target proteins directly to the lysosome. It is a pathway specific to mammalian cells and is involved in the selective breakdown of soluble proteins [248] there is no evidence to suggest that it is subject to regulation by mTORC1 so will not be reviewed here. Macroautophagy (referred to as autophagy from here on) is the process involving the breakdown of macromolecules, organelles and unwanted structures and is the only autophagy pathway to involve the autophagosome.

When autophagy is induced, a double membrane structure termed the 'phagophore' or 'isolation membrane' is formed, this process is referred to as nucleation and it is unclear how this occurs. Recent publications have indicated that early isolation membrane structures associate with the endoplasmic reticulum and that this may provide a source of lipids for the extension of the membrane [249]. It has also been suggested that the phagophore is formed in a small omega shaped compartment deemed the 'omegasome'. This is reportedly connected to the endoplasmic reticulum and contains high levels of phosphatidylinositol 3-phosphate ($PtdIns(3)P_1$) [250]. The phagophore envelopes a portion of the cytoplasm or targeted organelles, elongates and closes to form the autophagosome. The autophagosome membrane then fuses with a lysosome exposing the contents to hydrolases which degrade the inner membrane and its targeted contents. This produces amino acids which are released back into the cytosol by membrane permeases so they can be re-utilised by the cell [246, 247, 251].

Microautophagy is the transfer of cytosolic components directly into the lysosome via invagination of the membrane [252] however as with the CMA pathway, there is no evidence to suggest that it is regulated by mTORC1 and therefore will not be considered within this review.

1.6.2.1 Autophagy regulation in yeast

There has been a wealth of investigations regarding the induction of autophagy in yeast which has furthered our understanding of autophagy in mammalian cells. The process of macrophagy in yeast is very similar to the process of macrophagy in mammalian cells, it should be noted that the lysosomes are the equivalent of vacuoles in yeast (or can be considered that way for the purpose of this review [253]). In 1998, it was reported that rapamycin could induce autophagy in yeast grown in nutrient rich medium signifying that suppression of TOR is an initiating factor in the induction of autophagy [254]. It was later established that this is primarily regulated through the kinase activity of Apg1. A study by Kamada *et al.* demonstrated that rapamycin treatment caused an induction of Apg1 protein expression and activation. Furthermore they identified that associated proteins Apg13 and Apg17 were required for rapamycin induced Apg1 activation. They proposed a mechanism by which Tor phosphorylates Apg13 reducing its affinity for Apg1. Inhibition of Tor therefore results in an increase in hypophosphorylated Apg13

which is able to bind and activate Apg1 initiating autophagy [255]. It was knowledge of this pathway which led to several pivotal discoveries regarding the regulation of autophagy in mammalian cells which are outlined below.

1.6.2.2 Regulation of autophagy in mammalian cells

It is now known that the mammalian homologue of Apg1 is UNC-51 like kinase or ULK1. Since this discovery, three more Apg1 homologues have been identified in mammalian cells, namely ULK2, 3 and 4. There is evidence that ULK1, 2 and 3 are all involved in the regulation of autophagy within mammalian cells (see review [246]) whereas the role of ULK4 remains unknown. Little was understood about how the ULKs regulate autophagy in mammalian cells until the discovery of the mammalian homologue of Apg13, referred to as Atg13.

Since then, several research groups have demonstrated that Atg13 interacts with both ULK1 and ULK2 and that this interaction is required for the formation of the autophagosome. A screen for ULK1 binding proteins in mammalian cells identified focal adhesion kinase family interacting protein of 200 kD (FIP200) as being an interactor of both ULK1 and ULK2. FIP200 had previously been identified as a regulator of cell size and proliferation, Hara *et al.* indicated that under serum deprivation FIP200 re-localised from the cytoplasm to the phagophore. Furthermore, FIP200 deficient cells failed to induce autophagy after amino acid and serum deprivation or rapamycin treatment. FIP200-/- MEFs also exhibited defective ULK complex formation and showed a reduction in ULK1 phosphorylation and expression in comparison to wild-type confirming that FIP200 is required for autophagy [256]. It is now thought that the phosphorylation status of ULK1/2 regulates a complex of Atg13 and FIP200 to initiate autophagy. When mTORC1 is active, it phosphorylates ULK1/2 at Ser757 which inhibits autophagy. Under conditions of cellular stress or nutrient depletion, AMPK becomes activated and phosphorylates ULK1/2 at Ser317 and Ser777 to induce autophagy [257].

1.6.2.3 eEF2k and Autophagy

As shown in table 1.2, eEF2k is a downstream substrate of S6K1. S6K1 mediates the phosphorylation of eEF2k causing its inactivation. A recent study reported elevated levels of eEF2k during nutrient depletion induced autophagy, which may be expected given that mTORC1 is suppressed during nutrient deprivation. Interestingly

though it was demonstrated that knockdown of eEF2k by RNA interference inhibited autophagy in glioblastoma cell lines whilst overexpression of eEF2k enhanced it [258]. This implicates eEF2k with the regulation of autophagy, this seems plausible since ULK1 appears to be regulated in the same fashion. It has also been demonstrated that silencing of eEF2k blunted autophagy and relieved the suppression of protein synthesis which is usually coupled with autophagy in a breast cancer cell line. [259]. It is likely that the suppression is therefore in part mediated through disruption to the elongation phase of protein translation, which is regulated by eEF2. Since elongation is activated by dephosphorylated eEF2, knockdown of its kinase eEF2k will result in an accumulation of dephosphorylated eEF2 increasing protein synthesis even under nutrient deprivation. The role eEF2k may play in inducing autophagy itself, remains to be seen.

1.6.2.4 STAT3 and autophagy

A recent study of HIV-infected cells has highlighted the possibility of a role for STAT3 in the inhibition of autophagy. It was recently demonstrated that HIV-1 infected cells show defects in the process of autophagy, whereby the HIV-1 viral protein Nef functions as an anti-autophagic maturation factor to inhibit interactions with the regulator of autophagy, Beclin-1 [260]. One of the defining features in the pathogenesis of the HIV-1 virus is its ability to affect cells which are not actually infected with the virus. They demonstrated that HIV-1 infected cells exhibited defective autophagosome formation in both infected cells and bystander monocytic cells when treated with rapamycin. They showed evidence that this was due to the release of HIV-1 Tat. Tat is released by HIV-1 infected or dying cells and is known to be the primary activator of the virus. Van Grol *et al.* found that addition of Tat reduced autophagy in rapamycin treated MDM cells, importantly they demonstrated that this could be reversed by silencing of STAT3 or Akt [261]. As shown in figure 1.5, Akt inactivates the tumour suppressor TSC complex to activate mTORC1, in addition STAT3 is a thought to be active downstream of mTORC1. This evidence taken together suggests a role for STAT3 in the suppression of autophagy, it also adds support for the hypothesis that STAT3 is activated downstream of mTORC1. This was compounded by the finding that Beclin-1 expression was found to correlate inversely with phosphorylated STAT3 expression and Akt expression in malignant gliomas [262] confirming that STAT3 is a negative regulator of autophagy.

Furthermore, additional support for this can be found in a study by Aoki *et al.* Aoki investigated the effects of T-oligos on autophagy induction. T-oligos activate DNA damage response pathways in the absence of DNA damaging agents. The response to DNA damage appears to be cell type specific, in the case of glioma cells, treatment with t-oligos lead to the induction of autophagy. Aoki demonstrated that this could be enhanced with treatment of the JAK2 inhibitor, Ag490 or by mTORC1 inhibition with rapamycin [263]. This provides further evidence that STAT3 may play a role in the suppression of autophagy, the mechanism behind this however is yet to be determined.

1.6.3 Lipid Metabolism

The initial link between lipid biosynthesis and mTORC1 was based upon observations that mTORC1 modulation of sterol and regulatory element binding protein-1 (SREBP-1) [264, 265]. SREBP-1 is a member of the basic helix-loop-helix leucine zipper transcription factor family [266, 267]. It transduces the insulin signal to promote expression of genes regulating the synthesis of cholesterol, phospholipids, fatty acids and triglycerides [268] and is therefore a central player in the regulation of lipid metabolism (see review [267]). The SREBPs occur in three closely related isoforms, SREBP1a, 1c and 2, they are synthesised initially as inactive pre-cursors. For activation, SREBPs form a complex with SREBP-cleavage-activating protein (SCAP) at the endoplasamic reticulum membrane. This complex is recognised by sterol-regulated proteases and is subjected to a two step proteolytic cleavage, allowing the release of the mature SREBP form. The mature SREBP then translocates the nucleus where it binds to sterol response elements found within the promoters of target genes [269]. Porstmann *et al.* demonstrated that Akt activation resulted in SREBP nuclear accumulation in a rapamycin sensitive fashion. Furthermore mTOR over-expression activated SREBPs and its target genes whilst knockdown of SREBPs caused a significant reduction in cell size [264]. This suggests that mTORC1 is able to regulate cellular growth not only through protein synthesis but also through coordinated lipid metabolism.

Normally, insulin is released in response to elevated glucose levels. It functions to increase the synthesis of fats and reduce the synthesis of glucose in order to restore a homeostatic balance. However in the case of type II diabetes, partial insulin resistance develops whereby gluconeogenesis is not suppressed and

lipogenesis continues. Li *et al.* observed the effects of mTOR, Akt and PI3K inhibitors on gluconeogenesis and lipogenesis and demonstrated that the insulin signal transduction bifuricates upon Akt activation. The activation of Akt not only causes activation of mTORC1 as previously described, but also mediates the phosphorylation of FOXO1. FOXO1 activates the expression of genes required for gluconeogenesis, phosphorylation of FOXO1 by Akt results in its expulsion from the nucleus and hence repression of gluconeogenesis. We now know that Akt mediated activation of mTORC1 results in increased activation of SREBPs, promoting lipogenesis. This bifurification of the insulin signal transduction may explain how insulin resistant cells in type II diabetes are able to promote lipogenesis in response to insulin but not inhibit gluconeogenesis. Li's paper also showed evidence that S6K1 inhibition had no effect upon SREBPs activation indicating that it does not lie downstream of S6K1 [265] although S6K1 knockout mice do display signs of altered lipid metabolism indicating that mTORC1 is able to regulate lipid metabolism via several different mechanisms [148].

A recent publication indicated that mTORC1 is able to suppress the transcription of triacylglycerol hydrolases including ATGL (adipose triglyceride lipase) and HSL (hormone sensitive lipase). These enzymes facilitate the metabolism of triglycerides into glycerol and free fatty acids which can then be used as an energy source [270]. Chakrabarti *et al.* investigated the impact of activation and inhibition of mTORC1 upon ATGL and HSL. The study demonstrated that active mTORC1 represses the transcription of these enzymes. Suppression of ATGL and HSL limited the triacylglycerol hydrolase activity within the cell, promoting the accumulation of triglycerides.

It is thought that when nutrients are plentiful, active mTORC1 suppresses the breakdown of fats with the view of building energy stores. When nutrients become depleted however, suppression of mTORC1 results in a release of inhibitory activity towards ATGL (and to a lesser extent HSL) to facilitate the mobilisation of energy stores [271]. It is not clear from this study whether mTORC1 is able to inhibit the transcription of ATGL directly, or via a downstream effector. As stated earlier, S6K1 knockout mice exhibit altered lipid metabolism by a mechanism unrelated to SREBP expression so it is possible that ATGL may be regulated downstream of S6K1, although there is no direct evidence for this at present.

Bacquer *et al.* investigated the effects of a double knockout (DKO) of 4E-BP1 and 4E-BP2 on the fat metabolism of mice. In comparison to wild-type, 4E-BP DKO mice gained an average 29% more weight when fed on equal amounts of a high carbohydrate diet. Interestingly, when fed upon a high fat diet, DKO mice showed higher blood insulin levels in comparison to wild-type and although both mice types exhibited impaired glucose tolerance, the effects were accentuated in the DKO mice. Bacquer *et al.* saw a suppression of lipolysis in DKO mice, which suggests that mTORC1 mediated suppression of ATGL does not occur downstream of the 4E-BPs, although the levels of ATGL were not examined. Bacquer *et al.* did however report an increase in the expression of peroxisome proliferator-activated receptor gamma (PPARγ) and its upstream activator C/BP1δ. PPARγ is a key regulator of adipocyte differentiation and lipogenesis in adipose tissue [272] so elevated expression would explain why the DKO mice accumulated more fat. This may be a direct result of 4E-BP1 knockdown, or it may be due to enhanced mTOR/S6K1 signalling which occurs when 4E-BP1 is knocked down. It is evident from these reports however that lipid metabolism is under multi-faceted control from mTORC1 thus potentially implicating mTORC1 dysregulation with a number of pathologies caused by disrupted lipid metabolism [273].

1.6.4 Phosphatase regulation

As outlined earlier, phosphatases oppose the activity of kinases. In yeast, it is thought that TAP42, a regulatory subunit of the type 2A phosphatases, is complexed with either PP2A or SIT4 to mediate substrate dephosphorylation. It has been demonstrated that this complex formation is regulated by Tor within yeast, primarily via phosphorylation of a TAP42 binding partner, TIP41 [274].

In mammalian cells, there is limited evidence of phosphatase involvement in mTORC1 signalling despite the discovery of the mammalian homolog of Tap42, α-4 in 1998 [275]. One example has already been described, MAP4K3 which transduces amino acid input to regulate mTORC1 appears to be regulated by PP2A and its regulatory subunit PR61ε (see section 1.4.4.3). Downstream of mTORC1, it has been demonstrated that rapamycin can induce rapid dephosphorylation of S6K1 at all sites, despite the fact that only Thr389 phosphorylation is mediated by mTORC1 [65]. This could suggest that sites are phosphorylated in a sequential manner and phosphorylation of Thr389 acts as a priming event, however Thr389 appears to be

one of the latter residues to be phosphorylated making this unlikely. It could therefore be better explained by phosphatase activation induced by rapamycin treatment.

Peterson *et al.* compared the phosphatase activity *in vitro* of purified PP2A immune complexes from rapamycin treated and untreated cells towards pre-phosphorylated purified recombinant 4E-BP1. They revealed that PP2A phosphatase activity was elevated in rapamycin treated cells. Indicating that like in yeast, PP2A is activated when mTORC1 is repressed by rapamycin. An *in vitro* kinase assay indicated that mTORC1 could phosphorylate PP2A directly, strikingly, mTORC1 exhibited a higher level of affinity towards PP2A than to 4E-BP1 *in vitro*. They were also able to show direct PP2A interaction with S6K1 [155]. This study shows significant evidence that rapamycin treatment can induce phosphatase activity. A mechanism was proposed whereby mTORC1 phosphorylates PP2A to suppress it, therefore PP2A becomes active when mTORC1 signalling is inhibited.

Further evidence for rapamycin induced phosphatase activity comes from a more recent publication dissecting specific phosphorylation events of rpS6. Moore *et al.* demonstrated that rapamycin suppressed rpS6 phosphorylation at Ser235/236 and Ser240/244. This was then repeated in the presence of phosphatase inhibitors and strikingly rapamycin only caused repression of the Ser240/244 sites, indicating phosphatase activity towards the other residues. This type of regulation could extend to other substrates which have been linked to mTORC1 based upon studies with rapamycin, it is therefore no longer appropriate to assume that substrates dephosphorylated with rapamycin treatment are indeed downstream substrates for mTORC1. [157].

1.6.5 Rapamycin insensitive functions of mTORC1

In addition to this, recently developed inhibitors which specifically disrupt the kinase activity of mTOR have revealed some surprising results with kinase inhibitors suppressing cellular proliferation much more effectively than rapamycin.

Targeting the mTOR kinase domain also functions to perturb the signalling of mTORC2 so it was originally thought that the additional effects of mTOR kinase inhibitors compared with rapamycin were a result of simultaneous mTORC2 inactivation. Intriguingly however, the mTOR kinase inhibitors were still able to induce a more complete suppression of cell proliferation in Sin-1-/- MEFs lacking

mTORC2 activity. Feldman *et al.* reported that Sin-1-/- MEFs showed greater suppression of 4E-BP1 signalling in response to kinase inhibitors of mTOR than to rapamycin, suggesting that rapamycin may in fact be a selective substrate inhibitor of mTOR [276]. Rapamycin does not directly target the mTOR kinase domain; it is therefore looking increasingly likely that rapamycin only suppresses a subset of mTORC1 functions. Furthermore, the efficacy of rapamycin may be affected by other variables, such as FKBP12 availability, therefore specific kinase inhibitors may provide a more complete inhibition of mTORC1 (as well as mTORC2). This provides a case for utilising kinase inhibitors as a possible alternative to rapamycin derivatives for the treatment of TSC patients. The specific manifestations of suppressed mTORC2 signalling and potential rapamycin insensitive functions of mTORC1 are yet to be fully determined but are important considerations in the development of therapeutic strategy.

1.7 mTOR DYSREGULATION AND DISEASE

Rapamycin is used to suppress mTORC1 signalling and is already being utilised in a number of clinical trials, however the potential rapamycin insensitive functions of mTORC1 are becoming more apparent thus increasing the emphasis upon developing inhibitors which target specific facets of the signalling pathway. Some examples of how mTORC1 is dysregulated in disease are described below.

1.7.1 Inherited hamartoma disorders

The inherited hamartoma disorders usually arise due to loss of function mutations to tumour suppressor genes. Several of these have been linked to aberrant mTORC1 signalling and are currently undergoing clinical trials to investigate the efficacy of mTOR inhibitors as potential treatment. The most well described hamartoma disorders are described below.

1.7.1.1 Tuberous Sclerosis Complex

Dysregulation of mTOR signalling is a central facet of the inherited disorder TSC. TSC is an autosomal dominant disorder characterised by the formation of benign hamartomas primarily in the kidneys, heart, skin, lungs and brain. It affects approximately 1/6000 live births and is caused by a mutations to either the TSC1 gene, located on chromosome 9q34, or the TSC2 gene located on chromosome

16p13. TSC corresponds to the two-hit model of tumourigenesis, whereby hamartomas develop from cells which have suffered a second mutation to either the TSC1 or TSC2 gene resulting in a loss of heterozygosity and subsequent loss of tumour suppressor function.

As demonstrated in figure 1.5, the TSC1 and TSC2 gene products function together as a heterodimer exhibiting GAP activity towards the mTORC1 activator Rheb. This facilitates the conversion of GTP-Rheb to GDP-Rheb which is unable to activate mTORC1. As described in section 1.4, TSC2 is subject to regulation from a number of cell signalling pathways and is phosphorylated by multiple kinases in response to nutrients and growth factors. Loss of function to either TSC1 or TSC2 therefore removes the requirement for nutrients in the activation of mTOR. TSC1/2 deficient cells find themselves in a state of constitutive upregulation of cellular growth and proliferation pathways, resulting in the formation of hamartomas. Furthermore, constitutive activation of mTORC1 results in a general suppression of catabolic pathways, inhibiting autophagy and apoptosis promoting the survival of these mutated cells.

TSC can vary considerable in terms of its presentation and severity, with mutations to TSC2 generally corresponding to a more severe phenotype. Several functional domains have been characterised within the TSC2 protein however the significant variation that is observed in the presentation of the disease makes it almost impossible to find correlations between the genetic mutation and the severity of the phenotype - the manifestations associated with the disease are described in table 1.3 below. Patients may exhibit any number and combination of these symptoms, from minor skin involvement to severe mental and behavioural impairments, epilepsy and renal failure. In these instances,

TSC is a hugely debilitating disease having major impacts upon the individual and their family. The potential severity of the disease is to be expected when you consider the extensive signalling network which is governed by the tuberous sclerosis complex. Although we still have a long way to come in determining the full pathophysiology of TS, sufferers and their families can take solace in just how quickly the research has advanced in this field, particularly given the fact that it wasn't until 2003 that it was discovered that TSC1 and TSC2 function together to suppress mTORC1 signalling.

Table 1.3: Manifestations of Tuberous Sclerosis by organ system.

Organ	Symptom	Manifestations
Heart	Cardiac Rhabdomyoma	Reported to effect between 47-67% of patients, common in infancy but usually regresses by adulthood. Could lead to arrhythmia or heart failure.
Brain	Corical Tubers	Small areas of the cortex of the brain which do not develop properly, often resulting in epilepsy.
	Subependymal Nodules	Highly calcified nodules which are present around the wall of cerebral ventricles. Not thought to impact upon brain function at present.
	Subependymal giant cell astrocytomas (SEGAs)	Larger SEGAs can cause blockage of cerebral ventricles, preventing the flow of fluid in the brain causing increased intracranial pressure.
	Development delay and behavioural issues	It is unclear what causes this in TS however approximately 50-60% of TS patients exhibit learning difficulties. Around 25% of TS patients exhibit some form of autism; ranging from mild to severe. Attention deficit disorder and hyperactivity are common in children. Paranoia, depression and anxiety are common in adults.
Lungs	Lymphangioleiomyomatosis (LAM)	LAM almost exclusively seen in female sufferers with TSC. Characterised by abnormal alveolar smooth muscle proliferation resulting in cystic formation and progressive destruction of the lung parenchyma ultimately leading to respiratory failure and death.
Kidneys	Angiomyolipomas (AML)	Found in approximately 80% of TSC patients, abnormal growths consisting of smooth muscle, fat and abnormal vascular components. Often asymptomatic; however growth may effect renal function depending upon the size and location. Increased risk of haemorrhage due to micro and macro-aneurysms.
	Renal cysts and polycystic kidney disease (PKD)	Renal cysts incur the same risks as with AMLs, cysts however are more likely to impact upon renal function. TSC patients are also at increased risk of developing PKD since the PKD gene is adjacent to the TSC2 gene (see section ?? PKD).
	Renal cell carcinoma	TSC patients exhibit increased risk of developing renal cell carcinoma.
Skin	Facial angiofibromas	Hamartomatous development of dermal connective tissue and vascular elements effecting 70-80% of TSC sufferers.
	Shagreen patches	Areas of darkened thick skin resulting from an accumulation of collagen, described as having the appearance of orange peel.
	Periungual and ungual fibromas (Koenen tumors)	Smooth nodular lesions occurring adjacent to or underneath the nails, most common in adolescence, effects approximately 20% of TSC patients.
	Hypomelanotic macules	Small hypo-pigmented lesions present in approximately 90% of TSC patiemts; usually present from birth.

We now have advanced knowledge of mTORC1 signal transduction pathways, furthermore, the mTORC1 inhibitor rapamycin had already been clinically approved as an immunosuppressant for the use in renal transplant patients. This has accelerated it's progression to the pre-clinical trial phase for the treatment of tuberous sclerosis and has already shown some promising results with the reports of AML (angiomyolipomas – see table 1.3) shrinkage of 53.2±26.6% and increased lung capacity in LAM sufferers (see table 1.3) after 12 months of rapamycin treatment [277].

Our understanding of the signalling pathways downstream of TSC1/2 has advanced even during these clinical trials. We now know that TSC1 and TSC2 can also function to activate mTORC2 (see section 1.3.1), and that mTORC1 has rapamycin insensitive functions (see section 1.6.5). Furthermore there is also evidence that Rheb may have mTORC1 independent functions indicated by the recent discovery of 'Notch' signalling. Notch is a key regulator of cellular development and appears to be key in cell-fate selection, Notch signalling was recently shown to be dysregulated in TSC lesions [278, 279].

Interestingly, conflicting reports regarding the mechanism of this regulation were reported at around the same time, with one group showing evidence that Notch was regulated downstream of mTOR (likely via STAT3) [278] and one group arguing that Notch is regulated directly by Rheb [279].

At this stage it is unclear which is correct and it may be the case that both these mechanisms are contributing to Notch dysregulation, however if the latter is proved to be accurate then this reveals a branch of signalling dysregulated in TSC patients which would be unaffected by the use of mTOR inhibitors. These discoveries mean that we can assess the efficacy of the use of rapamycin or it's analogues for the treatment of TSC in a more enlightened and objective manner. Furthermore it may explain in part why although mTOR inhibitors have shown some success in clinical trials, the results have been less significant than first hoped. It is likely that TSC1, TSC2 and Rheb have functions independent of mTOR regulation which would not be targeted with mTOR inhibitors. Elucidating these mechanisms could provide new potential therapeutic targets for combinational therapy in conjunction with rapamycin.

Furthermore, when mTORC1 signalling is elevated through TSC1/2 loss, signalling upstream of mTOR is generally suppressed through compensatory

mechanisms (see section 1.4.6), treating with rapamycin is likely to therefore alleviate this suppression which may cause dysregulation of other signalling pathways (discussed further in context of cancer).

It may also be appropriate to utilise specific mTOR kinase inhibitors such as the recently described Torin-1, as an alternative which could be used to target rapamycin insensitive functions of mTORC1. All these factors may influence future development of treatment strategies for TSC patients, hopefully contributing to a brighter future for those sufferers and their families.

1.7.1.2 Neurofibromatosis type-1 (NF1)

NF1 shares several similarities with TSC, it is also an autosomal dominant disorder which affects multiple organ systems. It arises from mutations to the NF1 gene which encodes the tumour suppressor neurofibromin-1. The NF1 gene is highly susceptible to sporadic mutation and this contributes to a higher incidence rate than is seen with TSC, with 1 in 3,500 live births affected [280].

Its principal clinical manifestation is the development of benign peripheral nerve sheath tumours referred to as neurofibromas. In severe cases, patients can present with thousands of neurofibromas which can be painful and disfiguring, furthermore, NF1 is associated with cognitive defects as well as an increased risk of malignancy [281].

Neurofibromin is a GTPase activating protein expressed in neurons, glial cells, schwann cells and early on in melanocyte development. It is a tumour suppressor and functions to downregulate signalling downstream of Ras by converting active GTP-Ras to inactive GDP-Ras [280, 281]. As described in section 1.4.1, Ras is normally activated in response to growth factor stimulation and functions to activate PI3K signalling, (see figure 1.5) as well as the Raf/Mek1 signalling cascade disrupting formation of the TSC1/2 complex. NF1 patients exhibit constitutive activation of Ras in neuronal cell types, causing inappropriate cellular growth and proliferation. At present the only approved clinical treatment is surgical removal of neurofibromas, however the rapamycin analogue sirolimus is currently undergoing clinical trials as a potential treatment strategy [280]. A recent study indicated that mTORC1 may play a role in the development of NF1 associated malignancies [282]. Utilising a genetically engineered murine model, it was demonstrated that rapamycin could be utilised to suppress tumour growth.

Intriguingly however it was not via the usual mechanisms of mTORC1 mediated tumour suppression. Although tumour growth was inhibited, HIF-1α was not suppressed and Akt activity was also unaffected, even after long term rapamycin treatment. This may suggest that mTORC1 signalling differs in neuronal cells, or this may be a result of cross talk between other signalling components downstream of Ras. The role mTORC1 signalling has upon the pathophysiology of NF1 will become more apparent upon the completion of clinical trials using rapamycin and its analogues.

1.7.1.3 Peutz-Jeghers Syndrome

Peutz-Jeghers syndrome arises from mutations to the *LKB1* tumour suppressor gene. LKB1 is a ser/thr kinase which activates AMPK when ATP levels are depleted [283]. As described in section 1.4.3, activated AMPK functions to phosphorylate TSC2 at the Ser1345 residue. This, in combination with GSK3β mediated phosphorylation of TSC2 facilitates the formation of the TSC1/2 tumour suppressor complex, resulting in mTORC1 inhibition. AMPK is also thought to inhibit mTORC1 directly by phosphorylation of Raptor (see section 1.4.3). Therefore mutations to LKB1 result in inappropriate activation of mTORC1 in the absence of ATP levels. This manifests with the formation of intestinal polyps (similar to hamartomas), altered pigmentation of the mucas membranes and increased risk of malignancy [284, 285]. In addition to peutz-jeghers, LKB1 mutations are also associated with 30% of sporadic lung cancer cases, suggesting mTOR inhibitors may also have a role here. Mouse models have indicated that rapamycin may be effective at reducing tumour burden in peutz-jeghers, specifically through suppression of HIF-1α and it's gene targets [286].

1.7.1.4 Cowdens Syndrome

For the activation of mTORC1 by growth factors or insulin, activated PI3K must first catalyse the conversion of PIP2 to PIP3 in order to form a docking site for Akt (see figure 1.5). In the absence of such stimulation, PTEN, reverses this conversion, promoting the accumulation of PIP2 and preventing Akt activation [283, 287]. Therefore mutations to the PTEN gene also result in hyperactive mTOR signalling. There are a number of disorders associated with PTEN deficiency including

Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome and Lhermitte-Duclos disease, all of which carry an increased risk of malignancy [288].

Like the other familial-cancer disorders, Cowdens syndrome varies considerably in terms of severity and phenotypic presentation, with the most commonly associated manifestations being benign skin, uterine, thyroid and breast lesions. Cowdens syndrome is also associated with an increased risk of breast and thyroid cancer [287]. The use of mTOR inhibitors is currently being trialled for the treatment of Cowdens and other PTEN-related hamartomatous tumour syndromes.

1.7.2 Other associated disorders

1.7.2.1 LAM

As described in table 1.3, LAM is one of the complications associated with TSC however it does also occur sporadically. It is a progressive and debilitating disease characterised by uncontrolled proliferation of abnormal smooth muscle cells in the lung. This can cause serious deterioration of lung function, ultimately requiring lung transplantation for treatment. Sporadic LAM patients also often develop renal AMLs as seen with TSC patients. Although the pathogenesis remains unclear behind this disease, LAM cells do exhibit mutations to the TSC1 or TSC2 gene which is likely to contribute to the uncontrolled proliferation [289, 290]. In cases of sporadic LAM it is likely that the disease develops as a result of somatic mosaicism of TSC1/2 mutations which is confined to the lungs and kidneys [291]. There are multiple clinical trials on-going to establish how this disease could be treated with mTORC1 inhibitors.

1.7.2.2 Polycystic kidney disease (PKD)

Autosomal dominant PKD is a cystic disease of the kidneys. Multiple cysts form in the kidneys as a result of uncontrolled proliferation of renal epithelial cells. These highly proliferating cells gradually invade the kidneys over time, impacting upon renal function and inevitably resulting in renal failure. The disease occurs through mutations to the *PDK1/2* gene which encodes for polycystin-1/2, the gene is adjacent to the TSC2 gene on chromosome 16p13.3 so PKD can arise as a complication of TSC in cases of contiguous gene syndrome [292]. Polycystin 1/2 are integral cilia proteins and appear to mediate an influx of calcium in response to fluid flow [293] as sensed by primary cilium. Interestingly, loss of polycystin1/2 is also associated with

hyperactive mTOR signalling which is likely to be contributing heavily to cystogenesis in this disease [294]. The mechanisms behind this are not clear however the cytoplasmic c-terminal tail of polycystin-1 has been demonstrated to regulate the localisation of TSC2 to repress mTORC1 [295]. It has also been demonstrated that the mechanistic 'bending' of the cilia in response to urinary flow not only results in a calcium influx but also causes activation of LKB1, thus activating AMPK to negatively regulate mTORC1 [296]. Both mechanisms indicate ways in which mTORC1 signalling can be regulated mechanistically by extracellular cues.

1.7.3 Cancer

The list of publications citing mTOR involvement in the pathology of cancer is extensive. Many different cancer types commonly exhibit mutations to genes involved in regulating mTOR activity, for example, PTEN, a tumour suppressor which functions to dephosphorylate substrates of PI3K (see figure 1.5) is one of the most commonly mutated tumour suppressors. The *PTEN* gene is located on chromosome 10q23 and this locus is a hotspot for mutations in primary human cancer [297]. It is thought that PTEN is functionally haploinsufficient, therefore mono-allelic mutations are sufficient to reduce its expression. Strikingly, PTEN mutations are thought to occur in around 50% of endometrial cancer cases [298], approximately 24% of high-malignancy-grade gliomas, 10% of prostate carcinoma cases and 26% of endometrioid ovarian tumours [299].

It is also thought that mutations to the *PIK3CA* gene, which encodes the catalytic p110 subunit of PI3K, occur in around 15% of *all* cancer types, making it one of the most commonly mutated genes in the entire human genome [300]. mTOR also drives the activity of transcription factors HIF-1α and potentially STAT3, the gene targets of which are instrumental in the growth and spread of tumours.

Sato *et al.* recently reported two incidences of direct activating mutations to mTOR itself, one was found in a large intestine adenocarcinoma, the other being identified in renal cell carcinoma. These mutant forms of mTOR were found to be constitutively active regardless of nutrient status which would undoubtedly contribute to the cancer phenotype. Sato *et al.* suggest that mTOR may be directly activated in a wide range of cancers and could provide a potential therapeutic target [301].

Surprisingly however, rapamycin and its analogues have not been as successful in clinical trials as first hoped. In cell line models exhibiting PI3K

activating mutations, tumours were demonstrated to be hypersensitive to rapamycin inhibition but in patients the anti-tumour activity of rapamycin is much more modest. One reason for the limited success in patients was described by O'Reilly *et al.* who demonstrated that rapamycin treatment caused increased expression of IRS-1 and induced Akt activation in response to mTOR suppression [302]. Therefore rapamycin treated tumour cells would exhibit repression of mTOR signalling components but activation of Akt signalling [302]. Evidently TSC2 is not the only downstream substrate of Akt, it also suppresses the expression of the pro-apoptotic p53 and FOXO transcription factors, promoting cell survival. Akt, like mTORC1, is also thought to phosphorylate p27 and p21 to promote cell cycle progression and can even initiate the process of angiogenesis via activation of endothelial nitric oxide synthase [303]. These are only a few examples of how upregulation of Akt could function to counteract the anti-tumour properties of rapamycin and explains at least in part the limited efficacy of rapamycin in patients compared with disease models, it may therefore be appropriate to utilise Akt inhibitors in conjunction with mTORC1 inhibitors for maximal anti-tumour activity.

Furthermore, as described in section 1.3.5, rapamycin treatment also initiates Ras/raf signalling by alleviating suppression of the pathway from S6K1. Carracedo *et al.* demonstrated that the Mek1/2 inhibitor UO126 had a synergistic effect upon rapamycin mediated growth inhibition, compounding the theory that activation of Ras/Raf signalling was instrumental in reducing the anti-tumour effects of rapamycin [152].

These studies highlight the significance in determining feedback mechanisms which may be initiated or lost when the pathway is manipulated with therapeutics. The rapamycin insensitive functions of mTORC1 (see section 1.5.5) may also be subject to hyper-activation during rapamycin treatment as negative feedback mechanisms are initiated to counteract mTORC1 repression. mTOR kinase inhibitors may therefore be a more effective therapeutic strategy for diseases involving aberrant mTORC1 signalling.

1.7.4 Autoimmune diseases

Autoimmune diseases are triggered when T effector cells inappropriately target substances and tissues normally present in the body, therefore treatment options involve immunosuppression. Rapamycin is an effective immunosuppressant and

functions to block cytokine mediated proliferation through mTORC1 inhibition, it has therefore been considered a potential therapeutic in the treatment of several autoimmune diseases.

Rapamycin has the advantage over other immunosuppressant drugs such as cyclosporine because it allows the thymic generation and peripheral preservation of a specific subset of T-cell referred to as CD4+ CD25+ FoxP3+ T cells [304]. It has been reported that this particular subset of T-cells suppress autoimmunity and promote immune-tolerance [305]. Several autoimmune disease models have shown improvement with rapamycin treatment including type 1 diabetes [306], systemic lupus erythematosus (SLE) [307], autoimmune lymphoproliferative syndrome (ALPS) [308] and autoimmune uveoretinitis [309] indicating the potential applications for rapamycin in this field and also implicating mTOR in the development of autoimmune diseases.

1.7.5 Lipid signalling in disease

mTORC1 promotes lipogenesis by a variety of mechanisms which are described in detail in section 1.6.3. Dysregulation of lipid signalling pathways can contribute to the progression of a wide number of different diseases types, including, cancer, inflammation, autoimmunity, cardiovascular disease, some metabolic disorders and a number of degenerative diseases (for review see [310]). Therefore manipulation of mTORC1 signal transduction could potentially be utilised in the treatment of any of these diseases.

mTORC1 inhibition could also be used to target obesity where normal methods for weight loss have failed. In America, over 60% of the population are reported to be overweight or obese and there is a requirement for anti-obesity drugs due to the severe health risks which are associated with obesity, mTOR inhibitors have the potential to meet this criteria [311].

1.7.6 Diabetes

As described earlier, insulin stimulation in cells causes an initiation of signal transduction pathways activating Akt (see figure 1.5). At this point the insulin signal bifuricates, whereby Akt causes simultaneous activation of mTORC1 to promote lipogenesis and fat storage, as well as increasing glucose uptake by phosphorylation

of GLUT4, and suppressing gluconeogenesis through inhibition of GSK3β and FOXO1 [303]. This explains how insulin promotes the storage of energy.

Type II diabetes is characterised by partial insulin resistance, whereby insulin is still able to activate lipogenesis but cannot suppress gluconeogenesis or increase glucose uptake [265]. It is thought that type 2 diabetes develops due to overfeeding. Whereby continuous overfeeding increases glucose, insulin and amino acid levels causing hyper-activation of Akt and mTORC1 [312]. Hyper-activation of S6K1 downstream of mTORC1 induces a negative feedback mechanism to downregulate expression of IRS-1 (see section 1.4.6) causing insulin resistance. This is consistent with the observation that S6K1-/- mice exhibit enhanced insulin sensitivity even on a high fat diet [148]. Suppression of IRS-1 prevents insulin induced Akt activity, inhibiting glucose uptake and activating gluconeogenesis [303] aggravating the hyperglycaemic condition.

Interestingly in the case of type two diabetes, mTORC1 remains activated and IRS-1 expression remains repressed. It is unclear how mTORC1 remains hyperactive in the absence of PI3K/Akt signalling, however it may be a result of increased amino acid stimulation of mTORC1 due to overfeeding [312]. A recent study indicated that obese individuals had significantly higher levels of amino acids in their blood, particularly branched chain amino acids, with obese individuals exhibiting 14% higher levels of Leucine than leaner individuals [313]. As described in section 1.4.4.5, Leucine alone can induce mTORC1 signal transduction [128]. This evidence suggests that insulin sensitivity could be reversed with rapamycin treatment to alleviate S6K1 dependent suppression of IRS-1, however it may be more appropriate to address the problem with dietary improvements and exercise.

1.7.7 Neurological diseases exhibiting aberrant mTOR signalling

Two of the more common manifestations of TSC are epilepsy and impaired cognitive abilities, implicating mTORC1 signalling in brain and central nervous system function. Over the last five years, mTORC1 has been examined in a number of neurological disease models including Huntington's disease, Alzheimer's disease and Parkinson's disease with particular emphasis upon autophagy dysregulation. These links are outlined below.

1.7.7.1 Huntington's disease

In the case of Huntington's disease (HD), symptoms are caused by the accumulation of abnormally expanded polyglutamine proteins (in this case the accumulated mutant protein is Huntingtin) which form aggregates and impede neurological function, these aggregates cannot be degraded via the proteasome due to their long polyglutamine tracts. A recent study reported that rapamycin induced autophagy was effective at reducing these Huntingtin aggregates in a mouse model, furthermore rapamycin treatment caused a reduction in neurological dysfunction in a fly model for HD [314], indicating that induction of autophagy by mTOR suppression may be a useful therapeutic tool.

1.7.7.2 Parkinson's disease

Similarly in Parkinson's disease, the phenotype is associated with increased levels of α-synuclein and a mutant form of the protein. Mutant α-synuclein inhibits lysosome-mediated autophagy in Parkinson's sufferers, furthermore the ubiquitin-proteasome system for protein degradation is also dysregulated [315], leading to the accumulation of protein aggregates.

Rapamycin induced autophagy has been demonstrated to clear all known forms of α-synuclein in cell lines suggesting that rapamycin may be useful in the treatment of Parkinson's disease [316].

1.7.7.3 Alzheimer's disease (AD)

AD patients exhibit inappropriate activation of autophagy through decreased beclin-1 expression [317], this is coupled with increased synthesis of lysosomal components, premature initiation and defects in lysosomal maturation. This causes progressive accumulation of autophagic vacuoles and swelling of the neuritis. Importantly, these autophagic vacuoles secrete amyloid β-peptide (Aβ) [318]. Aβ is a toxic peptide which gradually accumulates in the AD brain, increasing oxidative stress ultimately inducing apoptosis and causing neurodegeneration [319].

As described in section 1.6.2.4, Beclin-1 expression was found to negatively correlate with expression of phosphorylated STAT3 and Akt in malignant gliomas, thus downregulation of mTOR and or STAT3 could function to increase Beclin-1 expression and may be a potential therapeutic target in AD.

Another hallmark of AD pathology is the accumulation of phosphorylated tau protein. It was recently reported that tau suppression could improve memory deficits and suppress neuronal death in a mouse model for AD [320]. Furthermore it was demonstrated that rapamycin treatment could enhance the clearance of tau and decrease it's toxicity, as well as increasing the lifespan of flies overexpressing tau [321]. This adds further support for the use of mTOR inhibitors in the treatment for AD.

1.7.8 Other neurological disorders associated with mTOR

A full review of the neurological disorders associated with aberrant mTOR signalling is not within the scope of this thesis. However it is pertinent to mention the potential associations. For instance cortical dysplasia, a malformation of the neurons in the cerebral cortex, is a common cause of intractable epilepsy in children and a recent study reported hyper-activation of mTOR within the dysplastic neurons associated with the disease [322]. This may suggest that epilepsy associated with TSC is a result of altered development of the cerebral cortex.

mTOR has also been associated with the processes of learning and memory recall, these processes rely upon long-term synaptic plasticity, whereby neurons which are used repeatedly, increase their affinity for each other at the synapse. mTOR is thought to control synaptic protein synthesis which is required for learning and memory [323]. This is likely to contribute to the cognitive defects common in TSC patients [324]. In addition, approximately 8-15% of cases of autism spectrum disorder (ASD) can be accounted for by singular genetic mutations, importantly more than half of these mutated genes are reported to be involved in the regulation of mTOR [324].

1.8 PROJECT AIMS

In order to understand the pathology governing diseases associated with mTOR, the signalling pathway must be further clarified. Research characterising mTORC1 as a regulator of protein translation has progressed quickly and revealed that mTORC1 signalling extends to regulate gene expression upon a transcriptional level as well. The central objective of this project is to further characterise the mechanisms behind the role of mTORC1 as a transcriptional regulator. The aims of this project were as follows:

1. To characterise mTORC1 dependent regulation of HIF-1α.
2. To examine HIF-1α regulation in the context of the disease Tuberous sclerosis.
3. To determine whether STAT3 is regulated by mTOR
4. To determine whether mTOR regulates STAT3 directly or via a downstream effector.
5. To investigate the relationship between mTOR, STAT3 and HIF-1α.

CHAPTER 2: METHODS & MATERIALS

2.1 SUPPLIERS

Materials used within this thesis were purchased from the following companies:

Abcam, Cambridge, UK

AbGene Surrey, UK

Amersham Life Sciences Ltd., Buckinghamshire, UK

Applied Biosystems, Cheshire, UK

ATCC, Middlesex, UK

BD Transduction Laboratories, Oxford, UK

Bibby Sterling, Staffordshire, UK

Bio-Rad Laboratories Ltd., Hertfordshire, UK

Calbiochem, Nottingham, UK.

Carl Zeiss MicroImaging GmbH, Jena, Germany

Cell Signaling Technologies, Danvers, USA

DAKO, Cambridgeshire, UK

Emscope, Kent, UK

Eurogentec, Hampshire, UK

Eurofins MWG Operon. Ebersberg, Germany

GE Healthcare, Buckinghamshire, UK

GIBCO, Paisley, UK

Helena Biosciences Europe. Gateshead, UK

Hoefer, Holliston, USA

Invitrogen Life Sciences Ltd. Paisley, UK

Millipore, Edinburgh, UK

National Diagnostics, Atlanta, USA

New England Biolabs Ltd., Hertfordshire, UK

Promega, Southampton, UK

Qiagen, West Sussex, UK

Roche Diagnostics, West Sussex, UK.

R & D Systems, Minneapolis, U.S.A.

Santa Cruz Biotechnology Inc., California, UK

Sigma-Aldrich Company Ltd. Dorset, UK

Starlabs, Milton Keynes, UK

Thermo Fisher Scientific, Surrey, UK
Vector Laboratories, Peterborough, UK
VWR International. Leicestershire, UK
Whatman International Ltd., Kent, UK

All standard laboratory chemicals were purchased from Sigma unless otherwise stated, tissue culture reagents were purchased from GIBCO, reagents for SDS gel electrophoresis and transfections were purchased from Invitrogen and DNA/RNA extraction kits were purchased from Qiagen.

2.2 BUFFERS AND SOLUTIONS

Buffers and solutions used in the compilation of this thesis are listed below. MilliQ grade water was used to prepare these buffers and solutions unless otherwise stated.

Luria Broth

15g Tryptone
7.5g Yeast Extract
15g NaCl
1.5g Glucose
1.5g Anhydrous MgCl
Combine with 1.5l of dH_20 and adjust to pH 7.0 before autoclaving.

Luria Agar

10g Tryptone
5g Yeast Extract
10g NaCl
1g Glucose
1g Anhydrous MgCl
Add 1l of dH_20 and adjust pH to 7.0 before adding:
15 g Agar
2ml of 1M NaOH
Autoclave for 12 min before pouring.

TBS-T

Add 2.42g Tris-base and 8g NaCl to 1l of dH_20. Adjust pH to 7.6 and add 1ml Tween-20 to give 0.1% (w/v) unless otherwise stated.

TAE Buffer

40 mM Tris acetate

1 mM EDTA

Blenis Lysis Buffer

10mM KPO_4

5mM EGTA pH 7.2

10mM $MgCl_2$

50mM β-Glycerophosphate

mTOR/Raptor Lysis Buffer

40mM HEPES pH 7.4

2mM EDTA

10mM β-Glycerophosphate

0.3% CHAPs

Rheb Lysis Buffer

40mM HEPES pH 7.4

10mM β-Glycerophosphate

5mM $MgCl_2$

0.3% CHAPs

Low Salt Wash Buffer

40mM HEPES pH 7.4

2mM EDTA

10mM β-Glycerophosphate

150mM NaCl

0.3% CHAPs

High Salt Wash Buffer

40mM HEPES pH7.4

2mM EDTA

10mM β-Glycerophosphate

400nM NaCl

HEPES/KCL Wash Buffer

25mM HEPES pH 7.4

20mM KCl

GTP Loading Buffer

25 mM HEPES pH 7.4

5 mM EDTA

0.5 mg/ml BSA

$MgCl_2$ Loading Buffer

25 mM HEPES pH. 7.4

5 mM $MgCl_2$

Rheb Storage Buffer

20mM HEPES pH 8.0

200mM NaCl

5mM $MgCl_2$

3x mTOR Kinase Buffer

75mM HEPES pH 7.4

60mM KCl

30mM $MgCl_2$

Start Buffer (mTOR kinase assay)

25mM HEPES pH 7.4

10mM $MgCl_2$

Buffer A – Hypotonic Buffer (Nuclear and cytoplasmic fractioning)

10mM HEPES pH 7.9

1.5mM $MgCl_2$

10mM KCl

Buffer C – Hypertonic Buffer (Nuclear and cytoplasmic fractioning)

20mM HEPES pH 7.9

25% Glycerol

420mM NaCl

1.5mM $MgCl_2$

0.2mM EDTA

Nuclear proteins lysis buffer

25mM Tris-HCL pH 7.5

150mM NaCl

1mM EDTA

10mM NaF

50mM β-Glycerophosphate

1% NP-40

5% Glycerol

NP-40 lysis buffer

20mM Tris – pH 7.4

150mM NaCl

1mM $MgCl_2$

1% Nonidet P-40

10% Glycerol

1mM DTT

50mM β-glycerophosphate

50mM NaF

Rheb exchange buffer

50mM HEPES – pH 7.4

1mM $MgCl_2$

100mM KCl

0.1mg/ml BSA

Rheb elution buffer

0.5mM GDP

0.5mM GTP

5mM DTT

5mM EDTA

0.2% SDS

IP wash buffer

20mM HEPES – pH 7.4

150mM NaCl

1mM EDTA

1% Nonidet-P40

1mM DTT

50mM β-Glycerophosphate

50mM NaF

Buffer A – S6K1 Kinase Assay

1% Nonidet P-40

0.5% Sodium Deoxycholate

100mM NaCl

1mM EDTA

Buffer B – S6K1 Kinase Assay

10mM Tris – pH 7.2

0.1% Nonidet P-40

0.5% Sodium Deoxycholate

1 M NaCl

1mM EDTA

ST Buffer – S6K1 Kinase Assay

50mM Tris-HCl – pH 7.2

5mM Tris-base

150mM NaCl

Cell Extraction Buffer

50mM β-Glycerophosphate

1mM EDTA

1mM EGTA

1% Triton X-10

Western Transfer Buffer x 10

144.07g Glycine

30.285g Tris-Base

2g SDS

Make up to 1l with dH_20

Running Buffer x 10

144.07g Glycine

30.285g Tris-Base

10g SDS

Make up to 1l with dH_20

Urea Sample Buffer

62.5 mM Tris-HCL pH 6.8

6M Urea

10% Glycerol

2% SDS

0.00125% Bromophenol Blue

5% ß-mercaptoethanol

Binding Assay Buffer

20mM HEPES

10 mM MgCl2

100 mM NaCl

Luciferase Reagent

50mM Tricine pH 7.8

15mM $MgSO_4$

15mM KH_2PO_4

4mM EGTA

2mM ATP

1mM Luciferin

2.3 METHODOLOGY

2.3.1 Plasmid details

Plasmid details, Myc-tagged mTOR/pRK5 (Addgene plasmid 1861) and HA-tagged raptor/pRK5 (Addgene plasmid 8513) plasmids were kindly obtained from Dr. D. M. Sabatini, referenced here [74]. HA-Raptor mutant four (mutant 4 $_{391}SQ_{392}$-PA) and the active mTOR mutants myc-L1460P and Myc-E2419K were generated using site-directed mutagenesis by Dr Elaine Dunlop [76]. pcDNA3.1, pRK7,Flag-Rheb/PRK7, Control 5'UTR bicistronic vector, 5'HIF-1αTOP reporter and GST-4E-BP1 were kind donations from Prof. John Blenis (Harvard University), GST-Flag-Rheb/PRK7, GST-Flag-Rheb-Q64L/PRK7, GST-4E-BP1-F1114A (TOS mutant), GST-4E-BP1-I15A and GST-4E-BP1-P16A were also generated using site directed mutagenesis by Dr. E. Dunlop.

pACATG, HA-HIF-1α HA-4E-BP1-YL>A were kind donations from Dr. Sonenberg (McGill University). HA-S6K1/pRK7, HA-S6K1-F5A/pRK7, HA-S6K1-F5A-R3A-pRK7 and HA-S6K1-F5A-R3A-E389-pRK7 were kindly donated from Dr. Stefanie Schalm [325].

pcDNA3.1, Flag-TSC2/pcDNA3, Flag-TSC2-R98W/pcDNA3, Flag-TSC2-L219P/pcDNA3, Flag-TSC2-L340P/pcDNA3, Flag-TSC2-L466P/pcDNA3, Flag-TSC2-N525S/pcDNA3, Flag-TSC2-K599M/pcDNA3, Flag-TSC2-R611Q/pcDNA3, Flag-TSC2-R905G/pcDNA3, Flag-TSC2-R905Q/pcDNA3 and Flag-TSC2-

R1720Q/pcDNA3 were provided by Dr. Mark Nellist for analysis in the HIF-1α transcriptional reporter assay.

Prof. Cheryl Walker's laboratory kindly provided the Flag-TSC2/pcDNA3.1, Flag-TSC2-R1743G/pcDNA3.1, Flag-TSC2-R1743Q/pcDNA3.1 and the Flag-TSC2-R1743W/pcDNA3.1 constructs.

Dr. E. Dunlop introduced mutations into the Flag-TSC2/pcDNA3.1 plasmid via site directed mutagenesis in order to produce TSC2-E92V, TSC2-R505Q, TSC2-H597R and TSC2-L1624P [326] (see below for site-directed mutagenesis methodology). 3xHA-HIF and the TOS mutant are described here [135] and were cloned into a GST-gateway vector (Invitrogen) in accordance with manufacturers protocol. The GST-gateway vector system from Invitrogen was also used to generate GST-Rheb-like-1 from pcDNA3.1-Rheb-L1 [62] and was utilised by Dr. E. Dunlop to create GST-STAT3 from a STAT3 cDNA purchased from ATCC. HIF-1α and STAT3 luciferase reporters were purchased from Promega (cat no.'s LR0128 and LR0077) and used in accordance with manufacturers protocol, where indicated an alternative HIF-1α luciferase reporter was utilised which is described here [135].

2.3.2 Antibodies

Clone 9E10 anti-Myc antibodies (Sigma) were used for immunoprecipitation, while clone 9B11 anti-Myc antibodies (Cell Signalling) were used for western blotting. Phospho-4E-BP1 (Thr37/45), phospho-4E-BP1 (Ser65), phospho-4E-BP1 (Thr70), Total 4E-BP1, TSC2, phospho-rpS6 (Ser235/236), β-actin, Lamin A/C, P-STAT3-Ser727, P-STAT3-Tyr705, Total STAT3, eIF4E, and p70 P-S6K1-Thr389 were all purchased from cell signalling. α-HA was purchased from Roche, α-GST was purchased from Upstate (Millipore), α-Flag was purchased from SIGMA. For detection of human HIF-1α, an antibody from BD transduction laboratories was utilised and a HIF-1α antibody from Abcam was purchased to detect HIF-1α from mouse cell lines. For immunohistochemistry, a VEGF antibody was purchased from Millipore.

2.3.3 Molecular Biology

All cloning was carried out using Gateway recombination technology to produce GST-tagged recombinant vectors in accordance with manufacturer's protocol. Vectors were then sent to be sequenced by Eurofins MWG Operon for verification.

PCR was utilised to amplify the DNA for cloning, all primers were purchased from Eurofins MWG Operon unless otherwise stated.

The following PCR reaction mix was prepared:

1. 50ng DNA template (5µl x 10ng/µl solution)
2. 125ng forward primer (5µl x 25ng/µl solution)
3. 125ng reverse primer (5µl x 25ng/µl solution)
4. 10µl of x5 GC buffer
5. 1µl dNTPs
6. 1.5µl of DMSO
7. 22.5µl dH_2O (> 50µl)
8. 0.5µl DNA phusion polymerase

Using the following cycle:

1. Denaturation at 98°C for 30sec
2. Annealing at 52°C for 30 sec
3. Polymerisation at 72°C for 3 min

This cycle was repeated 23 times.

2.3.3.1 Bacterial transformations

PCR products were separated on a 1% (w/v) agarose gel using x1 TAE buffer at 150V for 30 min to check the size, purity and approximate concentration of the product. A recombination reaction was performed to introduce the PCR product into the entry vector p-DONR221 (Invitrogen), this was carried out in accordance with manufacturer's protocol. The product was then transformed into ultra-competent *E.Coli* (Invitrogen) using the following heat shock protocol:

1. Chill DNA and thaw competent cells on ice.
2. Add DNA to cells at a ratio of 1:10 and gently agitate to mix.
3. Incubation on ice for 30 min.
4. Heat shock at 42°C for one min.
5. Recovery on ice for 5 min.

6. Add 200µl of SOC media (Invitrogen) to the mix – then incubate for 30 min at 37°C with gentle agitation.
7. Spread 20µl of the mixture onto agar plates containing the appropriate concentration of antibiotic and incubate overnight at 37°C.

The following day, colonies were picked and grown up in 5ml of LB media supplemented with antibiotics, DNA was extracted using the Qiagen Mini-prep kit and then sent for sequencing at Eurofins MWG Operon. Once the sequence was verified the DNA was cloned into the destination vector using the LR recombination reaction in accordance with manufacturer's protocol. This product was then transformed into the ultracompetent *E.Coli* and grown overnight in LB broth. Cloning was again confirmed with sequencing carried out by Eurofins MWG Operon.

For larger quantities of DNA, bacteria were grown in 250ml of LB broth and the Qiagen Maxi-Prep kit was utilised to extract plasmid DNA in accordance with manufacturer's protocol. DNA concentrations were checked using an ND-8000 8-sample Nanodrop spectrophotometer.

2.3.3.2 Site directed mutagenesis:

Mutations were introduced into vectors using site-directed mutagenesis using the same PCR mastermix as described above, however the following cycle was followed:

1. 98°C for 5 min (denaturation)
2. 98°C for 1 min (denaturation)
3. 52°C for 1 min (annealing)
4. 72°C for 15 min (polymerisation)
5. 72°C for 18 min (polymerisation)
6. 4°C ∞

Cycle was repeated 18 times.

Products were then treated with dPN1 (1µl/reaction) for 1h at 27°C and then transformed into one shot top 10 competent *E.Coli* (Invitrogen) using the heat shock

protocol described above, grown over night and extracted for sequencing using the Qiagen miniprep kit. Sequencing was carried out by Eurofins MWG Operon.

2.3.4 Tissue culture and cell lines

Human embryonic kidney 293 (HEK293) and Cancer research UK HEK293 cell lines were purchased from ATCC. TSC1-/-, TSC1+/+, TSC2-/- (p53-/-) and TSC2+/+ (p53-/-) mouse embryonic fibroblast (MEF) cell lines were kindly provided by Dr. D. Kwaitowski (Harvard University). All cell lines were cultured in $75cm^2$ flasks with Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% (v/v) Foetal Calf Serum (FCS) and 1% (v/v) Pen Strep. Cell lines were incubated at 37°C in 5% CO_2.

Once confluent, cells were passaged, HEK293 cell lines were washed twice in EDTA/Trypsin, this was removed via aspiration before a 5 min incubation at 37°C. Cells were then resuspended in DMEM and transferred to a new flask. MEF cell lines required 3 trypsin washes for removal.

For long term storage, cells were frozen down in freezing medium using cryogenic freezing container (FCS supplemented with 8% (v/v) DMSO) and stored in cryogenic vials in liquid nitrogen.

2.3.4.1 Serum starvation:

HEKCRUK293's were washed twice in Dulbecco's Phosphate Buffered Saline (D-PBS), the second wash included a 5 min incubation at 37°C before the media was replaced with DMEM supplemented with 1% (v/v) pen-strep (serum free media). MEF cell lines and HEK 293 cell lines were washed twice with serum free media.

Cells were insulin stimulated with 10µg/ml. This was administered 20 min prior to lysis, except when used in luciferase reporter assays where the treatment was given overnight.

Cells were treated with 50nM rapamycin, this was administered 1 h prior to lysis or overnight in the case of luciferase reporter assays.

MG132 was administered at a concentration of 50µM for 2 h prior to lysis.

2.3.4.2 Amino acid starvation and stimulation:

Cells were starved in D-PBS supplemented with:

(For 500mls)
50mg $CaCl_2$
0.05mg Ferric Nitrate
48.84mg Magnesium Sulfate
100mg KCl
1.85g Sodium bicarbonate
62.5mg Sodium phosphate monobasic
1.75g d-glucose
3.36mls Sodium Pyruvate

pH was re-adjusted to 8.060 and the media was filter sterilised. Media was then left in incubator overnight prior to use (37°C and 5% CO_2) to equilibrate the pH. Vitamin supplements (GIBCO) were added 25% (v/v). Cells were cultivated in this media for 4 h prior to lysis.

For amino acid-stimulation, cells were treated in the above modified D-PBS media containing 4% (v/v) MEM – amino acid solution (GIBCO). The MEM amino acid solution itself had also been further supplemented with 30 mg/l glycine, 42 mg/l l-serine, and 0.2 mM l-glutamine (GIBCO). Cells were stimulated for 4 h prior to lysis.

2.3.4.3 Cell lysis

To harvest the cells, plates were washed once in PBS before being resuspended in lysis buffer supplemented with protease inhibitors, 1mM Na_3VO_4, 2μM antipain, 10μM leupeptin, 1μg/ml pepstatin, 0.1mM PMSF, 1mM DTT and 1mM benzamidine (cells for GST-purification were lysed without the addition of DTT). Cells were incubated on ice for 20 min to aid lysis then centrifuged for 13.000rpm, 8 min at 4 °C.

Lysates for Q-PCR were lysed in RNA protect buffer (no protease inhibitors) then centrifuged at 5,000 rpm for 5 min at 4°C, supernatant was aspirated for removal and the pellet stored at -80°C until mRNA extraction.

2.3.4.4 Hypoxic incubation

For cells cultured under hypoxia, a binder CB-150 hypoxic chamber was utilised. Cells treatments/media changes were implemented, cells were then placed into the hypoxic chamber for the indicated times. The incubator was then adjusted to 1% O_2 for the duration of the experiments. The binder series of hypoxic incubators allows precise control of oxygen concentrations down to 0,2 Vol.-% so was appropriate for this study.

2.3.5 Transfections

For large scale transfections (6cm^2 plates and upwards) cells were split into plates on day one. DNA complexes were added to the cells 4 h later once they had re-adhered to the plate. Media was changed on day two and cells were harvested on day three.

DNA complexes were prepared by combining the DNA with milliQ water (for volumes see table 1.4 below) $CaCl_2$ was then added and the solution was vortexed. 2xBES solution was then slowly added dropwise whilst aerating the sample with a drawn glass pasteur pipette. Mixtures were left to stand at room temperature for 15-20 min (until precipitate becomes visible) then added dropwise to the cells.

Table 1.4: Mastermix volumes for calcium chloride transfection

	6cm^2 plate	10cm^2 plate	Elephant plate
DNA	5µg	10µg	40µg
dH_2O	225µl	450µl	1.8ml
$CaCl_2$	25µl	50µl	200µl
2xBES	250µl	500µl	2ml

For smaller scale transfections lipofectamine 2000 (Invitrogen) transfection reagent was utilised following manufacturers protocol, with 2.5µl of lipofectamine used per µg

of DNA. Cells were split into plates on day one. On day two transfection complexes were added to the cells and incubated for 4 h before changing the media. Cells were then harvested on day three.

2.3.6 Generating nuclear and cytoplasmic fractions

Cells were harvested in PBS containing protease inhibitors, then pelleted for 10 sec at 13,000rpm before re-suspension of the pellet in 400µl of hypotonic buffer A (see buffers and solutions 2.2.) supplemented with protease inhibitors. Cells were then incubated on ice for 10 min to allow swelling of the cells before a 10 sec vortex cycle on full power. Cells were centrifuged again for 10 sec at 13,000rpm and the supernatant retained for the cytoplasmic fraction. The remaining pellet was resuspended in chilled buffer C (hypertonic – see section 2.2) plus protease inhibitors and incubated on ice for 20 min for high-salt extraction. Cellular debris was removed by centrifugation for 2 min at 13,000 rpm.

2.3.7 Lysis protocol for detection of nuclear proteins

Cells were lysed in cell extraction buffer plus protease inhibitors and phosphatase inhibitors where indicated. Lysates were incubated on ice for 10 min before being passed three times through a QIA Shredder (invitrogen) using high speed centrifugation. Lysates were then subjected to GST-purification/immunoprecipitation or western blotting.

2.3.8 SDS-Page

The Invitrogen NuPage Novex gel systems and apparatus were used in accordance with manufacturer's protocol. 3-8% Tris-acetate gels were utilised for separation of larger molecular weighted proteins and 4-12% Bis-Tris gels were used for small to medium sized molecular weights.

Samples were prepared by dilution in 4 x NuPage LDS sample buffer (Invitrogen) and incubated at 70°C for 10 minutes. Gels were run in the respective Novex running buffer at either 150V for 1h (3-8%) or at 180V for 1h (4-12%).

The Protogel system was used as an alternative to visualise 4E-BP1 mobility shifts or in instances where samples were lysed directly in sample buffer, also in cases where more than 12 samples were run simultaneously. Small gels were run

for 1h at 200V, larger gels (up to 25 samples) were run at 225V and 45mA until dye front reached the bottom (3-4 h).

Samples were diluted in 2x Protein loading buffer blue (National Diagnostics) Then subjected to a pulse centrifugation up to 13,000rpm, denaturing at 95°C for 5 min and followed by a second pulse centrifugation. Gels were run with x10 running buffer (see section 2.2).

2.3.9 Electrotransfer

For gels with 12 or less samples:

Proteins were transferred to a polyvinylidene fluoride membrane (PVDF) (Immobilon-P, Millipore) at 25V for 2 h in transfer buffer (see section 2.2) using the hoefer miniVE vertical electrophoresis system, in accordance with manufacturer's protocol. Membranes were soaked in methanol for 1 min to increase porosity then equilibrated in transfer buffer prior to use.

The larger gel systems were transferred to membrane overnight (14h) at 25V using an omniPAGE maxi vertical electrophoresis system from Jencons.

2.3.10 Western blot analysis

After proteins were transferred, the membrane was blocked in 10mls of TBST plus 5% (w/v) non-fat milk powder for a minimum of 1h. The membrane was then incubated in primary antibody diluted 1/1000 (unless stated otherwise in manufacturers guidelines) in TBST, supplemented with 2% (w/v) BSA (Sigma).

The following day membranes were washed twice in TBST before a 30min incubation in secondary antibody (conjugated with horse radish peroxidase - SIGMA) diluted 1/10.000 in TBST and supplemented with 5% (w/v) non-fat milk powder.

Membranes were washed four times in TBST before being subjected to Enhanced Chemiluminesence (ECL), this involved a 1 min incubation in ECL western blotting reagents in accordance with manufacturers protocol.

Konica Medical Film was used to visualise the signal and the exposed films were developed using a Konica Minolta SRX-101A developer. Scanned images were analysed for densitometry using Image J. software (v.1.44) where indicated.

2.3.11 Far Western blotting

Raptor/Raptor mutant 4 lysates were produced by transient transfection of HEK293 cells using calcium chloride transfection (see section 2.3.5) with HA tagged Raptor/Mutant 4. Cells were then harvested in cell extraction buffer (see section 2.2) supplemented with protease inhibitors (see section 2.3.5) incubated on ice for 20 min before centrifugation for 8 min at 13,000rpm at 4°C.

PVDF membrane was soaked in methanol then washed in TBST. 25 ng of purified protein was dotted onto the membrane which was then incubated in TBST containing 5% (w/v) non-fat milk powder for a minimum of 1h. Each potential substrate was dotted onto 3 separate membranes, after blocking, one membrane was incubated in GST antibody overnight (antibody was diluted 1/10,000 into10mls TBST, 0.2% (w/v) BSA, 5% (w/v) non-fat powdered milk) to give total protein levels. The other two membranes were incubated in cell extraction buffer (see buffers and solutions section 2.2) containing 0.3% (w/v) CHAPs, 5% (w/v) non-fat milk powder, protease inhibitors and 4% (v/v) Raptor/Raptor mutant 4 lysate. Alternatively, substrates were separated out using SDS page and transferred (using electro-transfer, section 2.3.9) to a PVDF membrane before the blocking step,

After overnight incubation at 4°C, membranes incubated with raptor lysate were washed twice for 5 min each in TBS containing 0.2% Tween before incubation with HA primary antibody (diluted 1/3000 in TBS-T with 0.2% (w/v) BSA and 5% (w/v) non-fat milk powder.)

Membranes were subjected to three washes in TBS-T before incubation in secondary antibody and diluted 1/10,000 in TBST containing 5% (w/v) non-fat milk powder. Proteins were visualised following the western blotting ECL protocol (see section 2.3.10).

2.3.12 Immunoprecipitation

For each immunoprecipitation, lysates were diluted up to a 1ml volume in lysis buffer (for preparation of lysates see section 2.3.4.3) then rotated for 2 h at 4°C with 0.4% (v/v) antibody (unless otherwise stated). 40µl of a 50:50 slurry of protein-G beads was then added and samples were rotated for 1 h at 4°C. Beads were then washed 3 times in lysis buffer (plus protease inhibitors) before elution in 40µl of x1 sample buffer.

2.3.13 GST purification

For GST purifications, cells were harvested in Rheb Lysis Buffer (see 2.2 buffers and solutions) supplemented with 0.3% (w/v) CHAPs and protease inhibitors, excluding DTT. Cellular debris was removed by high centrifugation for 8 min at 4°C and remaining lysates was incubated for 2 h with 40µl of a 50:50 slurry of lysis buffer and glutathione Sepharose beads.

Immunoprecipitates were washed three times quickly with lysis buffer then a fourth time for 10 min at 4°C with rotation. They were then washed in Rheb storage buffer (plus protease inhibitors – excluding DTT) with a second 10 min incubation at 4°C with rotation. GST-bound proteins were eluted from the beads in Rheb Storage Buffer plus protease inhibitors, supplemented with 10mM Glutothione and adjusted to pH 8.

2.3.14 m^7-GTP-sepharose chromatography

For purification of eIF4E and its associated proteins, HEK293s were transfected with HA-4E-BP1 or the mutant constructs using lipofectamine 2000 transfection protocol (see section 2.3.5). Cells were lysed with cell extraction buffer supplemented with protease inhibitors, incubated on ice for 20 min then subjected to high speed centrifugation for 8 min at 4°C. 20µl of m^7-GTP-sepharose beads diluted to a 50:50 slurry in lysis buffer was added to the supernatant from each lysate. Lysates were then incubated for 4 h at 4°C with gentle rotation. Beads were washed three times in cell extraction buffer plus protease inhibitors before elution in 40µl Novex sample buffer supplemented with 0.2mM DTT. Proteins were visualised using SDS-PAGE with western blotting for detection.

2.3.15 mTORC1 kinase assay

2.3.15.1 Generating mTOR/Raptor complexes from HEK293 cells:

HEK293s were transfected using calcium chloride transfection (see section 2.3.5) with Myc-mTOR and HA-Raptor (or mutants) at a ratio of 4:1. One plate was utilised for three assays. Cells were serum starved and stimulated with insulin for 20 min prior to lysis. Cells were harvested in mTOR/Raptor lysis buffer (see 2.2 Buffers and Solution) containing 0.3% (w/v) CHAPs plus protease inhibitors. Cellular debris was removed by centrifugation for 8 min at 13,000rpm (4°C) and the remaining lysates was incubated for 2 h with 4µl of Myc or HA antibody per 1ml of lysate and rotated at

4°C. For an additional hour, a 50:50 slurry of protein G beads was added to the lysates (enough for 30µl per assay).

Immunoprecipitates were washed once with low salt wash buffer then twice with high salt wash buffer (both containing 0.3% (w/v) CHAPs plus protease inhibitors) and finally once in HEPES KCL wash buffer plus protease inhibitors (see 2.2 Buffers and Solutions.)

2.3.15.2 Generating GST-Rheb from HEK293 cells:

HEK293s were transfected with GST-Rheb (or constitutively active Q64L mutant) and grown in the presence of serum. Cells were then harvested in Rheb Lysis buffer (0.3% (w/v) CHAPs plus protease inhibitors excluding DTT) and subjected to GST purification (see section 2.3.13.)

2.3.15.3 Rheb loading with GTPγS or GDP

To load: 10µl of GTP loading buffer (see 2.2 buffers and solutions) was added to 10µl of purified Rheb protein (approximately 75ng/assay) plus 2µl of GTPγS of GDP (non-hydrolysable GTP analogue) the mixture was incubated at 37°C with agitation for 5 min. (GTPγS substituted for 100 µCi [α-^{32}P] GTP in radiolabelled assays).

To stop the reaction, 1µl of 0.5M $MgCl_2$ was added followed by 20µl of $MgCl_2$ loading buffer (see section 2.2).

2.3.15.4 Preparing the assays:

mTOR/Raptor complexes bound to protein G beads were split into the appropriate amount of reaction tubes and supernatant removed, 10µl of 3x mTOR kinase buffer was added to the beads in addition to 5µl of purified Rheb and 150ng of potential substrate. The reaction mix was made up to 30µl in dH_2O. A start buffer mix containing 10µl per reaction of Start buffer (mTOR kinase assay see section 2.2) supplemented with 500µM ATP (and [^{32}P] for radioactive assays) was added to initiate the reaction which was carried out at 30°C with gentle agitation for either 30 min or 1 h. The reaction was stopped with the addition of 13.3µl of x1 sample buffer (plus 200mM DTT for running on a Novex gel.)

Samples were analysed for phosphorylation of substrates and mTOR/Raptor levels using SDS page and western blotting (see sections 2.3.8-10).

2.3.16 mTORC1 Binding Assay

Active mTORC1 complexes were purified from HEK293 cells. Lysates were generated as described above for the mTORC1 kinase assay, immunoprecipitations were carried out using the HA-antibody conjugated to protein G beads (GE Healthcare) to purify the mTORC1 complex.

Immunoprecipitates were washed three times in mTOR/Raptor lysis buffer containing 0.3% (w/v) CHAPs and protease inhibitors. mTORC1 complexes captured onto protein G Sepharose beads were then divided into the appropriate number of reaction tubes. GST-Rheb was also purified from HEK293 cells and loaded with GDP/GTPγS also as described for mTORC1 kinase assay (see section 2.3.15.2-3) 5µg of GTP/GDP loaded Rheb was added to the reaction tubes. Potential substrates were purified utilising GST-purification system (see section 2.3.13 above) and 150ng of substrate was added to the reaction. Reactions were made up to a 30µl volume in binding assay buffer and assayed at 37°C for 20 min with gentle agitation. Protein G Sepharose beads were then washed three times again in mTOR/Raptor lysis buffer to remove non-specific interactions, proteins were eluted in 40µl of Novex sample buffer supplemented with DTT.

Substrate binding and mTOR/Raptor levels were visualised using SDS-PAGE and western blotting.

2.3.17 S6K1 Kinase Assay

Cells were transfected with HA-S6K1 or mutants using the lipofectamine 2000 transfection protocol (see section 2.3.5) and cultured in serum free media. Cells were then harvested in blenis lysis buffer plus protease inhibitors. After 20 min incubation on ice, cellular debris was removed via high centrifugation at 4°C for 8 min, a portion of lysate was retained to check protein expression levels using western blotting. The remainder of the lysate was incubated with 4µl of HA antibody (Roche, Switzerland) per 1ml of lysate for 2 h with rotation at 4°C. Protein G Sepharose beads were added for an additional hour at 4°C with rotation (40µl of a 50:50 slurry of beads and lysis buffer).

Immunoprecipitates were washed with 1ml each of buffer A, buffer B and ST buffer each containing protease inhibitors.

Kinase activity toward a recombinant GST-S6 peptide (32 final amino acids of ribosomal S6 – purified using GST purification – see section 2.3.13) in washed

immunoprecipitates was assayed in a reaction containing 20 mM HEPES, 10 mM MgCl2, 50 mM ATP unlabeled, 5 mCi of [g-^{32}P]-ATP (PerkinElmer Life Sciences), 3 ng/ml PKI, pH 7.2, for 12 min at 30 °C.

Reactions were subjected to 12% SDS-polyacrylamide gel electrophoresis, gels were then dried down using a BIORAD model 583 gel drier and the amount of [^{32}P]-radiolabel incorporated into recombinant GST-S6 protein was assessed by autoradiography. Exposed films were developed as described for western blotting in section 2.3.10.

2.3.18 Rheb GAP assay

TSC1/2 complexes were purified from HEK 293 cells transfected with pRK7, Flag-TSC1, Flag-TSC2, or Flag-TSC2-R1743Q on 10cm^2 culture dishes using calcium chloride transfection protocol (see section 2.3.5). 16 h after transfection, cells were treated with 100 nM wortmannin for 15 min before lysis in NP-40 lysis buffer (see buffers and solutions). Flag-tagged proteins were then immunoprecipitated for 2 h with 80 µl of an M2-agarose affinity gel slurry (Sigma). Immune complexes on beads were washed three times in IP wash buffer and once in 1 ml Rheb exchange buffer supplemented with protease inhibitors (see buffers and solutions). The washed beads were then separated into four aliquots. Three of these were used for separate GAP assays, and one was resolved by SDS-PAGE and immunoblotted to determine protein levels. GST-Rheb (10 µg) was loaded with 100 µCi [α-^{32}P]GTP (see section 2.3.15.3) or 10mM GDP. GAP assays were initiated by the addition of 20 µl GTP-loaded Rheb (approximately 1 µg GST-Rheb) to each aliquot of M2-agarose immune complexes described above. Assays were performed at room temperature with constant agitation for 20, 40, or 60 min. Reactions were stopped by the addition of 300 µl Rheb wash buffer containing 1 mg/ml BSA. M2-agarose immune complexes were removed by brief centrifugation, and nucleotide bound GST-Rheb was purified from the supernatant with 20 µl of a protein G slurry as described above. After three washes with Rheb wash buffer, radiolabeled GTP and GDP were eluted from Rheb with 20 µl Rheb elution buffer at 68°C for 20 min. Aliquots (1 µl) of each eluted reaction were resolved by thin-layer chromatography on PEI cellulose (Sigma) with KH_2PO_4 as the solvent. Dr. Andrew Tee assisted with these assays.

2.3.19 Transcriptional Luciferase Reporter Assays

Luciferase reporter constructs were utilised to determine the activity of transcription factors HIF-1α and STAT3. These vectors contain multiple copies of the cis-acting enhancer element for HIF-1α and STAT3 (see sequence below).

HIF-1α sequence (5'-3'): GTGACTACGTGCTGCCTAGGTGACTACGTG
CTGCCTAGGTGACTACGTGCTGCCTAGGTGACTACGTGCTGCCTAG

STAT3 sequence (5'-3'): TGCTTCCCGAATTCCCGAATTCCCGAATTCC
CGAATTCCCGAATTCCCGAACGT

Response elements are inserted upstream of a minimal TA promoter and the TATA box from the Herpes simplex virus thymidine kinase promoter. This promoter drives firefly-luciferase production which correlates with the DNA binding of the transcription factor (see below for vector map). The phrase "transcriptional activity" therefore refers to DNA binding activity of the transcription factor and thus correlates with target gene expression rather than expression of the transcription factor.

Cells were transfected with the reporter constructs alongside genes of interest, the optimum ratio of reporter DNA to additional vectors transfected was determined to be a 2:1 ratio favouring the reporter. To activate HIF-1α, cells were grown either in the presence of 1mM DMOG (dimethyloxalylglycine) or sealed in a Binder CB-150 hypoxic chamber set to 1% O_2 for 16 hours. DMOG was utilised in early experimentation before the acquisition of the hypoxic chamber to mimic hypoxic conditions (where indicated in figure legends). DMOG is an inhibitor of PHD enzymes required for oxygen-mediated degradation of HIF-1α (see introduction section 1.5.3) allowing its stabilisation in the presence of oxygen. This is not a perfect experimental model as PHD enzymes have roles outside oxygen sensing. Current evidence suggests that PHD activity is highly sensitive to amino acid levels. PHDs may therefore be involved in amino acid sensing and thus could signal to mTORC1 [327]. For this reason, the hypoxic chamber has been utilised where possible. Conversely, DMOG was used in experiments involving short term drug treatments, to avoid re-oxygenation upon opening the hypoxic chamber door. Re-oxygenation can cause a rapid induction of ROS which activate HIF-1α [328].

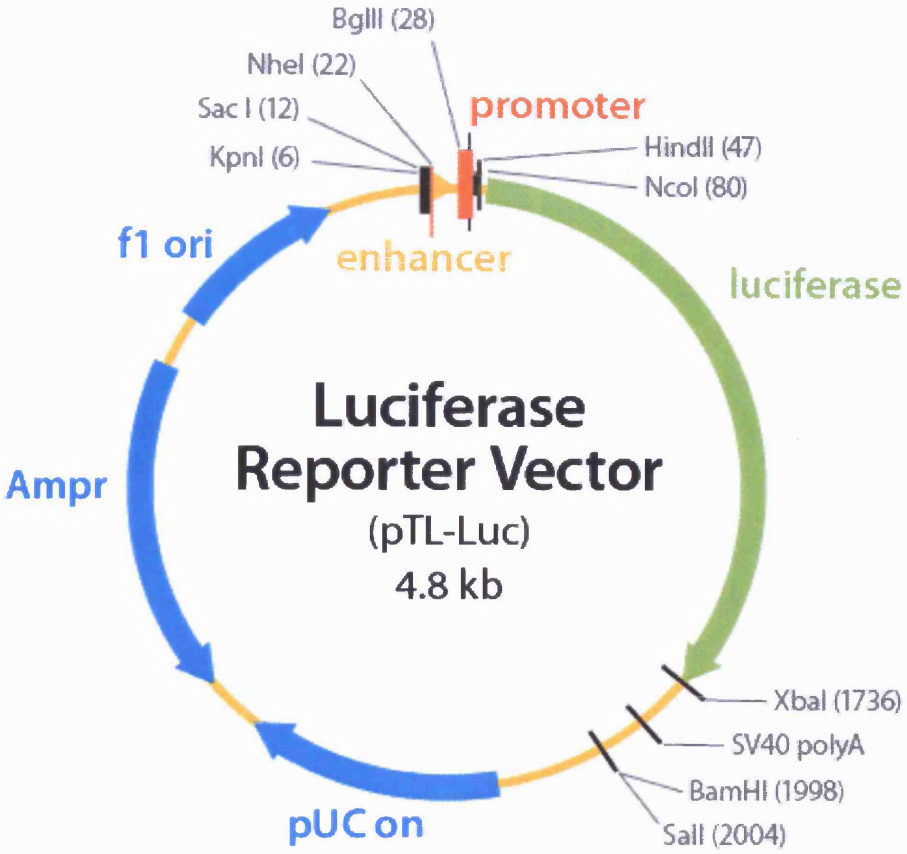


Figure 2.1 Luciferase reporter vector map (panomics).

The cytokine CNTF (ciliary neurotrophic factor) was utilised to activate STAT3, cells were cultured in the presence of 25ng/ml CNTF for 16 hours prior to lysis in order to allow the accumulation of luciferase over time.

Cells were harvested in blenis lysis buffer supplemented with protease inhibitors, incubated on ice for 20 min before centrifugation for 8 min at 13,000rpm. Luciferase activity was analysed using a TR717 Microplate Luminometer which was programmed to inject 50µl of luciferase reagent into each well containing 20µl of lysate, a measurement of the luminescence was taken 10 seconds later, data was collected using the Tropix WinGlow software. Experiments were performed in triplicate to check the consistency of the data, each lysate produced was also analysed in triplicate and an average was taken. Each experiment was repeated on at least three separate occasions. Luminescence was adjusted to total protein levels as determined by a Bradford protein assay (see below). Each lysate was measured three times for total protein levels, average luminescence was then divided by the average total protein to assess reporter activity.

2.3.20 Bradford protein assay

A standard curve was produced using BSA dissolved in dH_20 at the following concentrations, 0µg/ml, 0.25µ/ml, 0.5µg/ml, 1µg/ml, 2µg/ml and 4µ/ml. For each measurement, 2µl of the standard solution was diluted in 200µl of Bradford reagent and absorbance was measured using the Nanodrop spectrophotometer at 595nm. Three measurements were taken per concentration to generate a standard curve.

To analyse total protein, 2µl of sample was diluted into 200µl of Bradford reagent and absorbance measured and a standard curve generated using the Nanodrop software V.2.1.0.

2.3.21 Quantitative-PCR

For the extraction of mRNA, cells were harvested in RT-protect buffer and centrifuged at 5,000 rpm for 5 min to produce a pellet, the supernatant was discarded and the pellet saved for mRNA extraction.

mRNA was extracted using the Qiagen (West Sussex, UK) mRNA extraction kit in accordance with manufacturer's protocol, QIA shredders (Qiagen) were utilised

to homogenise the pellet. Resulting mRNA concentration was determined using the nanodrop spectrophotometer.

Total RNA from each sample (1 µg) was transcribed into complementary DNA using a Quantitect reverse transcription kit (Qiagen) in a thermal cycler (Applied Biosystems). The sequences of the VEGF-A primers used were forward 5'-GGAGAGCAGAAGTCCCATGA-3' and reverse 5'-ACTCCAGGGCTTCATCGTTA-3', as described in [329]. The following primer sets were purchased from Qiagen, who have the right to withhold primer sequence information: HIF-1α (cat no. QT01039542) and BNIP3 (cat no. QT00100233). Quantitative real-time PCR reactions were conducted in 96-well plates using appropriate primer assays and Sybr Green PCR Master mix (Qiagen). Assays were performed as follows:

1. Initial denaturation step (95 °C, 15 min),
2. 40 cycles of denaturation (94 °C, 15 s),
3. Annealing step (55 °C, 30 s)
4. Extension step (72 °C, 40 s).

The amplification products were quantified during the extension step in the fortieth cycle. The results were then determined using the ddCT (delta-delta-Ct) method, and standardised to β-actin. A dissociation step was performed, which verified that only one PCR product was produced with each primer set and shows their specificity. The correct size of PCR products was also verified by resolution on a 2% polyacrylamide gel with β-actin giving an amplicon length of approximately 77 bp, VEGF-A: 117 bp, BNIP3: 67bp and HIF-1α: 91bp. The efficiency of the primers was assessed by plotting ct values against the log concentration of the template, a linear trendline was applied. The Q-PCR standard curve slope to efficiency calculator (Stratagene) was used to assess the efficiency based on the equation: Efficiency = -1+10(-1/slope). A slope of −3.32 represents optimal efficiency (100%). Assays with amplification efficiencies of between 90 and 100% considered acceptable. All qPCR assays in this study were calculated to be at least 96% efficient.

2.3.22 Immunohistochemistry

Paraffin embedded kidney samples extracted from *Tsc1*$^{+/-}$ and *Tsc2*$^{+/-}$ mice were kindly donated from Prof. Cheadle's laboratory (Cardiff University). The *Tsc1*$^{+/-}$

mouse was previously designed by Wilson *et al.* [330] and contains a neomycin resistance cassette which replaces half of exon 6 and all of exons 7 and 8 of the *Tsc1* gene. The $Tsc2^{+/-}$ mouse was generated by Onda *et al.* [331] using homologous recombination to insert a neomycin resistance cassette into the second coding exon of *Tsc2.* Kidneys were collected from $Tsc1^{+/-}$ and $Tsc2^{+/-}$ mice and processed into paraffin wax by Miss Rebecca Harris.

Sections were cut at 4μm and floated on to poly-L-lysine treated glass slides and dried onto slides overnight at 45°C and stored at room temperature.

Immunohistochemistry analysis was carried out by Cardiff University Central Biotechnology Services and kidney sections from each genotype were stained for VEGF and phospho-S6 ribosomal protein (Ser240/244) using the following protocol:

1. Dewax and rehydrate -10mM Citrate buffer pH 6.0 100°C for 20min then washed thoroughly in H_2O.
2. Incubate in 20mM Tris + 0.9% NaCl pH 7.3 + 0.6% BSA (TBS/BSA) for 10 min.
3. Primary antibodies were diluted 1/25 or 1/50 rabbit anti-PS6 and 1/200 or 1/400 rabbit anti-VEGF in TBS/BSA, incubation for 60 min.
4. TBS/BSA washes, 3 x 1 min
5. Secondary antibody was diluted 1/150, goat anti-rabbit Ig horseradish peroxidase conjugate in TBS/BSA, incubation for 60min.
6. TBS/BSA wash, 1min
7. 50mM Tris pH 7.6 washes, 2 x 1min
8. 0.05% (v/v) DAB in 50mM Tris pH 7.6 for 3 min then slides were washed thoroughly in H_2O.
9. Incubation in 0.02% (v/v) methyl green for 5min
10. Dehydrate, clear and mount.

Immunohistochemical samples were viewed using an Olympus BX51 BF light microscope and photographs taken by Mr. Chris Von Ruhland.

2.4 ASSUMPTIONS

2.4.1. Luciferase reporter assays

The luciferase reporter assay system utilised in this project utilises a single reporter assay system as opposed to a more commonly implemented dual reporter system. A dual system requires a secondary Renilla based luciferase reporter which serves as a transfection control. These secondary reporters are primarily utilised to control for low and variable transfection efficiency, as well as variability in the cell lysis.

Initially, a secondary renilla reporter was co-transfected alongside the firefly reporters described, however the reporter appeared to be significantly affected by cellular treatments, specifically rapamycin. The use of a Renilla-luciferase reporter as an internal transfection control is entirely based upon the assumption that the Renilla luciferase reporter is constitutively expressed regardless of cellular treatments, therefore given that the two control reporters tested (pRL-TK and pRL-SV40 – panomics) did not meet this criteria, it was not considered feasible to utilise this as a normalising control as it lead to aberrant normalisation of results, as has been reported within the literature [332] [333]. Rather than use a transfection control, lipofectamine 2000 transfection reagent (Invitrogen) was utilised which offers high transfection efficiency across a broad range of cell lines (predicted 99% efficiency in HEK293s). This reduces the need for a secondary transfection control reporter. Secondly, all data presented within this thesis is a product of three individual experiments, all conducted in triplicate. Greater n-numbers were utilised to counteract the potential variation in the sample preparation, i.e., inconsistencies across cell lysis. Finally, all luciferase data was standardised to total protein levels, as determined by a Bradford assay. This normalises against variations in sample preparation, and also global effects treatments such as rapamycin may have upon protein synthesis. This data is based upon the assumption that modern transfection techniques, in combination with high n-numbers will account for variations in transfection efficiency which might occur between samples.

2.4.2 Densitometry analysis

Some densitometry analysis has been carried out throughout this thesis; however statistical analysis of these values was not carried out. It was not considered necessary to analyse the data in this manner as densitometry analysis is not a particularly reliable methodology. It is merely included in some studies to help the reader visualise the data and make comparisons between samples which are not adjacent to one another and should not be interpreted in any other way

2.4.3 Normoxia

This study examines the effects of hypoxia versus normoxia in various settings. For studies of normoxia, cells were cultured at 21% O_2 which is consistent with atmospheric oxygen and generally accepted practice. It is pertinant to note however that arterial blood oxygen partial pressure is likely to be significantly lower than atmospheric oxygen. It is therefore possible that the differences observed between hypoxic and normoxic conditions in this study may have been exagerated. For these reasons, within this study, normoxic conditions are used as a comparative control but little emphasis is placed upon differences between hypoxia and normoxia.

2.4.4. Statistical analysis

Numerical data obtained from luciferase assays and Q-PCR was analysed for statistical significance with a one-way anova using SPSS 16.0 software. A Levene's 'F-test' was utilised initially to determine whether equal variances were apparent. Tukeys post-hoc test was utilised to analyse multiple comparisons within the same data set. Initially, this data was analysed using a two sample t-test. Although this is an appropriate test for statistical comparisons of two means, it is not appropriate for multiple testing within the same data set. Therefore the data has since been reanalysed for statistical significance using the one-way anova. Adjustments have been made within the text to indicate changes.

CHAPTER 3: CHARACTERISATION OF mTORC1 DIRECTED REGULATION OF HIF-1α

3.1 INTRODUCTION

mTORC1 plays an extensive role in the regulation of protein translation through phosphorylation of its downstream substrates 4E-BP's and S6K's (see introduction sections 1.4.1 and 1.4.2). Signalling through mTOR exerts a multitude of cellular effects and the range of diseases linked to mTOR dysregulation is vast and rising. For mTOR to affect such a wide range of cellular processes, its role as a serine/threonine kinase must extend beyond its regulation of protein translation.

Recent work has identified several potential novel substrates for mTORC1 (see review [35]) including HIF-1α, STAT3 and YY1. This work aims to elucidate how mTORC1 may regulate the cellular response to oxygen deprivation, specifically through regulation of HIFs. HIF-1α is considered the master regulator of the hypoxic response [334]. Furthermore, Land *et al.* identified a putative mTORC1 signalling motif within the N-terminus of HIF-1α which is not found in the HIF-2α or HIF-3α isoforms, therefore HIF-1α will be the focus of this study.

There are several potential ways by which HIF-1α could be regulated by mTORC1.. mTORC1 may modulate HIF-1α via its transcription, translation, or by affecting its protein stability. mTORC1 may also directly phosphorylate HIF-1α to modulate its transcriptional activity. Alternatively, it is also possible that the roles of S6K's or 4E-BPs extend beyond the modulation of protein synthesis, therefore mTORC1 modulation of HIF-1α could be due to an indirect mechanism involving one or both of these substrates.

There has also been speculation that the unusual 5'-TOP (tract of polypyrimidine) structure of HIF-1α's 5'-UTR (untranslated region) could also be involved in the regulation of its translation. Long pyrimidine tracts contained within the 5'-UTR may confer preferential translation of HIF-1α during hypoxia whilst the phosphorylation of other mTORC1 substrates is suppressed [335, 336].

I hypothesised that mTORC1 directed regulation of HIF-1α is a multi-faceted process and therefore aimed to investigate the possible mechanisms governing this. Utilising a HIF-1α transcriptional luciferase based reporter construct to analyse HIF-1α mediated gene-expression, in conjunction with mTORC1 and S6K1 kinases assays, this chapter characterises the mTORC1-HIF-1α relationship.

3.2 METHODS

3.2.1 Analysis of active mutants of mTOR and luciferase assays

Cell culture including amino acid starvation and resupply was carried out as described in section 2.3.4.2. Cells were stimulated with 100 nM insulin (where indicated in fig 3.1) for 30 min prior to lysis in Blenis lysis buffer supplemented with protease inhibitors. SDS-PAGE was carried out as described in section 2.3.8 and 4E-BP1 mobility shift was visualised using the large scale omniPAGE vertical electrophoresis system. Luciferase assays was carried out as described in section 2.3.19. For HIF-1α luciferase assay, cells were transfected with the HIF-1α reporter described here [135], cells were amino acid deprived and insulin stimulated for 4 h prior to lysis to allow the accumulation of luciferase.

3.2.2 S6K1 kinase assay and luciferase assay

Cells were treated overnight with 50nM rapamycin and 100nM insulin prior to lysis and harvested in 200 µl of blenis lysis buffer. Of this, 100 µl was reserved for luciferase assays and western blotting of total lysates whilst 100 µl was utilised for the radioactive S6K1 kinase assay as described in section 2.3.17. Cells were cultured under normal O_2 tension but grown in the presence of 1 mM DMOG in order to stabilise HIF-1α.

3.2.3 Bicistronic reporter construct assay

Reporter assay carried out in same manner as the transcriptional luciferase assays (see section 2.3.19) but utilising Promega Dual Luciferase Reagent for analysis of Renilla and Firefly luciferase rather than standard Luciferase Reagent described in Materials and Methods.

3.2.4 Raptor/mTORC1 interaction studies

Overlay, binding assay and mTORC1 kinase assay were carried out as described in sections 2.3.15-2.3.16 of 'Materials and Methods'. For GST-purification of HIF-1α, cells were grown in the presence of DMOG and incubated in the hypoxia chamber for 4 h prior to lysis. Prior to lysis, cells were also treated for 2 h with 50 µM of MG132 to prevent proteasomal degradation of HIF-1α. Cells were quickly lysed in Rheb lysis buffer and sonicated for 3 x 5 s cycles on full power (30microns) before

centrifugation at 13,000rpm for 8 min at 4°C. Washes (as described in section 2.3.13) were executed as quickly as possible to preserve HIF-1α protein levels.

3.2.5 Quantitative-PCR

TSC1-/- MEFs or TSC2-/- MEFs and their wild-type counterparts were cultured overnight in serum free media under hypoxic or normoxic conditions in the presence and absence of 50nM rapamycin. mRNA was extracted as described in section 2.3.21 and analysed for HIF-1α mRNA levels using SYBR green detection. Results were standardised to β-actin expression and fold induction was calculated. (For primers see 2.3.21).

3.3 Results

3.3.1 HIF-1α transcriptional activity is upregulated by the expression of active mTOR mutants

The first aim of the study was to confirm that HIF-1α is regulated downstream of mTOR and to draw conclusions from comparisons to more bona fide mTORC1 substrates. To do this, two active mutants of mTOR were utilised (L1460P and E2419K). mTOR is a family member of PI3-kinase-related kinases. These kinases share several conserved domains which are central to their function. Of importance are the HEAT domain, FAT domain, kinase domain and FATC domain (see figure 1. 4). mTOR has an additional FRB domain which has been isolated as the binding site for the rapamycin:FKBP12 complex [337]. The active mutants used in this study were first employed by Urano *et. al.* Urano looked at Tor2 (the yeast homologue of mTOR) and found that point mutations to specific residues in the FAT domain and the kinase domain produced mutants that did not require Rheb for activation. Introduction of equivalent mutations in the mammalian homologue produced two mTOR mutants, L1460P and E2419K (mutations located in the FAT and kinase domains, respectively), which were constitutively active regardless of nutrient supply and in the absence of Rheb [338].

These mTOR mutants were generated and then utilised in this study to better characterise them against mTORC1 substrates. HEK293 cells were transfected with the mutant mTOR constructs under various conditions of nutrient-deprivation and insulin stimulation. The phosphorylation status of the direct mTORC1 substrate 4E-BP1 was then analysed.

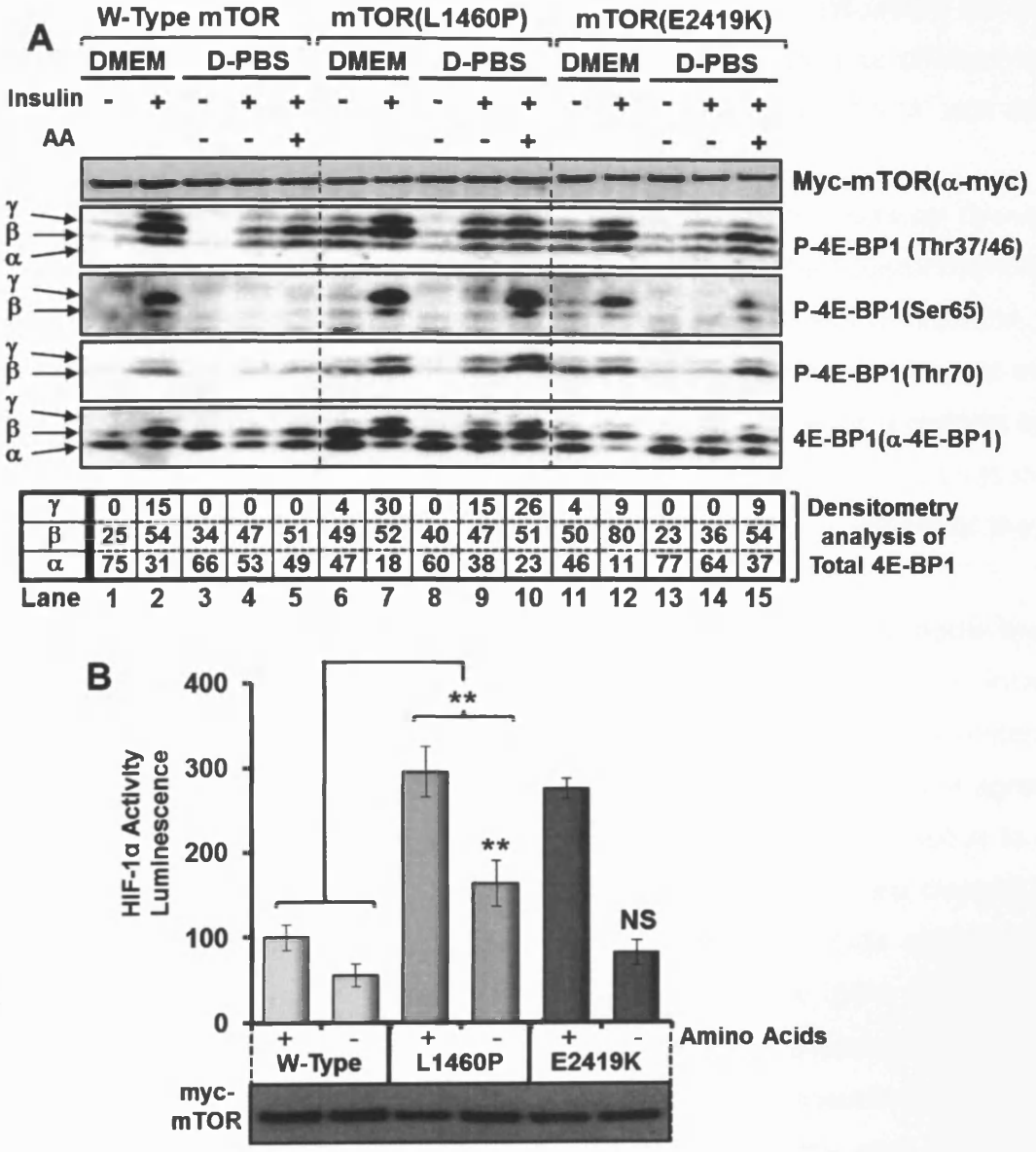


Figure 3.1 HIF-1α transcriptional activity is upregulated by active mTOR mutants: A: HEK293 cells transfected with Myc-4E-BP1 and Myc-mTOR (wild-type or mutants) were treated for 4 h in either serum-free DMEM or supplemented PBS in the presence or absence of amino acids (labelled 'AA') and insulin stimulated where indicated. 4E-BP1 activity was analysed using primary antibodies. The α-, β-, and γ-species of 4E-BP1 are labelled. Densitometry analysis was carried out on the α-, β-, and γ-species of 4E-BP1 using ImageJ where the protein abundance between these three species is set to 100% (labelled as '% 4E-BP1 Isoforms'). **B:** HEK293 cells transfected with the mTOR mutants as above in conjunction with the HIF-1α inducible luciferase reporter. Cells were starved of amino acids and grown in media containing 1mM DMOG for four hours, cells were stimulated with insulin 30 minutes prior to lysis. Luminesence was measured and standardised to total protein. Error bars indicative of standard deviation from three independent experiments. ** indicates significance <0.001. Under amino acid deprived conditions, only the L1460P mutation showed significantly increased HIF-1α activity upon insulin stimulation.

The extent of 4E-BP1 phosphorylation can be clearly visualised on a large gel, as the different phosphorylated isoforms of 4E-BP1 resolve as different bands. This change in the mobility of 4E-BP1 is demonstrated in figure 3.1 'A' with 4E-BP1 resolving as three different isoforms.

The least phosphorylated species of 4E-BP1 migrate the quickest through the gel (bottom band) and is referred to as the α-isoform while the hyperphosphorylated species of 4E-BP1 resolves as the top band and is referred to as the γ-isoform.

As expected, mTORC1 signalling was repressed under serum acid starved conditions, with 75% of 4E-BP1 present in the unphosphorylated α-isoform in cells expressing wild-type mTOR. Phosphorylation analysis revealed only a trace amount of phosphorylation at the Thr 36/45 site only which is likely to represent the slight resolving β band.

Both the L1460P and E2419K mutants demonstrated almost equal levels of the α and β isoforms (see densitometry figures for lanes 6 and 11). Increased phosphorylation at all phosphorylation sites in cells expressing the mutants (in comparison to wild-type) under serum-starvation was observed. This is in agreement with the work by Urano *et al.* indicating that these mutants were insensitive to serum withdrawal (compare lanes 1, 6 and 11). After insulin stimulation, the majority of 4E-BP1 was present in the phosphorylated β and γ isoforms for cells expressing both the wild-type and the two mutants. In the case of wild-type, 69% of 4E-BP1 was present in the β and γ isoforms combined whereas cells expressing L1460P and E2419K expressed 82% and 89% respectively in the phosphorylated β and γ isoforms. Interestingly, the L1460P mutant exhibited a 30% shift to the hyper-phosphorylated γ-isoform in comparison with 15% and 9% for the wild-type and E2419K respectively (see lanes 2, 7 and 12).

As expected, after amino acid deprivation, the majority of 4E-BP1 was shifted to the least phosphorylated α-isoform in all cases. Unexpectedly 4E-BP1 phosphorylation was enhanced upon insulin stimulation in cells expressing the L1460P construct even in the absence of amino acids (compare lanes 4, 9 and 14). The E2419K mutant only produced an 13% shift towards the β-isoform after insulin treatment whereas the L1460P mutant induced a 15% shift to the γ-isoform. This highlights differences between the two active mutants of mTOR, with the L1460P mutant negating the requirement for a permissive amino acid input in the insulin response. Given that the L1460P mutation resides within the FAT domain of mTOR,

this finding implicates the FAT domain in determining mTORC1 localisation (discussed later).

To further characterise these active mTOR mutants and to examine mTORC1 signalling towards HIF-1α, a HIF-1α transcriptional luciferase reporter was co-transfected alongside each of the active mutants in the presence and absence of amino acids (Figure 3.1 'B'). As predicted, both mutants caused an approximate 3-fold increase in the levels of HIF-1α transcriptional activity in the presence of amino acids. Similar to the effects observed on 4E-BP1 phosphorylation, cells expressing the L1460P mutant resulted in a higher level of HIF-1α activity which was partially resistant to amino acid deprivation (compare lanes 2, 4 and 6). Although expression of the E2419K mutant resulted in a higher level of HIF-1α activity, this activity was still sensitive to amino acid withdrawal. By employing these mTOR mutants, I have demonstrated that the activity of HIF-1α in cells is tightly regulated by signal transduction through mTORC1, suggesting that HIF-1α, like 4E-BP1, is a downstream target of mTORC1. This data supports the work carried out by Land and Tee which demonstrated that heightened mTORC1 signalling via Rheb over-expression led to enhanced HIF-1α activity [135].

3.3.2 Elevated HIF-1α activity in TSC2-/- MEFs is normalised by mTORC1 inhibition with rapamycin and abolished by re-expression of TSC2

By employing these active mTOR mutants, I revealed that increased signal transduction from mTOR potently activates HIF-1α in cells. However, to confirm that this mTOR-dependent regulation of HIF-1α involves Raptor and thus mTORC1 rather than mTORC2, a mouse cell line model for TSC was utilised. Mouse embryonic fibroblasts which are deficient of TSC2 (and also p53 to confer viability) were selected due to their elevated mTORC1 pathway status. These were utilised in conjunction with the HIF-1α transcriptional reporter construct. HIF-1α activity was assessed under conditions of, rapamycin treatment, TSC2 reintroduction, or expression of wild-type or mutant 4 Raptor (experiment carried out under hypoxic conditions to prevent O_2-dependent degradation of HIF-1α).

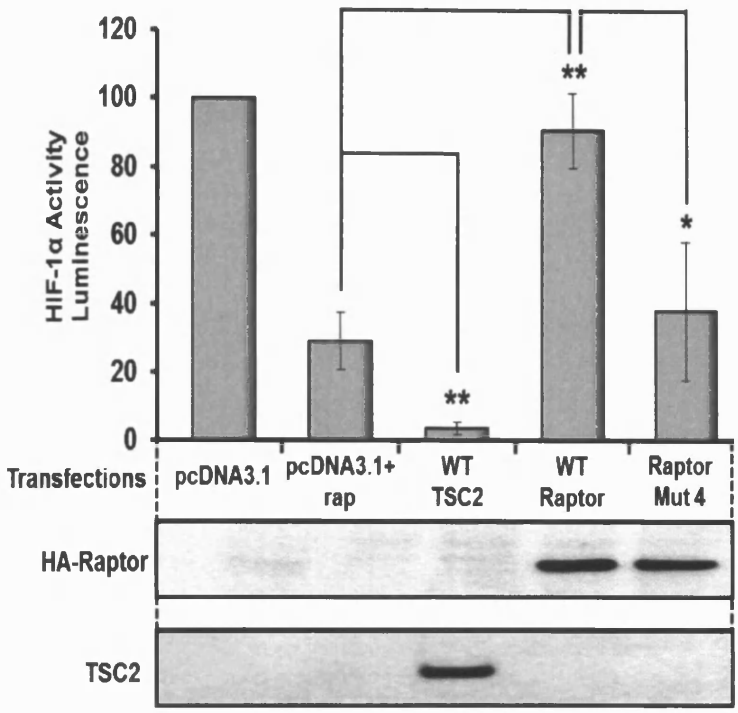


Figure 3.2 Elevated HIF-1α activity in TSC2-/- MEFs is normalised by mTORC1 inhibition with rapamycin and abolished by re-expression of TSC2 TSC2-/- MEFs were transfected as indicated alongside the HIF-α luciferase reporter. Cells were placed into serum free DMEM supplemented with 1mM DMOG and rapamycin treated where appropriate for 12 hours prior to lysis. Total lysate was analysed for HIF-1α transcriptional activity. The HIF1α activity from the pcDNA3.1 empty vector was standardised to 100%. Error bars indicate variation from three independent experiments. Western blotting was carried out to detect the expression levels of exogenously transfected TSC2 and HA-Raptor. * indicates p-value <0.05, ** indicates p-value of <0.001.

Raptor mutant 4 contains a point mutation within the RNC domain (see figure 1.4). Work carried out by Dunlop *et al.* demonstrated that Raptor mutant 4 co-purified with mTOR, however the mTOR/Raptor mutant 4 complex was unable to bind to 4E-BP1 and thus facilitate 4E-BP1 phosphorylation. Therefore, Raptor mutant 4 acts as a dominant inhibitor of mTORC1 substrate association and phosphorylation [76].

TSC2-/- cells expressing empty control vector alongside the HIF-1α reporter construct demonstrated high basal levels of HIF-1α transcriptional activity consistent with the elevation of mTORC1 signalling observed in these cells (see lane 1 of figure 3.2).

This elevated HIF-1α activity was sensitive to inhibition from the mTORC1 inhibitor rapamycin which caused an average 70.9% suppression of HIF-1α transcriptional activity. This supports the hypothesis that signal transduction through mTORC1 regulates HIF-1α.

Raptor mutant 4 expression was also able to inhibit HIF-1α transcriptional activation to a similar level of that of rapamycin (compare lanes 2 and 5 – no significant difference was seen between data sets) and demonstrates the dominant inhibition of mTORC1 by Raptor mutant 4 as predicted.

Interestingly, re-expression of TSC2 into these TSC2-deficient MEF cells completely abolished HIF-1α transcriptional activity. Rescued expression of TSC2 was significantly more effective at inhibiting HIF-1α than inhibition of mTORC1 via rapamycin or raptor mutant 4 expression. This suggests that TSC2 may inhibit HIF-1α via both mTORC1-dependent and independent mechanisms. Over-expression of wild-type Raptor serves as a control and as expected did not affect the activity of HIF-1α.

3.3.3 mTORC1 directed modulation of HIF-1α occurs independently of S6K1

S6K1 is a key effector of mTORC1 signalling, as a regulator of cellular growth and proliferation (see table 1.2 for list of downstream substrates of S6K1) it seemed logical to hypothesise that HIF-1α may be regulated downstream of S6K1. In order to investigate this, I utilised mutants of S6K1 with varying levels of activity.

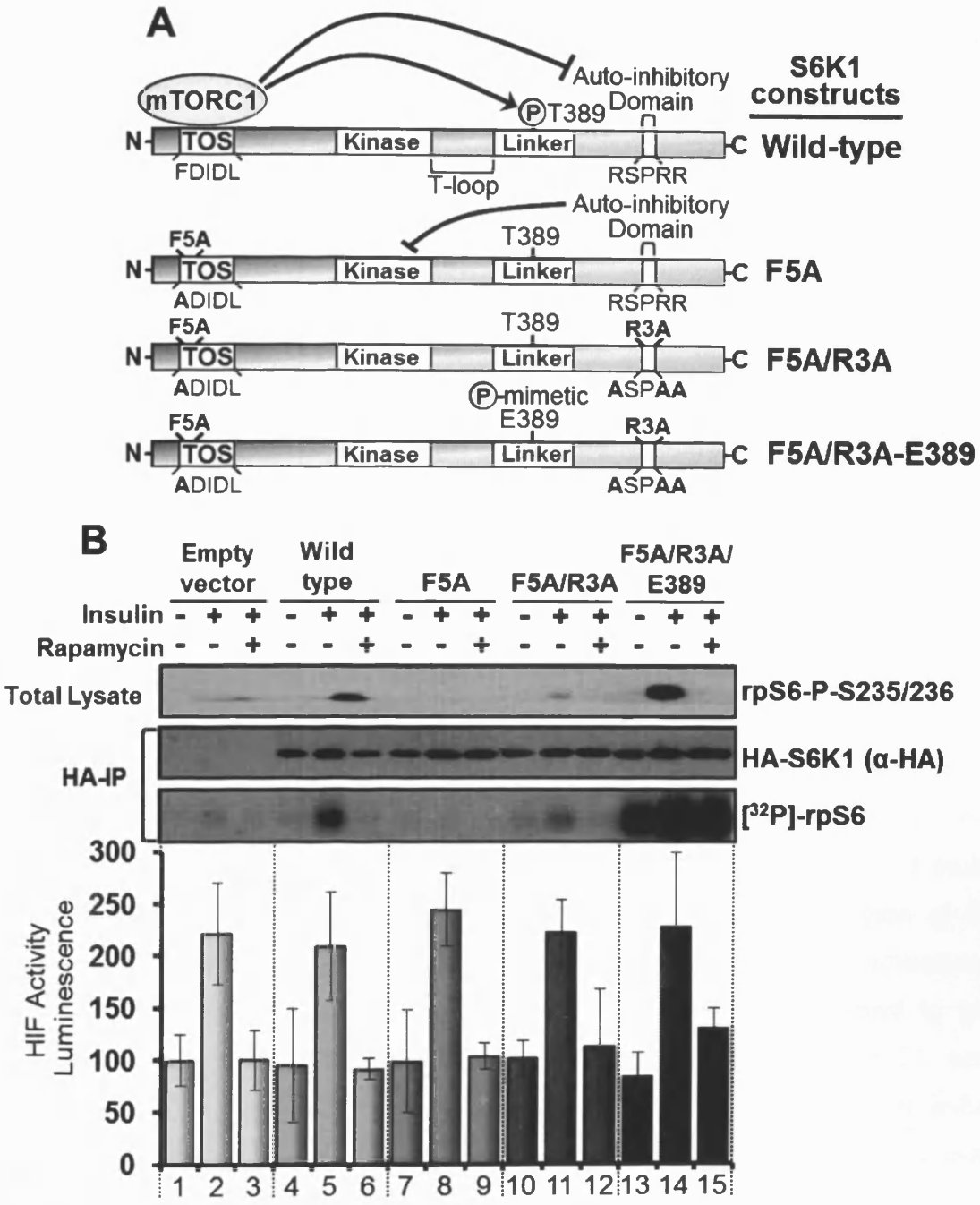


Figure 3.3: mTORC1 directed modulation of HIF-1α occurs independently of S6K1 A: Schematic showing S6K1 and mutant constructs. Domains: TOS refers to mTOR signalling motif, kinase domain, linker domain (containing mTORC1 directed phosphorylation site) and auto-inhibitory RSPRR domain. **B:** HEK293 cells were transfected with S6K1 constructs alongside HIF-1α luciferase reporter construct, grown in media supplemented with 1 mM DMOG, 100mM insulin and 50nM rapamycin treated overnight. 100 µl of total lysate was analysed for phospho-rpS6 and HIF-1α induced luciferase activity. The remainder of the lysate was subjected to a HA-immunoprecipitation and kinase activity towards recombinant rpS6 was analysed (see section 2.3.17). Error bars indicate standard deviation between three independent experiments. No significant difference was seen between S6K1 mutants.

The first mutant analysed was HA-S6K1-F5A which contains a point mutation to the phenylalanine residue contained within its mTOR signalling motif. In 2002, Schalm and Blenis [77] identified a conserved motif, which was later coined as the mTORC1 signalling (TOS) motif found in both S6K1 and 4E-BP1 (see table 1.1 for list of known TOS motifs). Substitution of the first phenylalanine within this TOS motif to an alanine residue results in a mutant of S6K1 which does not interact with Raptor (Fig 3.3 'A') and hence cannot be phosphorylated by mTORC1 [77, 79]. Lack of phosphorylation from mTORC1 renders this S6K1-F5A mutant inactive and unable to phosphorylate its downstream targets such as rpS6.

Further examination into functional domains of S6K1 by Schalm *et al.* also resulted in discovery of an auto-inhibitory domain contained within the C-terminus of S6K1 (5 amino acid motif 'RSPRR').

The 'RSPRR' motif mediates a rapamycin sensitive and thus mTORC1-dependent repression of S6K1 that likely involves a phosphatase [325]. The work by Schalm *et al.* demonstrated that substitution of the three arginine residues within this 'RSPRR' motif with alanines ('ASPAA') rescued the activity of S6K1-F5A to a level comparable to that of wild-type S6K1, this mutant 'S6K1-F5A-R3A was also utilised. Finally, a third mutant, S6K1-F5A-E389-R3A, which is constitutively active and insensitive to inhibition by rapamycin (see figure 3.3 'A' for schematic of mutants) was also used. In this case the Thr389 residue within the linker region of S6K1, which is directly phosphorylated by mTORC1 and is required for S6K1 activation was substituted for glutamic acid. This phospho-mimetic mutant was used to give a constituent level of S6K1 activation in cells regardless of mTORC1 activity.

Wild-type or mutant S6K1 was transfected alongside the HIF-1α inducible luciferase reporter construct in order to assay the activity of both HIF-1α and the S6K1 mutants (using rpS6 phosphorylation within the prepared lysate as a readout), as well as their phosphor-transfer activity (by examining activity *in vitro* activity against GST-S6 substrate) (Fig 3.3 'B').

Lysates were subjected to an S6K1 radioactive kinase assay (Fig 3.3 'B'). As expected, cells stimulated with insulin exhibited heightened activity of wild-type S6K1 and this activity was highly sensitive to inhibition of mTORC1 with rapamycin (rows 5 and 6). This result was also reflected in the phosphorylation of phospho-rpS6 within the lysates prepared. The activity of the TOS mutant of S6K1 ('S6K1-F5A') was not enhanced upon insulin-stimulation as expected and also dominantly impaired insulin

induced phosphorylation of rpS6 in cells (row 8). Insulin induced activation of S6K1 was partially recovered by the secondary mutation to the auto-inhibitory domain in support of the work of Schalm *et al.* (compare lanes 8 and 11).

The activity of the 'S6K1-F5A-E389-R3A' mutant, (lanes 13-15) in the *in vitro* kinase assay was high under all conditions however rpS6 phosphorylation was almost undetectable under serum-starved or rapamycin treatment in the equivalent total lysate samples. This data suggests that serum-starvation and treatment of cells with rapamycin is causing activation of a phosphatase that is directed towards rpS6. Indeed a rapamycin sensitive phosphatase towards rpS6 has been shown [157] and is discussed in more detail in section 3.4.

Figure 3.3 'B' shows a comparison of S6K1 activity and HIF-1α activity, there is evidence that both S6K1 and HIF-1α are regulated downstream of mTORC1 since both display increased activity upon insulin stimulation in a rapamycin sensitive fashion. However, despite the variation in S6K1 activity seen with expression of the S6K1 mutants, HIF-1α transcriptional activity remains consistent whether cells are expressing empty vector or the highly active HA-S6K1-F5A-R3A-S6K1. This data indicates that HIF-1α is being mediated in an mTORC1 dependent manner independently of S6K1.

3.3.4 Pyrimidine tracts found in HIF-1α 5'UTR do not confer preferential translation

Initially, S6K1 was thought to be involved in the regulation of a subset of mRNAs which contain additional long oligopyrimidine tracts in the 5'-terminus untranslated region of their mRNA. This subset of mRNAs are referred to as 5'-TOP mRNA, HIF-1α is often considered a member [339] [340]. It has been shown that 5'-TOP mRNAs are preferentially translated under conditions of nutrient deprivation when translation via the 5'-UTR has been shut down due to energy depletion or hypoxia [341]. It has also been demonstrated that this regulation is sensitive to rapamycin therefore may be an mTORC1 dependent mechanism [336]. However, more recent data suggests that there is not a cause and effect relationship between S6K1 and the translation of 5'-TOP mRNAs [4, 15], it is now considered that the translation of 5'-TOP mRNA is independent of S6K1 [335, 342]. Subsequently, the question of how 5'-TOP mRNAs are translated during a general 'shut down' of the translational machinery during hypoxia remains unanswered. The next phase of this study was therefore to try and determine the significance of the 5'-TOP tract found in HIF-1α mRNA. To do this a bicistronic reporter construct was employed containing the 5'-TOP sequence of HIF-1α upstream of a renilla luciferase reporter, with a firefly luciferase reporter located downstream of the IRES (see figure 3.4 'A'). A second control reporter containing the HIF-1α 5'UTR without the 5'TOP motif was also utilised.

In order to see if the 5'TOP sequence could confer a translational advantage to HIF-1α, the luciferase activity under hypoxia was examined. Figure 3.4 'B' demonstrates that over-expression of Rheb under hypoxia caused a general rapamycin sensitive increase in the translation of *both* reporters, indicating that the 5'TOP sequence is unable to confer any advantage to HIF-1α when mTORC1 is activated. Translation initiated at the IRES remained consistent under all conditions and is not regulated in an mTORC1 dependent fashion (figure 3.3 'B').

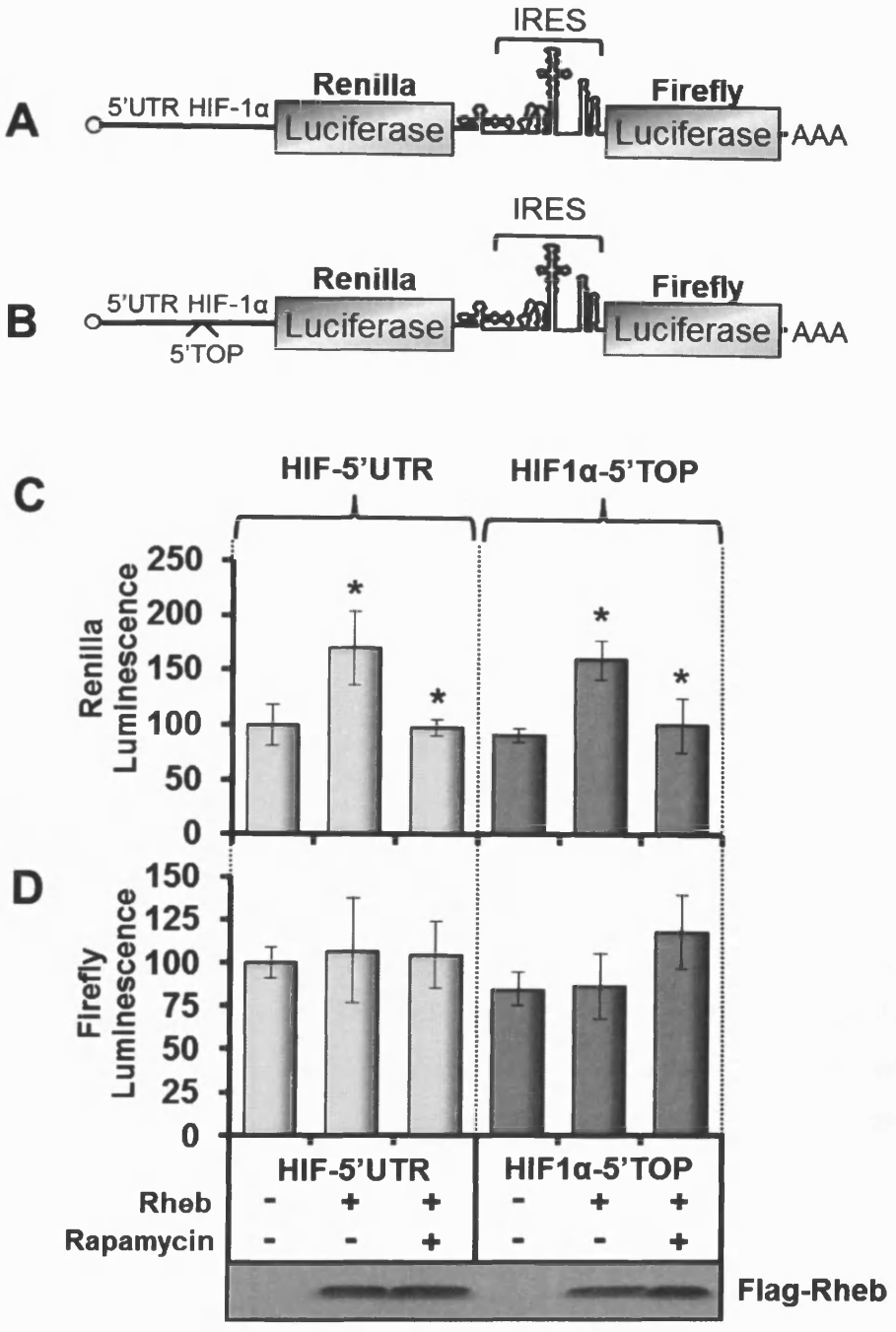


Figure 3.4 Pyrimidine tracts found in HIF-1α 5'UTR do not confer preferential translation A: Schematic of HIF-5'UTR reporter, contains the HIF-1α 5'UTR without the 5'TOP motif upstream of a Renilla luciferase reporter with a firefly reporter downstream of the IRES. **B:** The HIF-1α-5'TOP reporter is identical except it contains the complete HIF-1α 5'UTR including the 5'TOP motif. **C:** Renilla luminescence shows rate of translation during hypoxia, under conditions of Rheb over expression and rapamycin inhibition. **D:** Firefly luminescence is indicative of translation mediated via IRESs, no significant difference was seen under any conditions. Total lysate was also analysed to ensure consistent levels of Rheb expression.

3.3.5 mTOR regulates HIF-1α at a translational level

The next logical step of this study was to establish whether HIF-1α is regulated via the other well characterised mTORC1 effector, 4E-BP1. 4E-BP1 acts as a translational repressor in its hypo-phosphorylated state by binding to and inhibiting eIF4E. eIF4E forms part of a heterotrimeric initiation complex which associates with the 5'-cap structure which is present on all eukaryotic cellular (except organellar) mRNAs (see introduction section 1.2.2) [343]. When mTORC1 phosphorylates 4E-BP1, it causes its dissociation from eIF4E and cap-dependent translation is initiated [344-347]. The first step of this investigation was to characterise this interaction further. A far western approach was utilised to demonstrate Raptor interaction with 4E-BP1. Fig 3.5 'B' shows clear binding of raptor to purified wild-type 4E-BP1. A TOS mutant of 4E-BP1 with a single alanine substitution to the phenylalanine at position one of the TOS motif 'FEMDI' was also utilised. It was previously demonstrated that this particular phenylalanine was crucial to the function of the TOS motif and that mTORC1 signalling towards 4E-BP1 was disrupted if it is mutated to an alanine [80].

A second regulatory conserved motif was also identified in 2002 by Tee *et al.* which was located in the N-terminus of 4E-BP1, it is thought that this motif was essential for optimal phosphorylation of 4E-BP1 and was deemed the 'RAIP' motif after its amino acid sequence [169]. In order to further characterise the function of the 'RAIP' motif, I15A and P16A mutants were prepared from cells and used as a substrate for Raptor interaction alongside the wild-type and TOS mutant of 4E-BP1. It was previously demonstrated that complete mutation of the RAIP motif resulted in a mutant of 4E-BP1 which could not be phosphorylated at mTORC1 sensitive sites [169, 171]. Therefore, single point mutations were introduced to the last two amino acids to determine whether these were critical residues within the motif (site directed mutagenesis carried out by Dr Elaine Dunlop).

Both of the RAIP mutants demonstrated binding to wild-type Raptor, see figure 3.5 'B', in the Raptor overlay assay. To look at this interaction in more detail, larger amounts (3 µg compared to 50 ng) of purified 4E-BP1 (wild-type/mutants) protein were separated by SDS-PAGE then transferred to a PVDF membrane (figure 3.5 'C') before incubation with Raptor expressing lysate.

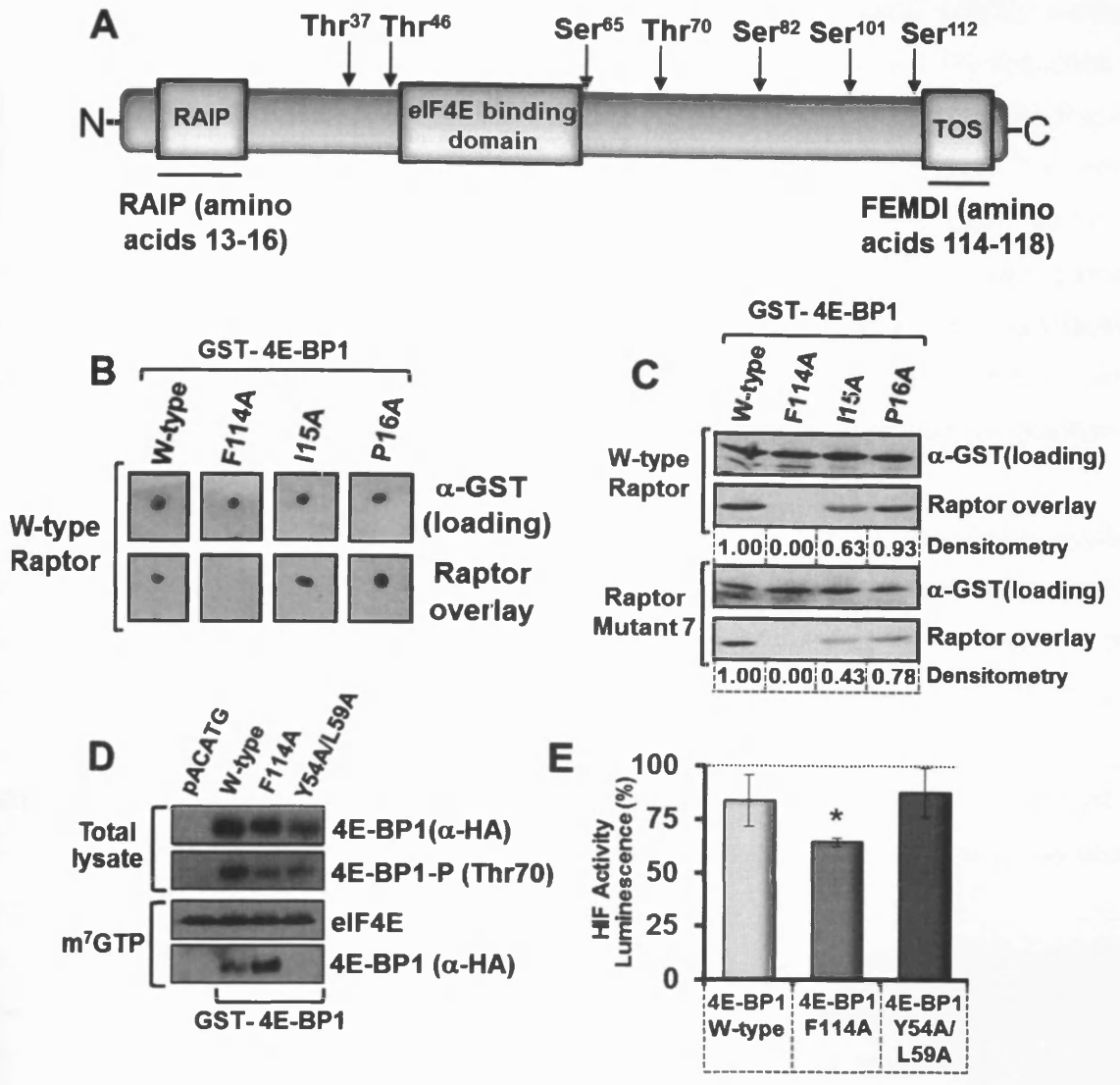


Figure 3.5 mTOR regulates HIF-1α at a translational level A: Schematic showing conserved domains of 4E-BP1 which are mutated. **B:** Far western, mutants of 4E-BP1 were purified from HEK293 cells using GST-purification (see section 2.3.13), a far western was carried out using HA-Raptor expressing lysate generated from HEK293 cells. **C:** To increase the 4E-BP1 protein load, substrates were instead separated using SDS-PAGE and transferred to a PVDF membrane using electro-transfer. Far western was then repeated using lysates generated from HEK293 cells expressing either wild-type Raptor or Raptor mutant 7. **D:** m^7-GTP-sepharose chromatography was utilised to purify eIF4E from TSC2-/- MEFs over-expressing the different mutants of 4E-BP1 alongside the HIF-1α luciferase reporter cultured for 12 hrs under hypoxic conditions. Western blotting was used to determine levels of 4E-BP1 co-purifying with eIF4E. Total lysate was analysed for total and phospho-4E-BP1 to indicate activity. **E:** Total lysates produced from 'D' were also analysed for HIF-1α transcriptional activity, the HIF-1α from cells expressing pACATG empty vector was standardised to 100%. Error bars indicate variation across three independent experiments. * denotes p-value, 0.05.

Interestingly the I15A and P16A forms of 4E-BP1 showed slightly weaker interaction with Raptor when compared to that of wild-type (37% and 7% reduction in binding, respectively). The binding was reduced even further when wild-type Raptor was substituted with Raptor mutant 7, despite the fact that Raptor mutant 7 showed complete binding to wild-type 4E-BP1. Raptor mutant 7 contains a point mutation situated close to the conserved HEAT repeats, studies utilising this mutant carried out within our research group demonstrated that it was able to form an active complex with mTOR which could mediate 4E-BP1 phosphorylation in the *in vitro.* mTORC1 kinase assay [76]. This implicates the HEAT repeats within Raptor in mediating 4E-BP1 interactions.

Due to the fact that mTORC1 was still able to interact with and phosphorylate the RAIP mutants of 4E-BP1, an alternative mutant was examined in the context of HIF-1α activation. Work by Tee *et al.* demonstrated that a double mutation to the eIF4E-binding domain resulted in a mutant of 4E-BP1 which was unable to bind to and inhibit eIF4E (4E-BP1-Y54A/L59A) [344]. Therefore, this was used in conjunction with the TOS mutant (4E-BP1-F114A) of 4E-BP1 which in contrast *is* able to bind and inhibit eIF4E but is not phosphorylated by mTORC1 and therefore its inhibitory activity cannot be relieved.

Taking a similar approach to figure 3.4, the effects of these 4E-BP1 mutations upon HIF-1α transcriptional activity were examined. It was postulated that if mTORC1 was regulating HIF-1α levels via cap-dependent translation, the TOS mutant of 4E-BP1 which has a stronger binding affinity for eIF4E, would inhibit HIF-1α transcriptional activity. To investigate this, the TSC2-/- MEFs were transfected with the HIF-1α inducible luciferase reporter construct alongside either wild-type/mutant 4E-BP1. The lysates were analysed for HIF-1α transcriptional activity (figure 3.5 'E') and their binding ability (to eIF4E) was examined using m^7GTP affinity chromatography (figure 3.5 'D').

In concordance with work by Tee *et al.*[344], increased levels of the TOS-mutant of 4E-BP1 co-purified with eIF-4E. This indicates a stronger binding affinity than that of the wild-type (lanes 2 versus 3), hence the TOS mutant of 4E-BP1 is a more effective inhibitor of cap-dependent translation.

In contrast, the Y54A/L59A mutant was unable to bind to eIF4E so was ineffective at inhibiting translation (lane 4). These samples were then analysed for HIF-1α transcriptional activity (figure 3.5 'E'). As before, high levels of HIF-1α

activation were observed in the cell line so activity was standardised to 100%, whereby 100% was the average HIF-1α activity seen in TSC2-/-cells expressing empty vector. Expression of the Y54A/L59A mutant of 4E-BP1 had no significant effects upon HIF-1α activity as was the case for wild-type 4E-BP1. Over-expression of the inhibitory TOS-mutant of 4E-BP1 resulted in a significant (p-value of 0.05) decrease in HIF-1α transcriptional activity (figure 3.5 'E'). This indicates a potential mechanism whereby mTORC1 regulates HIF-1α activity by inducing cap-dependent translation and triggering the formation of the eIF4F complex. It could be argued that if HIF-1α translation was being regulated by mTORC1 in this manner then over-expression of wild-type 4E-BP1 would also inhibit HIF-1α activity. However, it is likely that the inhibitory effect of over-expressing wild-type 4E-BP1 was diminished by the high level of 4E-BP1 phosphorylation (see figure 3.5 'D') resulting from the high basal activity of mTORC1 signalling within these TSC2 -/- cells.

3.3.6 mTORC1 binds to HIF-1α and this binding is augmented by the presence of Rheb

The next phase of the study was to establish whether or not HIF-1α could be directly phosphorylated by mTORC1. Land and Tee first postulated that as HIF-1α is a phospho-protein, it is possible that mTOR may phosphorylate HIF-1α directly to promote its function as a transcription factor [135]. In figure 3.5 'B', it was demonstrated that Raptor could bind to 4E-BP1 in an overlay assay, it was hypothesised that if HIF-1α was a direct substrate of mTOR then Raptor would bind to HIF-1α but not the inactive TOS-mutant of HIF-1α under the same conditions, Raptor mutant 4 was employed as a negative control (as stated earlier, raptor mutant 4 co-purifies with mTOR but cannot facilitate substrate recognition [76]). However as the overlay assay in figure 3.6 'A' demonstrates, neither wild-type HIF-1α nor the inactive TOS mutant showed interaction despite strong binding between Raptor and 4E-BP1.

This may reflect weakness in the methodology since the overlay assay only appears to detect strong interactions, evidenced by the fact that this technique is unable to show Raptor binding to S6K1 (data not shown) despite the fact that S6K1 is a known interactor [77].

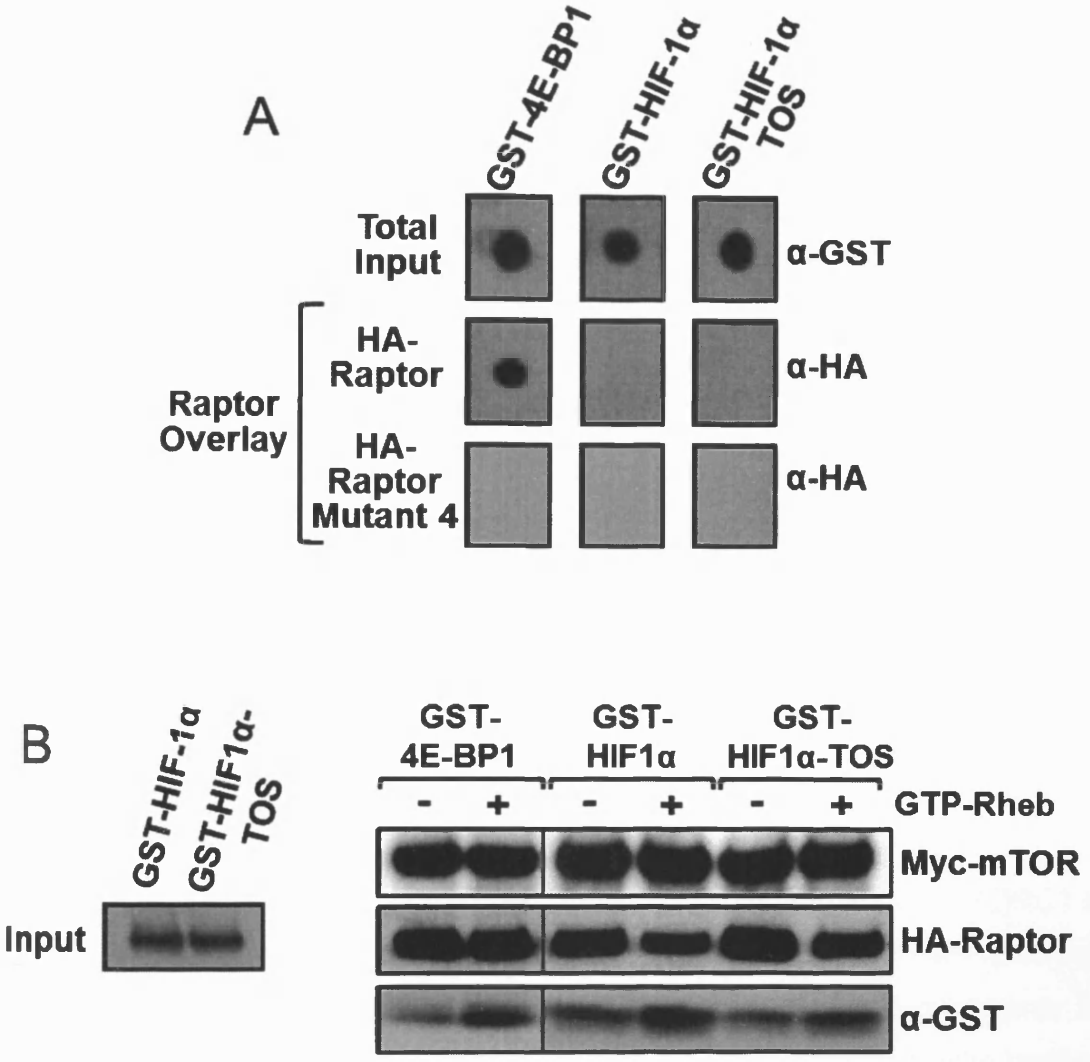


Figure 3.6 mTORC1 binds to HIF-1α and this binding is augmented by the presence of Rheb A: HEK293s expressing GST-4E-BP1/HIF/HIF-TOS were harvested and subjected to a GST-purification (cells expressing HIF proteins were cultured under hypoxia for 12 h prior to lysis). Purified protein was dotted onto PVDF membrane and a far western was carried out using lysates extracted from HA-Raptor or HA-Raptor mutant 4 expressing HEK293 cells. α-GST primary antibody was used to determine substrate levels. **B:** An active mTORC1 complex was purified from HEK293 cells over-expressing HA-raptor and myc-mTOR, GST-HIF/GST-HIF-TOS were purified from HEK293s using a GST-purification and confirmed with western blotting (input). Purified mTORC1 complexes were then incubated with the substrates in the presence and absence of purified GTP-loaded Rheb, non-specific interactions were then removed with washes. Samples were then analysed for mTOR/Raptor expression and substrate binding using α-GST antibodies.

Furthermore, Land and Tee demonstrated that mTORC1 bound more readily to 4E-BP1 than to HIF-1α [135], indicating that perhaps this assay is not sensitive enough to show Raptor interactions with HIF-1α.

Sato *et al.* previously observed that Rheb enhanced recruitment of substrates to mTORC1 *in vitro* [59], therefore to address the potential weaknesses in the far western methodology, I devised a similar technique to enhance Raptor-substrate interactions. This involved purification of mTORC1 complex for incubation with substrates in the presence and absence of a constitutively active mutant of Rheb (Q64L). This was followed by a series of washes to remove non-specific binding.

Results indicate that binding of mTORC1 to the substrate 4E-BP1 could be augmented with the inclusion of purified Rheb protein in the assay in concordance with the work by Sato *et al.* It also demonstrates binding of the mTORC1 complex to HIF-1α which was again further augmented with Rheb inclusion, unexpectedly however, the TOS mutant also demonstrated binding (although to a lesser extent) to the mTORC1 substrate.

3.3.7 HIF-1α could not be phosphorylated by mTORC1 in an *in vitro* kinase assay

The next stage of this line of experimentation was to implement an mTORC1 kinase assay to see if a purified mTORC1 complex could phosphorylate HIF-1α or the TOS mutant *in vitro.* Work by Kim *et al.* in 2002 utilising non-ionic detergents for the purification of mTOR lead to the discovery of Raptor. Therefore a similar method was utilised for the purification of an mTOR/Raptor complex [74].

Later work by Sancak *et al.* revealed that under conditions of insulin deprivation, PRAS40 binds to and inhibits the mTORC1 complex (see section 1.3.6). Sancak demonstrated that PRAS40 could be removed from the complex by high salt concentrations so a high salt wash was integrated into the methodology.

Sancak *et al.* also utilised $MgCl_2$ based buffers to purify the active mTOR complex, previously used mTOR kinase assays had used $MnCl_2$ to artificially increase the weak phospho-transfer activity seen when mTORC1 was purified. A similar methodology was employed to purify a physiologically relevant and highly active mTORC1 complex. In addition to this, GST-Rheb was also purified from mammalian cells and added to the assay to enhance the specificity of substrate binding and facilitate phosphorylation.

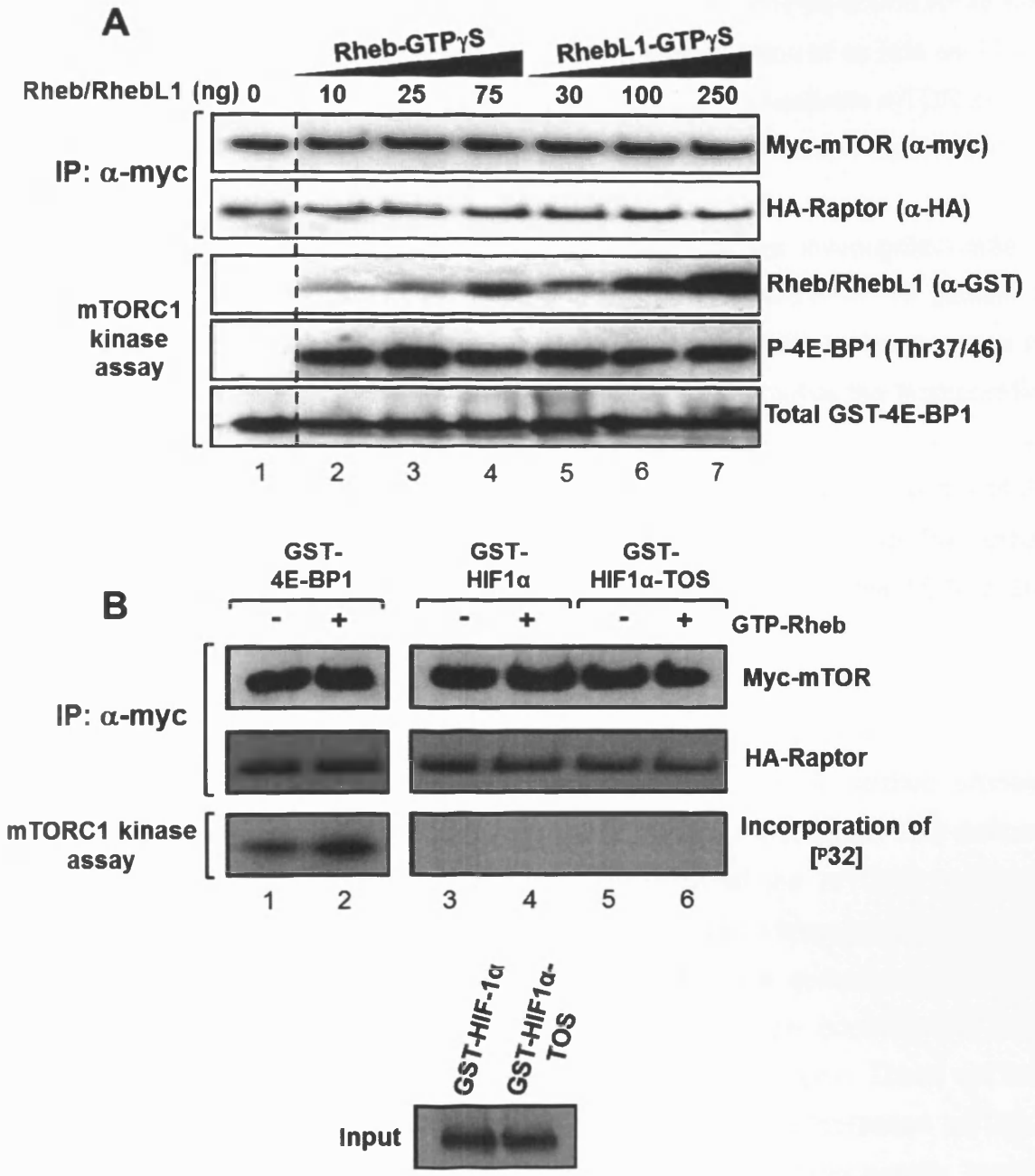


Figure 3.7 HIF-1α could not be phosphorylated by mTORC1 in an *in vitro* kinase assay A: An mTOR kinase assay was performed from unstimulated Myc-mTOR and HA-Raptor transfected HEK293 cells (see section 2.3.15) with the addition of increasing amounts of Rheb (10–75 ng) or RhebL1 (30–250 ng). mTOR activity was determined by analysis of 4E-BP1 phosphorylation on Thr37/46. **B:** A second mTORC1 kinase assay was performed in the same manner to evaluate kinase activity of mTORC1 towards purified GST-HIF-1α or GST-HIF-1α-TOS in the presence of GDP or GTP bound Rheb. GST-HIF-1α and GST-HIF-1α-TOS were purified using a GST-purification (section 2.3.13) from HEK293 cells cultured under hypoxia for 12 h. α-GST antibodies were used to check the purification (shown – input).

Figure 3.7 'A' demonstrates how during optimisation, GTPγS-bound Rheb was able to potently activate phosphorylation of 4E-BP1 with addition of as little as 10 μg, it also confirmed that the Rheb-like-1 protein was also able to activate mTOR *in vitro* supporting the work of Tee *et al.* in 2005 which identified Rheb-like-1 as an activator of mTORC1 and a substrate of the TSC1/2 complex [62].

After optimisation of the assay, the next stage in the investigation was to establish whether mTORC1 could phosphorylate purified GST-HIF-1α protein *in vitro*. Potential mTORC1 directed phosphorylation sites of HIF-1α have yet to be identified, I therefore utilised a radioactive kinase assay to visualise the incorporation of [^{32}P]-radiolabel into purified GST-HIF-1α or the TOS mutant. 4E-BP1 was utilised as a positive control and was phosphorylated by mTORC1 (see lanes 1 and 2 of 2.7 'B'), this was augmented by inclusion of GTPγS-bound Rheb into the assay. However as shown in figure 3.7 'B', neither wild-type HIF-1α nor the TOS mutant were phosphorylated in this assay.

3.3.8 HIF-1α mRNA is regulated in an mTORC1-dependent fashion

It is likely that mTOR directed regulation of HIF-1α is a multi-faceted process, therefore it is important to explore each angle of potential modulation. TSC deficient cells lines were implemented to see if up regulation of the mTORC1 pathway resulted in upregulation of HIF-1α mRNA levels as well as its translation and activity under various conditions. TSC1-/- and TSC2-/- MEFs were compared for HIF-1α mRNA levels (standardised to β-actin) against their wild-type counterparts under hypoxia or normoxia and in the presence and absence of rapamycin. These cell lines are particularly useful in this context because they exhibit unsuppressed mTORC1 signalling so HIF-1α is elevated, furthermore HIF-2α is transcriptionally inactive within MEFs therefore the hypoxic response is primarily mediated via HIF-1α [348]. Interestingly, the mRNA levels of HIF-1α were not regulated in a hypoxia dependent fashion. Induction of hypoxia caused no significant difference in the HIF-1α mRNA between the TSC1-/- cells or the TSC2-/- cells. HIF-1α mRNA however did seem to be subject to rapamycin inhibition. In the case of the TSC1-/- MEFs, rapamycin caused repression of HIF-1α mRNA under all conditions, however the reduction was only significant under hypoxia.

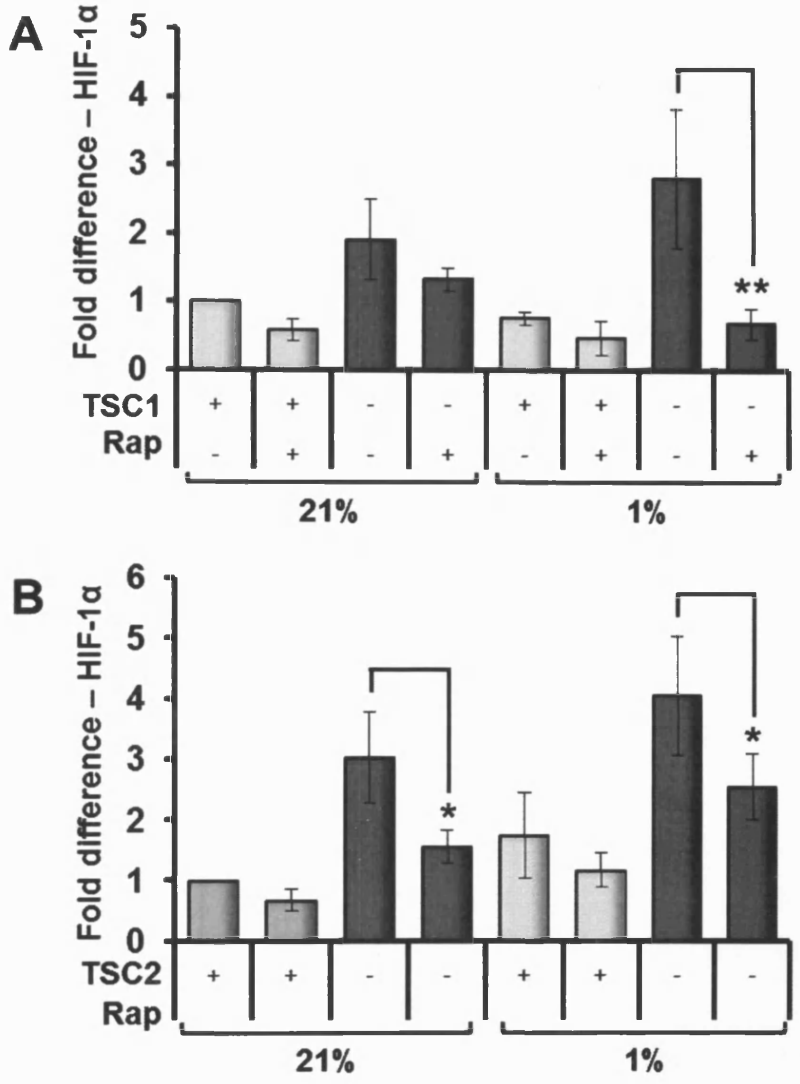


Figure 3.8 HIF-1α mRNA is regulated in an mTORC1-dependent fashion
Untransfected TSC1-/- MEFs **(A)** or TSC2-/- MEFs **(B)** and their wild-type counterparts were cultured overnight in serum free media under both hypoxic and normoxic conditions in the presence and absence of rapamycin as indicated. mRNA was then extracted and quantitative-PCR was utilised to determine HIF-1α mRNA levels and standardised to β-actin. Significant differences indicated, * = $p<0.05$. ** = $p>0.001$.

HIF-1α mRNA levels were significantly higher in both the TSC1 and TSC2 deficient cells lines compared with the wild types MEFs, indicating that mTORC1 is able to modulate HIF-1α mRNA synthesis or stability, as well as regulating it's translation. Therefore TSC patients are likely to exhibit increased HIF-1α mRNA levels as well as an increased rate of its translation. This will no doubt be contributing to the highly vascularised tumours which develop when mTORC1 signalling is unrestrained.

3.3.9 Rapamycin does not alter HIF-1α protein stability

During this chapter I have explored how mTORC1 regulates HIF-1α mRNA and it's synthesis as well as its transcriptional activity through potential phosphorylation events. As discussed earlier, HIF-1α is primarily regulated in terms of oxygen content, whereby the HIF-1α protein is rapidly degraded under normoxia by PHD enzymes. It is therefore possible that mTORC1 is also able to regulate the stability of the HIF-1α protein, either by modulating the activity of PHDs or by directly enhancing the stability of HIF-1α to protect it from PHD mediated degradation. To investigate this possibility, I utilised the bacterial derived toxin cyclohexamide. Cyclohexamide reversibly interferes with ribosomal translocation to prevent protein translation, by blocking the synthesis of HIF-1α, I was able to observe the degradation of the protein over time in the presence and absence of rapamycin.

As figure 3.9 demonstrates, rapamycin caused no significant impact upon the degradation of HIF-1α in the presence of cyclohexamide. This indicates that mTORC1 is not able to regulate the stability of the HIF-1α protein.

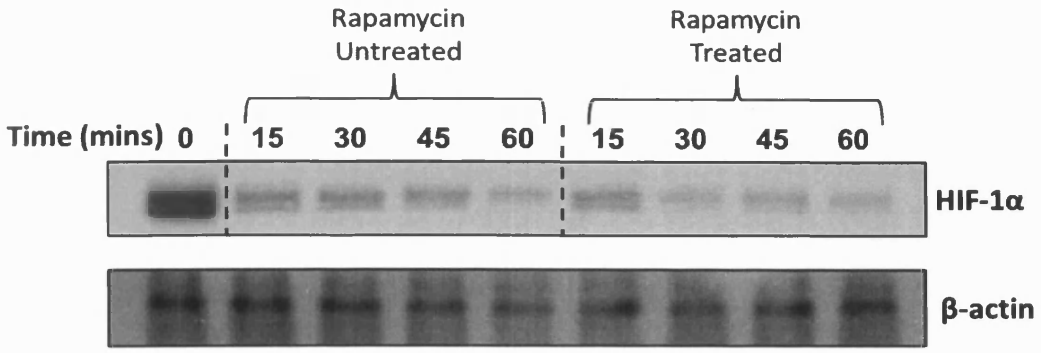


Figure 3.9 HIF-1α protein stability is unaffected by rapamycin treatment
HEK293 cells were simultaneously treated with 10μg/ml cyclohexamide and 50nM rapamycin where indicated. Cells were then cultured in the hypoxic chamber at 1% O_2 for the indicated time points prior to lysis. Lysates were sonicated and analysed for HIF-1α and β-actin protein levels using western blotting. Result is representative of three independent replicated experiments.

3.4 DISCUSSION

This study aimed to characterise mTORC1 dependent regulation of HIF-1α. It involved taking a varied approach, where I systematically investigated different ways in which mTORC1 could enhance HIF-1α activity.

Initially I was able to show that the active mutants of mTOR could enhance the transcriptional activity of HIF-1α supporting previous work by Land *et al.* and validating HIF-1α as a potential therapeutic target in diseases where mTORC1 signalling is abberant.

Urano *et al.* demonstrated that the mutants of mTOR were constitutively active under conditions of nutrient starvation. Figure 3.1 supports this work to an extent however by using a wider range of conditions of nutrient starvation and resupply it demonstrates previously unseen subtle differences between the L1460P mutant and the E2419K mutant. In the cases of wild-type and the E2419K mutant, insulin stimulation was able to enhance the phosphorylation of downstream substrates to a much greater extent in the presence of amino acids (figure 3.1 'A' compare lanes 4 and 5 with 14 and 15). Conversely, the L1460P mutant was highly responsive to insulin stimulation even in the absence of amino acids. This was demonstrated more clearly in the HIF-1α transcriptional reporter assay, whereby the L1460P mutant was able to propagate much higher levels of HIF-1α activity in the absence of amino acids than both wild-type and the E2419K mutant. This demonstrates that HIF-1α is as responsive as the mTORC1 substrate 4E-BP1 to changes in mTOR activity. It also indicates the potential significance of the FAT domain of mTOR (where the L1460P mutation is situated).

In a continuation of this work, Dunlop *et al.* was able to demonstrate that this L1460P mutant was resistant to inhibition of mTOR by inactive Rag complexes. As explained in the introduction, active Rag complexes are thought to localise mTOR to the lysosomes where Rheb is also contained [76, 116]. Sancak *et al* recently demonstrated that when mTORC1 is forced to the lysosomes (through addition of an intracellular targeting sequence to Raptor) it loses sensitivity to amino acid withdrawal, creating a constitutively active complex. This is very similar to the signalling pattern I observed with the L1460P mutant of mTOR. This may therefore indicate that the L1460P mutant is mislocalised to the lysosomal membrane causing constitutive activation. This would explain why it is still activated by insulin in the absence of amino acids, and also why it was insensitive to inhibition from the

inactive Rag complexes. This evidence therefore may hold clues as to how the mTORC1 complex is localised to and from the lysosomal membrane, however further work is required to elucidate the mechanisms behind this.

After confirmation of a correlation between mTOR activity and HIF-1α, the next logical step was to investigate the dependence upon signal transduction through mTORC1. This was carried out in a cell line model for TSC with the view of establishing whether mTORC1 inhibitors may be effective in normalising HIF-1α activity. If the high level of tumour vascularisation seen in patients with TSC could be therapeutically targeted in this way, it could significantly reduce the capacity for growth of the tumour. As predicted, rapamycin and expression of the dominant mTORC1 inhibitor Raptor mutant 4 caused a similar level of inhibition. With an average 70.9% reduction in HIF-1α transcriptional activity upon rapamycin treatment, and an average 61.9% reduction in activity with raptor mutant 4 expression (no significant difference between the two means). However re-introduction of TSC2 into the cell line caused a much more substantial 96.3% reduction in HIF-1α transcriptional activity, this was significantly more inhibition than was seen with mTORC1 inhibitors (p-value 0.001).

While it is possible that TSC2 was over-expressed to an unphysiological level within these cells causing a more complete inactivation, this seems unlikely given that over-expression of the dominant inhibitor Raptor mutant 4 did not have the same effect despite being over expressed to a similar level (see expression control blots figure 3.2). A more logical explanation may therefore be that TSC2 is also able to inhibit HIF-1α in an mTORC1-independent manner which may be contributing to the pathology of TSC. This hypothesis is supported by evidence within the literature indicating that both TSC1 and TSC2 have independent functions outside their role as a heterodimer tumour suppressor which may not involve mTORC1 [349]. It has also been well documented that TSC2 can modulate transcriptional events regulated by steroid nuclear receptor function, extending its role to transcriptional regulation [350-352]. This evidence strengthens the possibility of a TSC2 dependent, mTORC1-independent mechanism of HIF-1α regulation and instigated the series of experiments shown in the fourth chapter of this thesis.

It is thought that mTORC1 regulates cellular growth primarily through regulation of S6K1 or 4E-BP1. Given that HIF-1α regulates processes involved in cell

growth such as glucose transport and angiogenesis, I hypothesised that HIF-1α may be regulated downstream of one of these mTORC1 effectors.

It has been previously postulated that HIF-1α translation under hypoxia may be regulated via S6K1 [340, 353]. It was thought that S6K1 could up-regulate 5'TOP mRNA translation during hypoxic conditions. However, this correlation has more recently been dismissed [335, 342, 354]. This study therefore aimed to establish whether S6K1, 4E-BP1 or the 5'TOP motif were required for mTORC1 regulation of HIF-1α.

Initially, S6K1 mutants were utilised to see whether they could modulate the activity of HIF-1α. Figure 3.3 clearly demonstrates the varying degrees of activity demonstrated by the different S6K1 mutants employed. Intriguingly, the incorporation of [^{32}P]-radiolabel into ribosomal protein S6 as observed in the *in vitro* S6K1 assay did not entirely mirror the levels of phospho-S6 seen *in vivo*. Schalm *et al.* demonstrated that the HA-S6K1-F5A-3A-E389 mutant was rapamycin resistant, however figure 3.3 'A' shows that this rapamycin resistance was only seen *in vitro* and did not translate to phospho-rpS6 levels observed in the total lysate (lane 15). The basal activity seen under serum starvation conditions was also significantly lower *in vivo* (lane 13). This is likely to be a result of phosphatase activity towards rpS6. It is known that both rapamycin treatment and conditions of serum deprivation can induce the phosphatase PP2A to act within cells causing its dephosphorylation [155, 157]. This could explain the differences seen *in vivo* as purification of HA-S6K1 for the kinase assay would have removed any phosphatases from the reaction. Within cells however, there is an on-going homeostatic balance of fine tuning occurring between phosphatases and kinases to regulate the phosphorylation status of rpS6. Conditions of serum starvation and rapamycin treatment cause the balance to favour phosphatases such as PP2A, whereas conditions of insulin stimulation favour S6K1 kinase activity.

Interestingly, it has been shown that hypoxia mediated suppression of mTORC1 has far greater suppressive effects upon S6K1 and rpS6 phosphorylation than 4E-BP1 [355]. This is consistent with the findings of this study since there are differences in the way HIF-1α and S6K1 are regulated despite them both showing mTORC1 dependence, it also supports the evidence in this study demonstrating that the translation of HIF-1α is regulated by eIF4E availability. This may be one of the

ways in which mTOR is able to maintain HIF-1α production during a general suppression of protein synthesis during hypoxia.

Although it was originally thought S6K1 regulated the translation of 5'TOP mRNAs via phosphorylation of rpS6 [356], the involvement of S6K1 in this process was later dismissed [342, 356]. Given that HIF-1α has previously been considered a 5'TOP mRNA and also appears to be regulated independently of S6K1, I wanted to determine whether the 5'TOP motif was essential for HIF-1α translation. HIF-1α had been considered a 5'TOP mRNA due to the presence of long pyrimidine tracts contained within the HIF-1α 5'UTR. Thomas *et al.* showed evidence that mTORC1 did regulate HIF-1α via the 5'TOP, demonstrating that VHL-resistant HIF-1α mutant cDNAs lacking the 5' TOP sequence were resistant to a CCI-779 (a rapamycin analogue) induced growth suppression. Since they had also demonstrated that CCI-779 did not affect the protein stability of HIF-1α (in concordance with the results of this study) they attributed mTORC1 dependent regulation of HIF-1α to translation mediated by the 5'TOP and predicted S6K1 involvement. Conversely, Karni *et al.* showed that the HIF-1α 5'-UTR is not involved in the enhancement of translation by Src, which is an upstream activator of mTOR and that over-expression of eIF4E could augment HIF-1α protein levels, in concordance with the results of this study. More recent studies have also indicated that HIF-1α is not a true 5'TOP mRNA and cannot be translated via the 5'TOP message [355, 357].

The results of this study clearly demonstrate that Rheb expression is able to enhance HIF-1α translation in a rapamycin sensitive manner, however the presence of the 5'TOP message did not seem to influence the rate of translation under any conditions. This conflicts with the observations of Thomas *et al.* [340]. The reasons for this are unclear since the same reporter constructs were utilised, it may indicate that different cell-types are able to differentially regulate HIF-1α translation. However, given the fact that Thomas *et al.* showed a loss of sensitivity to CCI-779 in the context of an over-expressed mutant which could not be degraded through ubiquitination or be translated specifically via a 5'TOP message, it is difficult to dissect whether it is the stability of the mutant or the 5'TOP sequence which is causing the effect. Furthermore, in an earlier experiment Thomas *et al.* reported that CCI-779 caused a 38% decrease in the protein levels of HIF-1α without the 5'TOP compared to a 65% in HIF-1α with the 5'TOP. If this was the case then it would be

expected that CCI-779 was still able to cause some inhibition of the translation of the HIF-1α mutant cDNAs lacking the 5' TOP sequence, yet this was not apparent.

Furthermore, it does appear that although the cells expressing these mutants were insensitive to growth suppression, GLUT-1, a HIF-1α gene target does appear to show reduced protein levels upon CCI-779 treatment suggesting that it is not completely recalcitrant to CCI-779 as suggested (see Glut-1 protein levels, figure 3 [340]). By over-expressing Rheb in this context I was able to look at the rate of translation whilst mTORC1 signalling was enhanced as opposed to in Thomas *et al.'s* study where loss of VHL may have had effects on other signalling pathways. This is therefore a more appropriate model for investigating mTORC1 signalling. It may be the case that HIF-1α translation is only initiated via the 5'TOP message under certain conditions of nutrient/O_2 availability, it appears however that when mTORC1 is active (through Rheb over-expression), HIF-1α is not primarily regulated by the 5'TOP message during hypoxia.

The presence of a conserved putative TOS motif within HIF-1α implies that mTORC1 could directly phosphorylate HIF-1α and may provide an additional governing mechanism contributing to mTORC1-directed regulation of HIF-1α. It is likely that the regulation of HIF-1α is multi-faceted given the fact that the TOS mutant of 4E-BP1 only repressed the activity of HIF-1α by approximately 40% (figure 3.5) whereas rapamycin/raptor mutant 4 expression caused an average 70.9% reduction (figure 3.2).

Land *et al.* found that the expression of a mutant of HIF-1α lacking the TOS motif (F99A mutant) resulted in dominant inhibition of HIF-1α activity [135]. In order to further characterise the TOS motif of HIF-1α, purified recombinant GST-HIF-TOS was utilised as a substrate for the Raptor overlay assay. No binding was seen between HIF-1α and Raptor using the far western approach, this was surprising since Land *et al.* had previously demonstrated a weak Raptor interaction. However, it was also demonstrated that Raptor bound more readily with 4E-BP1 suggesting a hierarchy of mTORC1 substrate phosphorylation (where Raptor may preferentially bind to 4E-BP1 over HIF-1α). Alternatively it could suggest that the binding between mTORC1 and HIF-1α is a more transient interaction. The Raptor overlay assay in figure 3.5 'A' signified no difference between the binding of the RAIP mutants and the wild-type, calling into question the sensitivity of this assay. To address this, a more sensitive methodology was employed (as described in section 3.3.6). I was

then able to demonstrate an interaction between Raptor to HIF-1α, which, in support of Sato's work, was further augmented with the addition of GTP-bound Rheb into the assay.

Surprisingly, the TOS mutant of HIF-1α also exhibited Raptor binding, the interaction observed between mTORC1 and HIF-1α-TOS was weaker indicating that this motif is influential in mTORC1 mediated substrate binding but not essential. This was somewhat unexpected as characterisation of more bona fide mTORC1 substrates have indicated that mutations to their TOS motifs could not be tolerated and caused complete disruption to Raptor binding. Although in this assay there was a distinct reduction in the binding of the TOS mutant compared to wild-type HIF-1α (compare lanes 1 to 3 and 2 to 4), it was still a significant interaction within both upon addition of GTP-Rheb. It is therefore possible that the TOS motif found within HIF-1α functions differently to the TOS motif found in 4E-BP1 or S6K1. This theory is supported by the fact that substrate binding is not as strong to HIF-1α as it is to 4E-BP1. Also work by Land *et al.* demonstrated that the TOS mutant of HIF-1α demonstrated reduced binding to the transcriptional co-activator p300, this suggests differing functions of this TOS motif relating to transcriptional activation.

The binding of HIF-1α was still significantly increased after Rheb incubation indicating a probable direct interaction between mTOR and HIF-1α. Land *et al.* saw small amounts of Raptor co-purified with the TOS-mutant of HIF-1α, supporting the notion that Raptor may be able to form interactions with the TOS-mutant of HIF-1α. However, it is also possible that the wash steps within the assay after substrate incubation were not sufficient to remove all non-specific binding. Conversely, if the binding between mTORC1 and HIF-1α-TOS was non-specific, you would not expect inclusion of GTP-Rheb to increase the interaction. This study does therefore provide evidence of an interaction between mTORC1 and the purified HIF-1α complex, however further characterisation is required to determine the role of the TOS motif.

The implementation of an *in vitro* mTOR kinase assay was utilised to try and confirm this interaction and phosphorylation of HIF-1α, however no incorporation of [^{32}P] was seen. This may indicate that p300 interaction is required for phosphorylation by mTOR and since HIF-1α was purified after treatment with rapamycin, it is unlikely that p300 would have co-purified with it. Alternatively, it may be the case that mTOR does not directly phosphorylate HIF-1α but simply facilitates the interaction with p300. It may also be possible that mTORC1 directly

phosphorylates p300 itself, although it is unclear from the literature how the function of p300 is related to its phosphorylation status [358]. However, since the assay is carried out *in vitro* and it has been previously demonstrated that mTORC1 more readily binds to 4E-BP1 than HIF-1α, the possibility that mTORC1 can directly phosphorylate HIF-1α can not be ruled out on the basis of this data.

Prof. John Blenis carried out a SILAC (Stable isotope labeling with amino acids in cell culture) screen of potential phosphorylation sites which are altered in response to rapamycin and revealed Thr798 as a possible site of modulation (unpublished data – personal communication). Intriguingly this site is located directly within the transcriptional activation domain of the HIF-1α subunit so it is possible that mTORC1 regulates the transcriptional activity of HIF-1α via phosphorylation at this site. Generation of antibodies against this site could provide a more insightful look into mTORC1 dependent HIF-1α regulation in the future.

Finally, this study demonstrated that HIF-1α mRNA levels are also regulated by mTORC1. At this stage the mechanism behind this modulation can only be speculated. Previous work has identified several putative hypoxia response elements (HREs) in the HIF-1α promoter, this could imply an auto-regulatory loop whereby HIF-1α expression is able to up-regulate synthesis of its own mRNA [359, 360]. This may also contribute to the continued activation of HIF-1α under hypoxia when other substrates of mTOR show suppression. S6K1 activates 45s ribosomal gene transcription by phosphorylation of the rDNA transcription factor UBF which indirectly leads to an increase in transcription (see introduction table 1.2). A similar mechanism may be in place here, whereby mTORC1 is able to modulate HIF-1α mRNA by phosphorylation of upstream transcription factors. Alternatively it may be possible that mTORC1 directly or indirectly modulates the stability of the HIF-1α mRNA as opposed to its synthesis, however further research in this area is required to determine this mechanism.

Intriguingly, it was recently demonstrated that STAT3 was required for HIF-1α mRNA expression [361], furthermore there is evidence that STAT3 can be modulated by mTORC1 (see introduction section 1.5.4) so mTOR may regulate HIF-1α mRNA synthesis downstream of STAT3.

mTORC1 directed HIF-1α regulation is likely a multi-faceted process, I therefore wanted to explore different potential mechanisms of mTORC1 directed HIF-1α regulation. A crucial factor governing HIF-1α activity is the stability of the

protein, Land *et al.* showed evidence that mTORC1 regulated HIF-1α in an VHL-independent manner. This evidence further supports this hypothesis and clearly demonstrates that rapamycin does not affect the stability of the HIF-1α protein.

The aim of this study was to characterise the mechanism behind mTOR directed regulation of HIF-1α. There has been a high degree of conflicting evidence indicating potential mechanisms involving S6K1 and the 5'TOP structure of HIF-1α mRNA (see review [35]), this study presents clear evidence disputing the involvement of either. Although this study could not demonstrate direct phosphorylation from mTORC1, it has shown interaction with Raptor indicating the potential for an additional mechanism of regulation via mTOR directed phosphorylation. It has also clearly demonstrated that mTOR can modulate the translation of HIF-1α through the phosphorylation of the 4E-BPs in addition to regulating HIF-1α mRNA expression.

There are increasing reports showing evidence for mTORC1 as a transcriptional regulator and widening the implications of mTOR dysregulation in disease, HIF-1α mediates expression of a number of genes which contribute to the pathology in diseases where mTORC1 is dysregulated. This study has answered questions regarding this regulation, dispelling myths related to S6K1 regulation and how the 5'TOP-like message is interpreted by mTORC1. Just as importantly however, it has raised questions about how HIF-1α functions independently of mTORC1 and how HIF-1α mRNA levels can also be modulated, thus providing a foundation for further research in this area.

CHAPTER 4: HIF-1α IN THE CONTEXT OF TUBEROUS SCLEROSIS

4.1 INTRODUCTION

Research over the last decade has indicated that both TSC1 and TSC2 appear to be multi-functioning proteins, with evidence for over 50 binding partners for TSC2 and or TSC1 (see review [1]). There is growing evidence to suggest that TSC2 in particular has significantly more mTOR independent roles with repeated studies demonstrating its involvement in transcriptional events relating to steroid/nuclear receptor signalling [350-352, 362]. This evidence is compounded by the fact that TSC patients arising through TSC2 mutations tend to present with a more severe phenotype than those with TSC1 mutations [363]. This has implications particularly for TSC patients as current clinical trials are utilising mTOR inhibitors by means of potential treatment. Such therapy would be ineffective at treating symptoms within TSC patients arising from mTOR independent functions of TSC1/2.

In chapter 3, it was demonstrated that re-introduction of TSC2 back into TSC2-deficient cells was significantly more effective at inhibiting HIF-1α than the potent mTORC1 inhibitor rapamycin, suggesting an additional role for TSC2 in the modulation of HIF-1α. TSC patients develop highly vascularised tumours and demonstrate high levels of VEGF secretions [135, 364] where the mTORC1-independent mechanism(s) relating to loss of TSC2 function may be a contributing factor to these effects. Previous work suggested that TSC2 may regulate VEGF via mTORC1-independent mechanisms, however it was thought that this was independent of HIF-1α [365]. I utilise TSC2 mutational analysis in conjunction with both TSC1-deficient and TSC2-deficient cell lines to attempt to further characterise mTORC1-dependent and independent regulation of HIF-1α.

4.2 METHODS

4.2.1 HIF-1α Luciferase Assays

TSC2-/- MEFs were co-transfected with the HIF-1α luciferase reporter (purchased from Panomics Ltd.) alongside empty vector, wild-type or mutant TSC2. Cell were cultured under serum starved conditions under hypoxia or normoxia for 12 h prior to lysis and treated with rapamycin where indicated. Cells were lysed as described in section 2.3.4.3 with Blenis lysis buffer supplemented with protease inhibitors. 100 µl of lysate was extracted for protein detection (where indicated) via western blotting

and each sample was sonicated for three cycles of 5 s on maximum power (30microns) incubating on ice in between sonications. Lysates were then subjected to high centrifugation for 2 min at 4 °C, diluted in Invitrogen SDS-page sample buffer and subjected to separation by SDS-PAGE using the 3-8% novex gel system from Invitrogen.

Luciferase assays carried out as previously described in section 2.3.19, results shown are either comprised of or representative of three independent experiments (where indicated)

4.2.2 Quantitative-PCR

Cells were lysed in RNA protect lysis buffer then pelleted at 5,000 rpm for 5 min before mRNA extraction using Qiagen mRNA extraction kit in accordance with manufacturers protocol. 1 µg of each mRNA extraction was converted to cDNA using Qiagen reverse-transcriptase kit and analysed as described in section 2.3.21. Each cDNA was analysed in triplicate for β-actin, VEGF, BNIP3 or HIF-1α and fold difference was calculated using the ddCT (delta-delta-Ct) method (results standardised to β-actin). Results are comprised of three independent experiments.

4.2.3 S6K1 kinase assay of TSC1-/- and TSC2-/- MEFs

Cells were transferred to serum free media containing 1 mM DMOG and rapamycin treated overnight before lysis in Blenis lysis buffer (see section 2.2) supplemented with protease inhibitors. S6K1 kinase assay and HIF-1α luciferase assay were carried out as described in section 2.3.17 and 2.3.19 respectively.

4.2.4 GTPase-Activating Protein Assay

See section 2.3.18. Dr. Andrew Tee assisted with these assays.

4.2.5 Nuclear and Cytoplasmic Fractioning

Localisation of TSC2 was analysed after TSC2-/- MEFs expressing either pRK7, wild-type TSC2 or TSC2-R1743G were lysed in PBS supplemented with protease inhibitors then subjected to nuclear and cytoplasmic fractioning as described in section 2.3.6. Nuclear and cytoplasmic fractions were analysed with western blotting for detection of TSC2 expression using the Novex gel system as previously described (see section 2.3.8).

4.2.6 Immunohistochemistry

Kidney samples extracted from TSC1 and TSC2 heterozygous mice and embedded in formaldehyde blocks were kindly donated from Prof. Jeremy Cheadle's Laboratory, 30 x 0.4 μM sections were taken from 3 different mice of each genotype and staining for VEGF was carried out as described in section 2.3.22 by Cardiff University Central Biotechnology Services. Pictures were taken using light microscopy to visualise the sections.

4.3 Results

4.3.1 Rapamycin treatment of TSC2-/- only results in partial inhibition of HIF-1α transcriptional activity

The initial stage of this line of investigation was to analyse the transcriptional activity of HIF-1α in the context of cell line models for TSC. TSC2-/- MEFs were first utilised to examine changes of HIF-1α activity in the presence or absence of TSC2. This was carried out in both hypoxic and normoxic conditions to establish whether the effect was mediated by O_2 levels. The effects of rapamycin treatment upon this activity were also examined. These cell-lines are particularly useful for specifically dissecting HIF-1α signalling as HIF-2α is not transcriptionally active in MEFs and therefore the hypoxic response is mediated via HIF-1α only [348, 363]. Confirming this, I did not observe HIF-2α expression in these cells (data not shown).

The data clearly demonstrates that HIF-1α activity is enhanced in the absence of TSC2 with an almost 10-fold increase in activity (compare lanes 5 and 7 of figure 4.1). Transfection of TSC2 back into these cells resulted in an average 89.7% reduction in HIF-1α activity, this result was highly significant with a p-value of 0.001 (compare lanes 5 and 7). Rapamycin treatment however only caused an average reduction of 61.1% (p-value 0.001). HIF-1α activity was significantly higher in rapamycin treated TSC2-/- cells demonstrating at least an average three-fold increase over the rapamycin untreated cells expressing TSC2 (p-value 0.05, compare lanes 5 and 8).

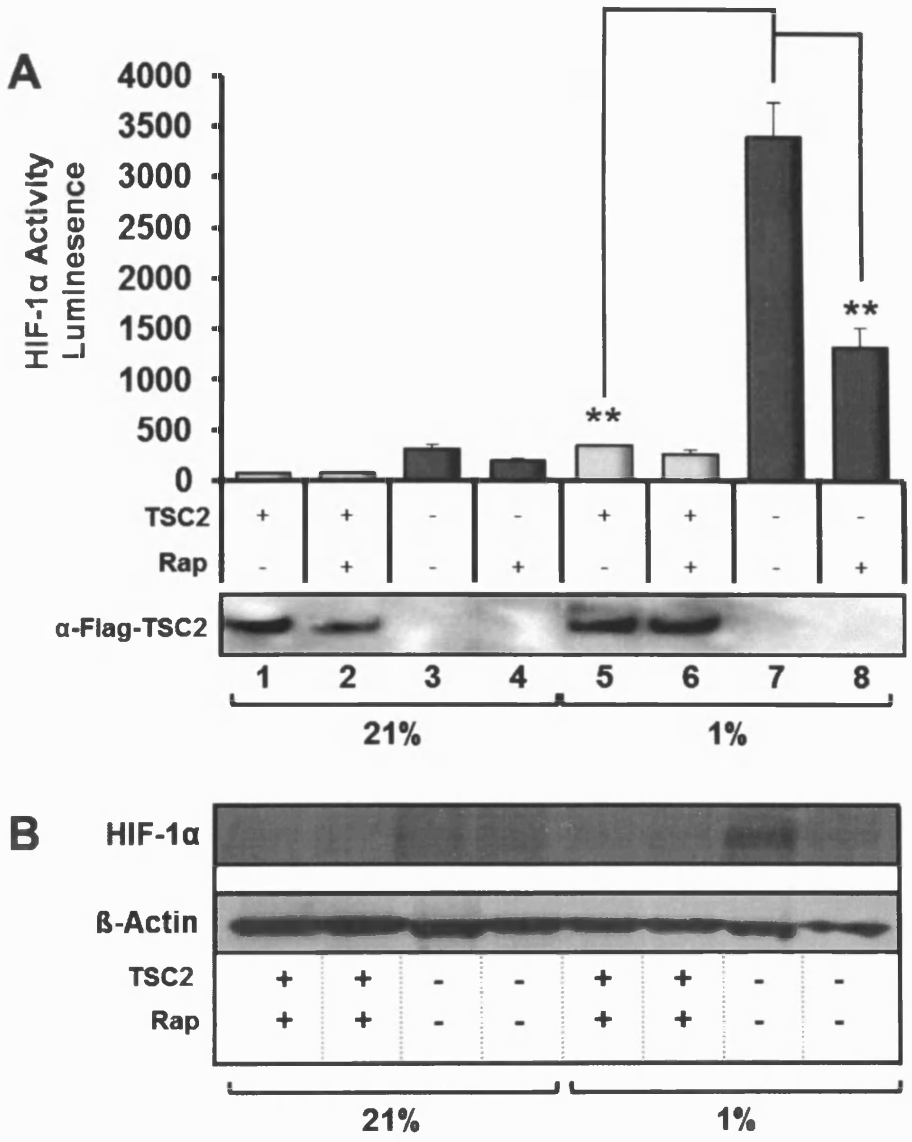


Figure 4.1: Rapamycin treatment of TSC2-/- only results in partial inhibition of HIF-1α transcriptional activity (A) TSC2-/- MEFs were transfected with the HIF-1α-inducible luciferase reporter (Panomics) alongside either wild-type TSC2 or empty vector. Cells were serum starved and cultured under hypoxic (1%) or normoxic (21%) conditions in the presence and absence of rapamycin as indicated. Cells were then harvested in Blenis lysis buffer supplemented with protease inhibitors and total lysate was analysed for HIF-1α transcriptional activity. Results are representative of three independent experiments, and standardised to total protein levels as determined by a Bradford assay. Error bars indicative of standard deviation from three independent experiments. * indicates p-value significance <0.05, ** indicates significance <0.001, NS indicates not significant. **(B)** Untransfected TSC2-/- MEFs and their wild-type counterparts were cultured under same conditions described in 'A', however cells were harvested directly in sample buffer, sonicated and then analysed for HIF-1α protein levels and β-actin as a loading control.

Analysis of HIF-1α protein levels in the TSC2-/- MEFs compared to the wild-types (figure 4.1 'B') demonstrates that HIF-1α protein levels appear to be highly sensitive to inhibition with rapamycin, consistent with the results of chapter 3 indicating that mTORC1 regulates the translation of HIF-1α mRNA. Interestingly the more sensitive transcriptional assay reveals that under hypoxia, HIF-1α transcriptional activity is still elevated after rapamycin treatment in the TSC2-/- MEFs when compared with the wild-types (compare lanes 5 and 8). The continued elevation of HIF-1α activity in the presence of rapamycin suggests that TSC2 is able to regulate HIF-1α independently of mTORC1.

4.3.2 TSC2-/- MEFs are less sensitive to rapamycin inhibition of HIF-1α mediated gene expression than TSC1-/- MEFs

After demonstrating that TSC2 re-introduction could suppress HIF-1α activity to a greater extent than rapamycin inhibition, it was important to confirm that this was not a consequence of over-expressing TSC2 to a level that would be unphysiological. I also wanted to clarify whether specific loss of the TSC2 protein (and not the TSC1 protein) was incurring mTORC1-independent inhibition of HIF-1α. Finally, I needed to confirm that this increased activity of HIF-1α translated to elevated gene-expression of HIF-1α targets. To address these points, q-PCR was utilised to analyse the gene-expression of two HIF-1α targets, VEGF-A and BNIP3.

The data includes a comparison of the gene-expression of these targets in both TSC1- and TSC2-deficient MEFs, in comparison to their wild-type counterparts. VEGF-A was selected as a target due to its association with pathogenic angiogenesis in TSC patients. BNIP3 (Bcl-2/adenovirus E1B 19-kDa interacting protein 3) was also selected as a HIF-1α target. BNIP3 is a member of the Bcl-2 pro-apoptotic family. Although traditionally BNIP3 was thought to regulate apoptosis, it has more recently been demonstrated to induce autophagy and necrosis (see review [366]). Shackleford *et al.* demonstrated that BNIP3 is upregulated in an LKB1 mouse model of Peutz-Jeghers syndrome in a rapamycin sensitive manner and it is therefore appropriate for this study [286].

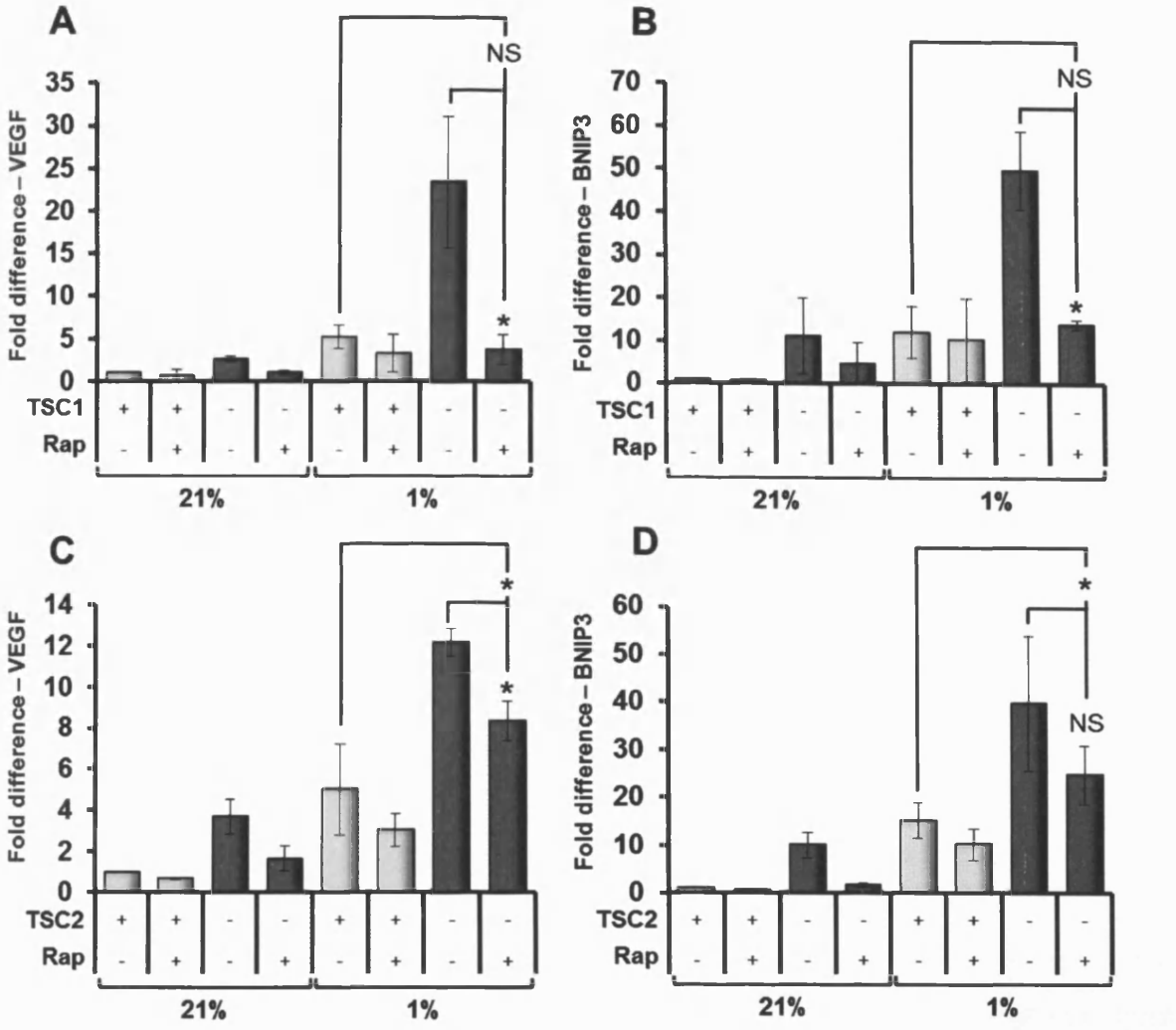


Figure 4.2 MEFs are less sensitive to rapamycin inhibition of HIF-1α mediated gene expression than TSC1-/- MEFs: TSC1-/- (A and B) or TSC2-/- MEFs (C and D) and their wild-type counterparts were cultured under serum starved conditions under normoxia (21% O_2) or hypoxia (1% O_2) for 12 h in the presence and absence of rapamycin. Cells were then lysed in RNA protect buffer, mRNA was extracted, VEGF or BNIP3 mRNA levels were quantified using SYBR green detection as described in section 2.3.21. Fold difference was calculated using the ddCT method. Error bars indicative of standard deviation from three independent experiments. * indicates p-value significance <0.05, ** indicates significance <0.001, NS indicates not significant.

It is notable at this point that BNIP3 also plays a role in the suppression of mTORC1 under hypoxia by interacting with Rheb, which leads to a reduction in the phosphorylation of 4E-BPs and S6Ks (see section 1.4.5.4) [146]. Paradoxically, it may actually contribute to the survival of TSC-deficient cells by suppression of mTORC1 signalling and induction of autophagy under additional hypoxic stress.

Gene expression was analysed under both hypoxic and normoxic conditions and in the presence and absence of rapamycin. Figure 4.2 'A' and 'B' demonstrate the gene-expression of VEGF-A and BNIP3, respectively in the TSC1-/- MEFs and their wild-type counterparts. Both HIF-1α target genes were upregulated by hypoxia as expected and were further augmented by TSC1 loss. The upregulation of HIF-1α activity seen in the TSC1-/- cells was highly sensitive to rapamycin with VEGF-A expression being inhibited by an average of 84.9%. BNIP3 showed an average reduction of 72.9% after rapamycin treatment (compare lanes 8 and 9 on both figure A and B).

Statistical analysis revealed that there was no significant difference in VEGF-A or BNIP3 induction when comparing the untreated TSC1+/+ cells and the rapamycin treated TSC1-/- cells under hypoxia. Therefore as expected, rapamycin normalises the elevation of HIF-1α activity induced by TSC1 deficiency. In contrast, this was not the case in the TSC2-/- cell lines. VEGF-A induction was elevated in the absence of TSC2 (under hypoxia) and although it was subject to inhibition from rapamycin, rapamycin caused an average inhibition of 31.6% (compared with 84.9% in the case of the TSC1-/- cells), giving a p-value of 0.05. BNIP3 was reduced by an average 32.4% with rapamycin treatment and this reduction was not statistically significant. Consistent with the transcriptional assay shown in figure 4.1, VEGF-A levels were significantly higher in rapamycin treated TSC2-/- MEFs than in the untreated TSC2+/+ MEFs giving a p-value of 0.05. Although intial statistical analysis indicated that BNIP3 was also higher in rapamycin treated TSC2-/- MEFs than in the untreated TSC2+/+ MEFs, once the data had been corrected for multiple testing using the one-way anova, the significant difference was lost. Although a similar trend is apparent therefore this is likely to be due to the increased experimental variation between the BNIP3 and VEGF-A analysis. . The VEGF-A Q-PCR and luciferase assays however still support the conclusion that rapamycin treatment normalises HIF-1α activity in the absence of TSC1 but not in the absence of TSC2. This suggests that in the TSC1-/- cells, the enhanced activation of HIF-1α induced gene

expression observed is primarily through enhanced mTORC1 signalling. In the TSC2-/- MEFs, elevation of mTORC1 certainly accounts for a substantial increase in HIF-1α activity, however loss of TSC2 appears to cause an increase in HIF-1α activity which is also independent from mTORC1.

4.3.3 TSC1-/- MEFs exhibit similar mTORC1 activity and sensitivity to rapamycin as TSC2-/- MEFs

After demonstrating that there appeared to be differences in HIF-1α activity and rapamycin sensitivity between the TSC1 and TSC2-/- MEFs it was important to confirm that the differences in HIF-1α sensitivity to rapamycin was not due to differences in mTORC1 signalling between these cell types. To access the mTORC1 activity in these cells, an S6K1 kinase assay was implemented alongside the HIF-1α reporter assay. Total lysates were analysed for both the activity of HIF-1α and the phosphorylation of ribosomal protein S6 at Ser235/236 in the presence of rapamycin. In addition to this, HA-S6K1 was immunoprecipitated from the total lysate and its kinase activity measured towards recombinant ribosomal protein S6 *in vitro.*

Results demonstrate once again that the TSC1-/- MEFs were significantly more responsive to rapamycin induced down-regulation of HIF-1α activity than TSC2-/- MEFs (p-value 0.05) – figure 4.3 'A'. Importantly, this experiment also confirms that the treatment of these cells lines with rapamycin was sufficient to block mTORC1 signalling, as observed by reduced S6K1 activity and rpS6 phosphorylation in rapamycin treated cells. This indicates that the TSC1-/- MEFs and TSC2-/- MEFs are equally as sensitive to rapamycin-mediated inhibition of mTORC1 signal transduction. They are not however equally sensitive to rapamycin induced suppression of HIF-1α, adding further support to the hypothesis that TSC2 can also act independently of TSC1 and mTORC1 to regulate HIF-1α transcriptional activity.

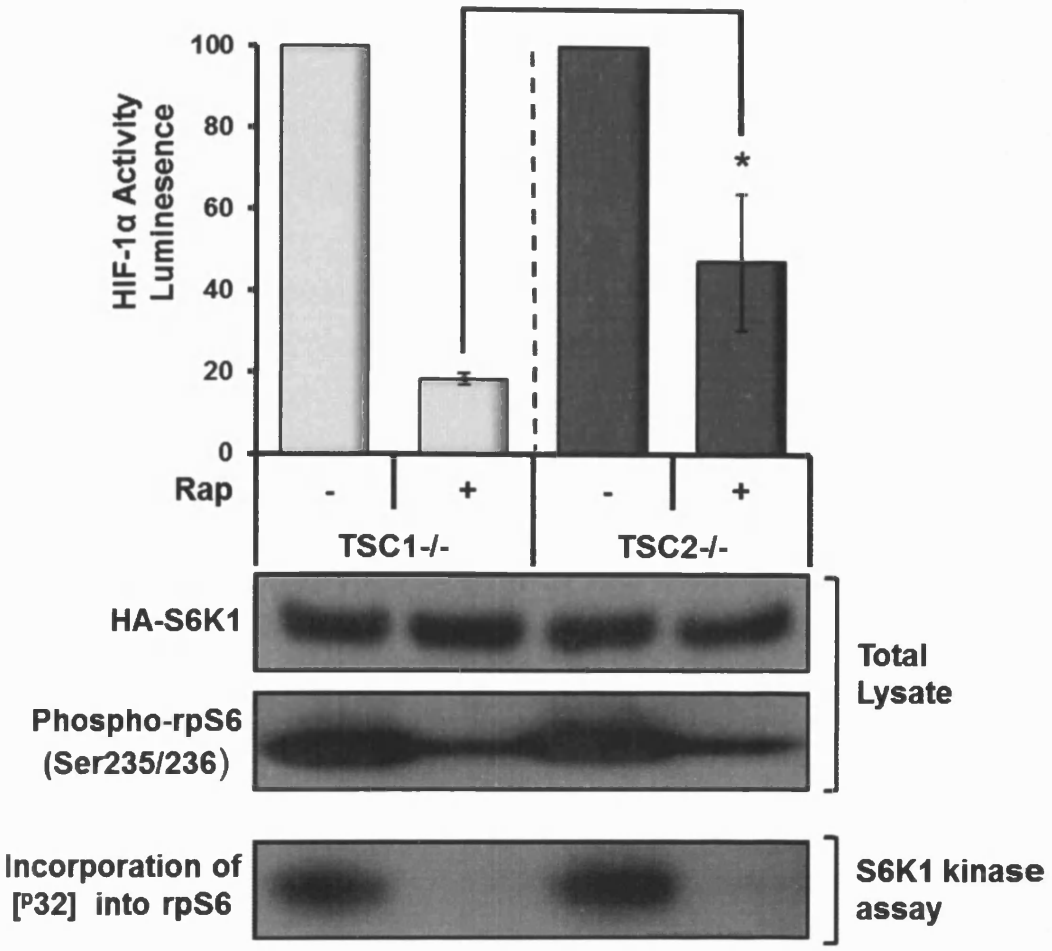


Figure 4.3 TSC1-/- MEFs exhibit similar mTORC1 activity and sensitivity to rapamycin as TSC2-/- MEFs TSC1-/- MEFs and TSC2-/- MEFs were transfected with HA-S6K1 alongside the HIF-1α luciferase reporter construct. Cells were cultured in the presence and absence of rapamycin in serum free media supplemented with 1 mM DMOG. Cells were lysed in Blenis lysis buffer supplemented with protease inhibitors. Total lysate was analysed for HIF-1α transcriptional activity which was standardised to total protein levels as determined by the Bradford assay. Phospho-rpS6 levels and total HA-S6K1 expression was determined by western blotting. The remaining lysate was subjected to an HA-immunoprecipitation and radioactive S6K1 kinase assay utilising purified recombinant GST-rpS6 as a substrate. Error bars indicative of standard deviation from three independent experiments. * indicates p-value significance <0.05.

4.3.4 TSC2 mutational analysis

The disease TSC results in the development of benign tumours across multiple organ systems including the brain, heart, kidneys, lungs and skin. Some TSC patients present with severe autism, whereas some patients may suffer no neurological impairments. The development and incidence of the tumours are highly variable and thus the severity of the disease is equally variable. It is thought that the severity of the disease may be affected by positioning of the genetic mutation to either the TSC1 or TSC2 genes. The Uniprot website lists over 50 different naturally occurring point mutations within the TSC2 gene alone which have been reported to cause TSC. It has therefore been speculated that the position of the mutation may be a determining factor in the penetrance of the disease. Since mutation to the TSC2 gene tends to manifest with more severe presentation of the disease than with TSC1 mutations [367], I postulated that this may be contributed in part by loss of TSC2 - mediated mTORC1 independent inhibition of HIF-1α.

The next stage of this study was to determine what impact TSC2 mutations could have on the modulation of HIF-1α. In order to do this, Dr. Mark Nellist (Erasmus Medical Centre) kindly provided a panel of mutants of TSC2 constructs, containing mutations within various conserved domains along the gene. I utilised the TSC2-/- MEFs to determine what how effective these disease causing mutations were at suppressing HIF-1α activity.

Results showed a good deal of variation in the degree of inhibition of HIF-1α, indicating that the R98W, L340P, N525S and K599M mutants were all able to inhibit HIF-1α activity to a level which was not significantly different to wild-type (see figure 4,.4 'B'). Conversely, there are reports in the literature that the R98W mutant is less able to repress S6K1 activity in comparison to wild-type [368]. Nellist's research group also identified the L340P mutant as being pathogenic and consistent with this, the L340P mutant is unable to suppress Thr389 phosphorylation of S6K1 (unpublished data – personal communication). It is likely that these discrepancies are caused through differences in TSC2 expression levels as these studies utilised different expression vectors. From my earlier studies using the pcDNA3.1/Flag-TSC2 vector I observed that the Flag-TSC2 expressed weakly, approximately three times lower than was seen with these TSC2 mutant constructs.

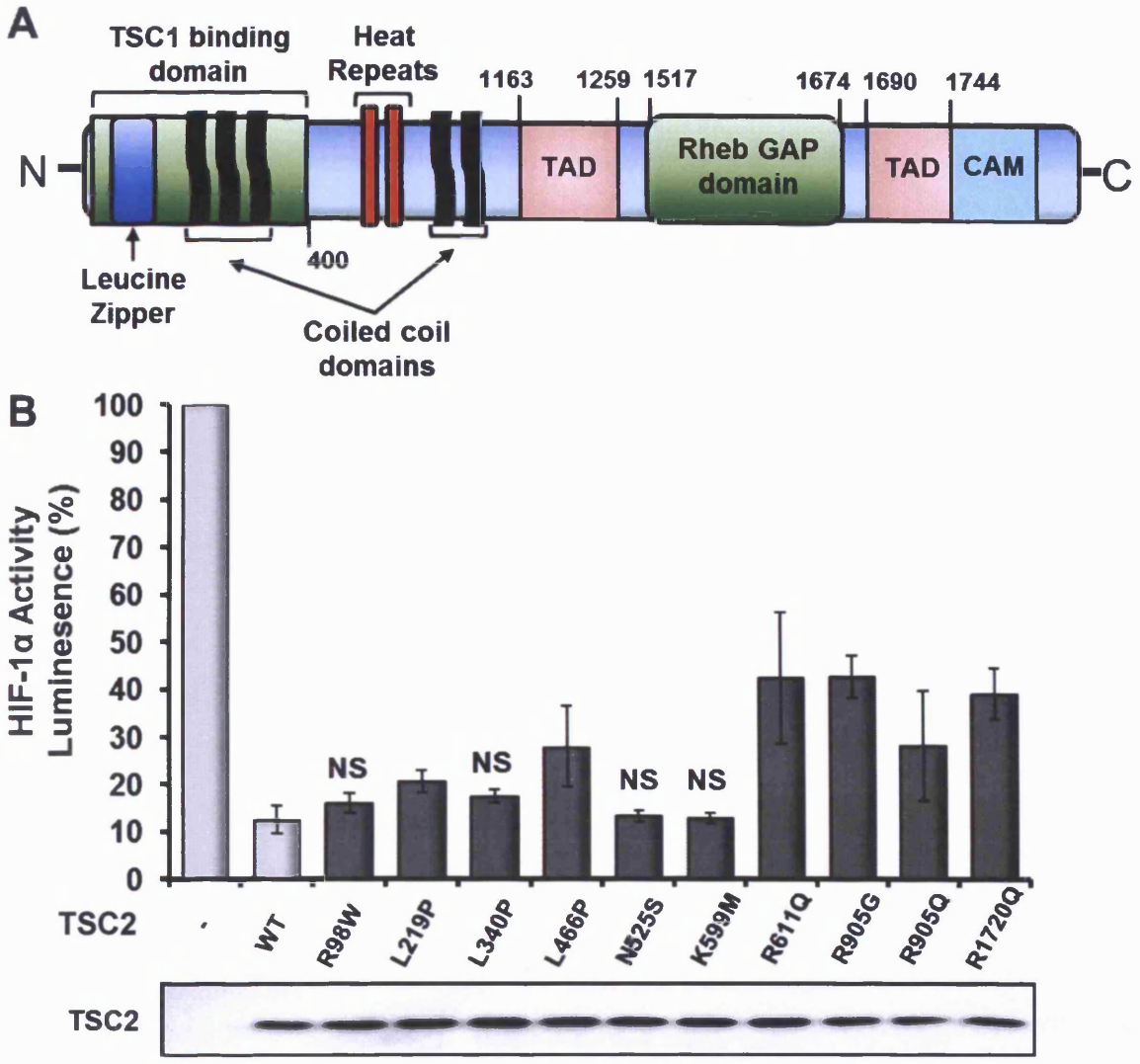
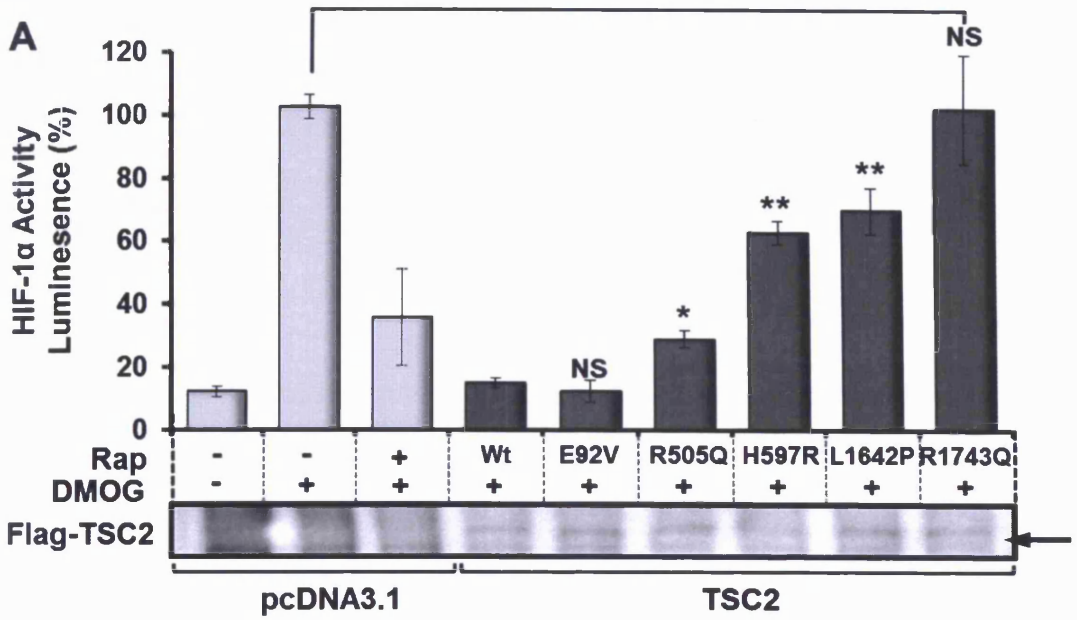


Figure 4.4 TSC2 mutational analysis: A: Schematic of TSC2 demonstrating known functional and conserved domains. **B:** TSC2-/- MEFs were transfected with the HIF-1α luciferase reporter construct alongside empty vector, wild-type TSC2 or mutant TSC2. Cells were cultured under serum starved conditions in media supplemented with 1 mM DMOG for 12 h prior to lysis. Cells were harvested in Blenis lysis buffer and analysed for HIF-1α transcriptional activity, this was standardised to total protein levels as determined by a Bradford assay. The remainder of the lysate was analysed for TSC2 expression levels by western blotting. Error bars indicative of standard deviation from three independent experiments. NS indicates 'Not significantly different to wild-type.

TSC2 mutants provided by Mark Nellist were generated into the original but discontinued pcDNA3 expression vector. Western blotting of TSC2 levels confirmed that the mutants were expressing at a much higher level. See TSC2 expression blot (figure 4.4 'B'), whereby the control blot shown only required a 10 second exposure after ECL Furthermore the R1720Q mutation, which has previously been shown to be pathogenic and completely ineffective at inhibiting downstream mTORC1 substrates [368], caused a 60.6% inhibition of HIF-1α. It is probable that therefore these TSC2 mutants are expressing at a higher level which is not physiologically relevant, this may be causing artificial inhibition of HIF-1α due to excess protein levels. In order to confirm this, a second panel of mutations was analysed which were cloned into the pcDNA3.1 expression vector to express the TSC2 protein at a lower level.

4.3.5 Point mutations to TSC2 confer differential abilities to inhibit HIF-1α when TSC2 is expressed at a lower and more physiological level

Once again, mutational analysis was carried out to analyse the inhibitory effects of TSC2 towards HIF-1α. These particular TSC2 missense variants were selected from a database of mutations identified in patient samples which were referred to the clinical genetics service laboratory in Medical Genetics, Cardiff University (with the exception of the wild-type TSC2 vector and the R1473Q mutant which were kind donations from Prof. Cheryl Walker). Five different patient derived mutations were selected from various domains within the TSC2 gene and introduced into the Flag tagged wild-type TSC2 construct using site directed mutagenesis (site-directed mutagenesis work carried out by Dr Elaine Dunlop). Figure 4.4 'A' indicates the conserved regions of TSC2. To analyse the effects of these mutations, TSC2 mutant constructs were once again transfected into TSC2-/- MEFs alongside the HIF-1α transcriptional reporter. An additional rapamycin treated condition was included so that the inhibition levels could be compared as a control. Western blotting analysis revealed that these mutants were expressing at much lower levels making the results more physiologically relevant unlike the previous experimentation (figure 4.4) where the TSC2 protein was grossly over-expressed. As figure 4.5 shows, the results were much more comparable to the empty vector sample than was seen in figure 4.4. This is likely to be due to the reduced expression levels.



B

Amino Acid Change	Functional region	Polyphen prediction	SIFT prediction
E92V	Leucine zipper (TSC1 binding domain).	Possibly damaging	Affects protein
R505Q	Conserved	Benign	Tolerated
H597R	Conserved	Probably damaging	Affects protein
L1624P	Rheb GAP domain	Probably damaging	Affects protein
R1743Q	TAD/CAM	Benign	Affects protein

Figure 4.5 Point mutations to TSC2 confer differential abilities to inhibit HIF-1α when TSC2 is expressed at a physiological lower level A: TSC2-/- MEFs were transfected with the HIF-1α luciferase reporter construct alongside empty vector, wild-type TSC2 or mutant TSC2. Cells were cultured under serum starved conditions in media supplemented with 1 mM DMOG for 12 h prior to lysis. Cells were harvested in Blenis lysis buffer and analysed for HIF-1α transcriptional activity, this was standardised to total protein levels as determined by a Bradford assay. The remainder of the lysate was analysed for TSC2 expression levels by western blotting. Error bars indicative of standard deviation from three independent experiments. NS indicates Not significantly different from wild-type. * indicates p-value of 0.05, ** indicates p-value of 0.001. **B:** Chart detailing variants examined, Polyphen and SIFT programmes were used to indicate predictive effects of mutations upon protein function. Arrow indicates WT/TSC2 mutant construct expression.

Interestingly, the patient derived mutations did not exhibit an on/off effect of inhibition, instead, many of the mutants were able to inhibit HIF-1α to varying degrees with the exception of the R1473Q mutant which will be discussed in more detail later.

The E92V mutant was able to inhibit HIF-1α activity to the same degree of wild-type TSC2 and statistical analysis confirmed that there was no significant difference between the average inhibition from wild-type and E92V indicating that the E92V mutation may not be pathogenic. Polyphen analysis indicated that the mutation maybe 'possibly damaging' and SIFT analysis indicated that the protein function would be affected (see figure 4.5 'B') however it is clear from figure 4.5 'A' that this E92V mutant is fully functional at rescuing the heightened activity of HIF-1α activity in these TSC2-/- MEFs.

Each mutant of TSC2 produced a different level of inhibition of HIF-1α, which interestingly gives insight into the variation upon function a single point mutation can have to the TSC2 gene. This mutational analysis also revealed that the R1743Q mutant was unable to suppress HIF-1α transcriptional activity. This mutant is equivalent to the R1720Q mutant analysed in figure 4.4 and therefore confirms that expression of the mutants at more physiological levels makes the inhibition levels much more comparable to empty vector. Work by Dunlop *et al.* demonstrated that both the L1624P mutant and the H597R mutant had no GAP activity towards Rheb *in vitro* and were unable to suppress phosphorylation of S6K1 or 4E-BP1 *in vivo*. However, both mutants suppressed HIF-1α transcriptional activity by an average of 31.7% and 30.1% respectively. This supports the hypothesis that TSC2 may be able to influence the transcriptional activity of HIF-1α independently of mTORC1.

Interestingly the R1743Q variant is completely non-functional at suppressing HIF-1α. The R1743Q mutation is situated within the Ca^{2+}-dependent calmodulin (CaM) binding domain of TSC2 which interestingly overlaps a transcriptional activation domain (TAD), see figure 4.4 'A'. This region has previously been identified by Noonan *et al.* as essential for TSC2 mediation of transcriptional events relating to steroid hormone receptors [351]. Later work indicated that the oestrogen receptor binds directly to TSC2 via this domain which enables its regulation at a transcriptional level [350]. This could suggest that the Arg1743 residue of TSC2 is involved in mTORC1 independent modulation of HIF-1α transcriptional activity.

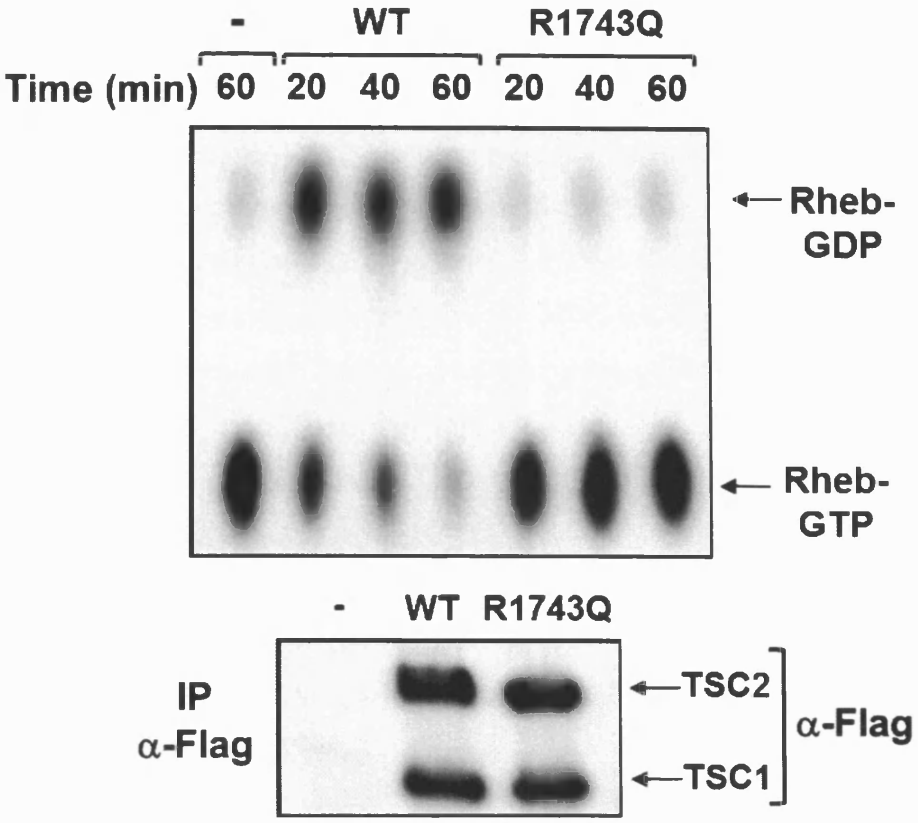


Figure 4.6 R1743Q-TSC2 has no GAP activity towards Rheb: TSC1/2 complexes were purified from HEK 293 cells transfected with pRK7, Flag-TSC1, Flag-TSC2, or Flag-TSC2-R1743Q. 16 h after transfection, cells were treated with 100 nM wortmannin for 15 min before lysis in NP-40 lysis buffer. Flag-tagged proteins were then immunoprecipitated for 2 h with 80 µl of an M2-agarose affinity gel slurry. Immune complexes on beads were washed three times in IP wash buffer and once in 1 ml Rheb exchange buffer supplemented with protease inhibitors. The washed beads were then separated into four aliquots. Three of these were used for separate GAP assays, and one was resolved by SDS-PAGE and immunoblotted to determine protein levels. GST-Rheb (10 µg) was loaded with 100 µCi [α-^{32}P]-GTP or 10 mM GDP. GAP assays were initiated by the addition of 20 µl GTP-loaded Rheb (approximately 1 µg GST-Rheb) to each aliquot of M2-agarose immune complexes described above. Assays were performed at room temperature with constant agitation for 20, 40, or 60 min. Reactions were stopped by the addition of 300 µl Rheb wash buffer containing 1 mg/ml BSA. M2-agarose immune complexes were removed by brief centrifugation, and nucleotide bound GST-Rheb was purified from the supernatant with 20 µl of a protein G slurry. After three washes with Rheb wash buffer, radiolabeled GTP and GDP were eluted from Rheb with 20 µl Rheb elution buffer at 68°C for 20 min. Aliquots (1 µl) of each eluted reaction were resolved by thin-layer chromatography on PEI cellulose (Sigma) with KH_2PO_4 as the solvent.

4.3.6 R1743Q-TSC2 has no GAP activity towards Rheb

Since Dunlop *et al.* did not analyse the R1743Q variant it was important to determine whether it was defective at repressing mTORC1 signalling as seen with the L1460P and H597R variants. The R1743Q mutant was therefore assessed in terms of its status as a GAP towards Rheb using an *in vitro* GAP assay (GAP assays assisted by Dr. Andrew Tee). As figure 4.6 demonstrates, Rheb stayed predominantly in its GTP-bound state during incubation with the R1743Q mutant. This indicates that the purified TSC2-R1743Q mutant protein has no measurable GAP activity towards Rheb *in vitro*. If HIF-1α was being regulated downstream of mTORC1 alone, then you would expect to see similar levels of inhibition with L1460P, H597R and R1743Q since they all show similar defects in their GAP activity towards Rheb. As figure 4.5 'B' indicates, the R1743Q mutant is not able to inhibit HIF-1α whereas expression of both the H597R and L1460P mutants results in significant inhibition of HIF-1α (p-value 0.05). This adds further support to the hypothesis that TSC2 can impact HIF-1α activity independently of mTORC1.

4.3.7 The CaM-binding domain/TAD of TSC2 is necessary for direct TSC2 mediated inhibition of HIF-1α

There are two reports within the Uniprot database concerning additional mutations within the Arg1743 site. SIFT analysis predicts mutation at that particular site to 'affect protein function' and POLYPHEN suggests that mutation to this site to be 'benign' (see figure 4.5 'B'). I demonstrated that a glutamine substitution of arginine at position 1743 abolishes the GAP activity of TSC2. On the Uniprot website, R1743Q is a known variant but there is limited information upon the effects of this point mutation. Prof. Cheryl Walker's research group was also investigating these mutants but in the context of their localisation. They kindly provided three TSC2 R1743X mutant constructs for analysis. These mutants were assayed for HIF-1α activity in the same manner to confirm the importance of this Arg1743 residue within the CaM binding domain of TSC2 with respect to HIF-1α regulation. As expected, none of the three Arg1743 site mutants were able to inhibit HIF-1α and the effect of introducing these mutants to the TSC2-/- MEFs was not significantly different to introducing empty vector. The GAP mutant of TSC2 (L1462P) however was able to inhibit HIF-1α activity by an average of 39.7% (p-value 0.05) despite its lack of ability to inhibit mTORC1 as was shown by Dunlop *et al.* [326]

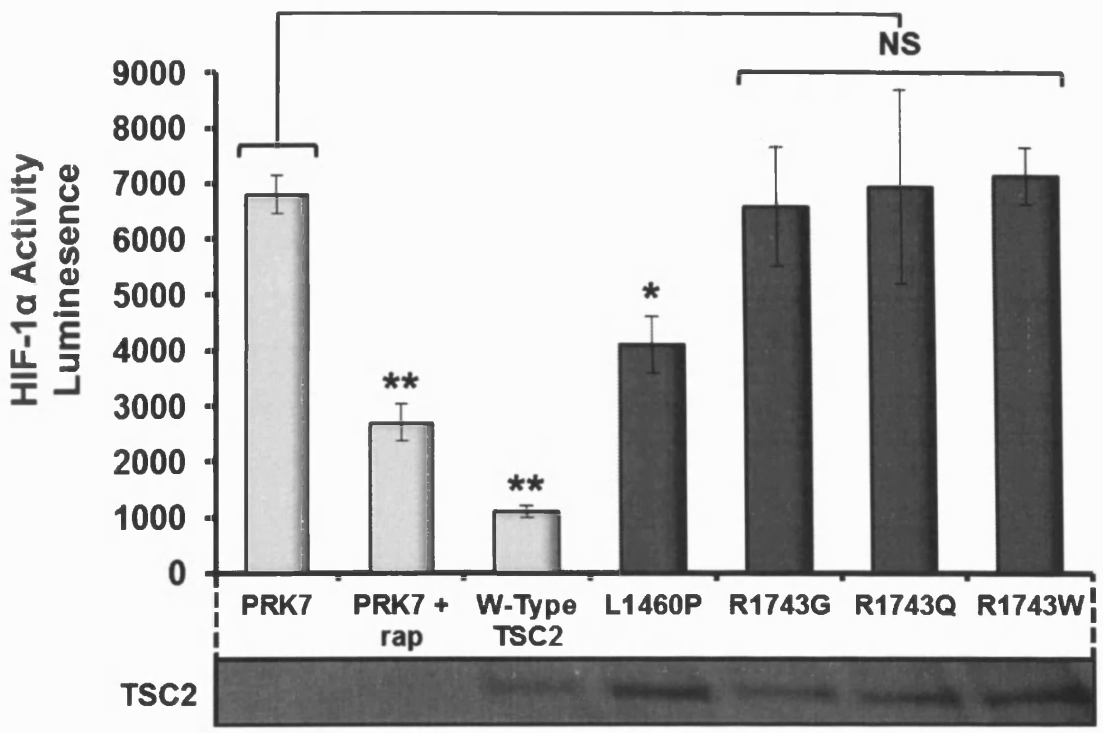


Figure 4.7 The CaM-binding domain/TAD of TSC2 is necessary for direct TSC2 mediated inhibition of HIF-1α TSC2-/- MEFs were transfected with the HIF-1α luciferase reporter construct alongside empty vector, wild-type TSC2 or mutant TSC2. Cells were cultured under serum starved conditions in media supplemented with 1 mM DMOG for 12 h prior to lysis. Cells were harvested in Blenis lysis buffer and analysed for HIF-1α transcriptional activity, this was standardised to total protein levels as determined by a Bradford assay. The remainder of the lysate was analysed for TSC2 expression levels by western blotting. Error bars indicative of standard deviation from three independent experiments. NS indicates Not significantly different from wild-type. * indicates p-value of 0.05, ** indicates p-value of 0.001. PRK7 indicates empty vector transfection control.

This suggests that the Arg1743 residue is critical for HIF-1α regulation, It is likely from this data that no substitution can be tolerated at this site, signifying the importance of this residue in the functioning of TSC2.

4.3.8 Nuclear localisation is not significantly altered by mutation at the Arg1743 site

It is well documented that TSC2 can translocate to the nucleus upon serum withdrawal where it interacts with and modulates the activity of several steroid receptor family members [350-352, 362, 369, 370]. The results of this study indicate that this regulation may extend to HIF-1α as well. So far I have demonstrated the significance of the Arg1743 residue in this activity, intriguingly, Arg1743 is the first amino acid of what York *et al.* identified as being a nuclear localisation sequence. Given that activated HIF-1α translocates to the nucleus (see section 1.5.3) it may be possible that TSC2 is able to regulate HIF-1α within the nucleus as is seen with other transcription factors. I hypothesised that the Arg1743 mutants may therefore have lost their ability to translocate to the nucleus and hence were unable to suppress HIF-1α.

To investigate this, TSC2-/- MEFs were transfected with the wild-type TSC2 or the mutant construct and cultured under serum starved condition. Nuclear and cytoplasmic fractions were prepared and western blotting was utilised to confirm the localisation of TSC2. However it is clear from figure 4.8 that the R1743G mutant has retained its ability to translocate to the nucleus. This confirms that the mutation to the Arg1743 site does not impair TSC2 nuclear localisation and the hypothesis is rejected.

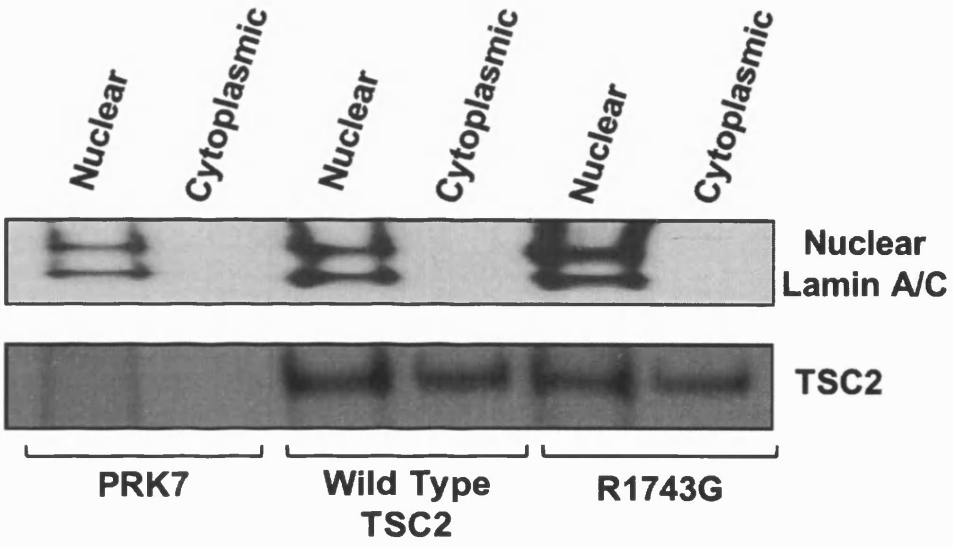


Figure 4.8 Nuclear localisation is not significantly altered by mutation at the Arg1743 site: TSC2-/- MEFs were transfected with either empty vector, wild-type or mutant TSC2 and cultured under serum starved conditions. Cells were then lysed in PBS and nuclear and cytoplasmic fractions were generated as described in section 2.3.6. Western blotting was then utilised to determine the localisation of TSC2.

4.3.9 Immunohistochemical analysis of HIF-1α targets

This study has so far demonstrated that rapamycin is able to normalise HIF-1α activity and VEGF expression in the absence of TSC1 but not TSC2. This suggests that TSC patients with TSC2 mutations may be less responsive to rapamycin treatment and may also exhibit higher levels of vascularisation than those with TSC1 mutations. This is consistent with a more severe phenotype which appears to present with patients exhibiting TSC2 mutations. To investigate this further, immunohistochemical techniques were employed to see if there was differential expression of HIF-1α targets between TSC1 and TSC2 heterozygous mice.

TSC mouse models were engineered in Prof. David Kwiatowski's lab to generate mice with heterogenous inactivating mutations to either the TSC1 or TSC2 genes [331, 371]. Kidney samples extracted from these mice which had been embedded in paraffin blocks were kindly donated by Prof. Jeremy Cheadle's laboratory. Sections were taken from both TSC1-/+ and TSC2 -/+ mice and then stained with VEGF-A antibodies.

Results demonstrated a fair degree of peripheral staining of the tubules which is to be expected as VEGF-A is thought to play a role in the maintenance of glomerular capillary endothelial fenestrations [372]. Unfortunately as figure 4.9 demonstrates, staining of VEGF-A was highly variable between samples of the same genotype. It was, therefore, not possible to make justified comparisons between them. It is likely that the variable staining is a result of varying degrees of hypoxia within each region of the tissue. This may have produced the localised spots seen randomly across both genotypes (arrows in figure 4.9 indicate VEGF staining hotspots) or it may be indicating areas where mTORC1 activity is increased. However, due to the variable nature of the results, it was decided that this route of enquiry would not be continued.

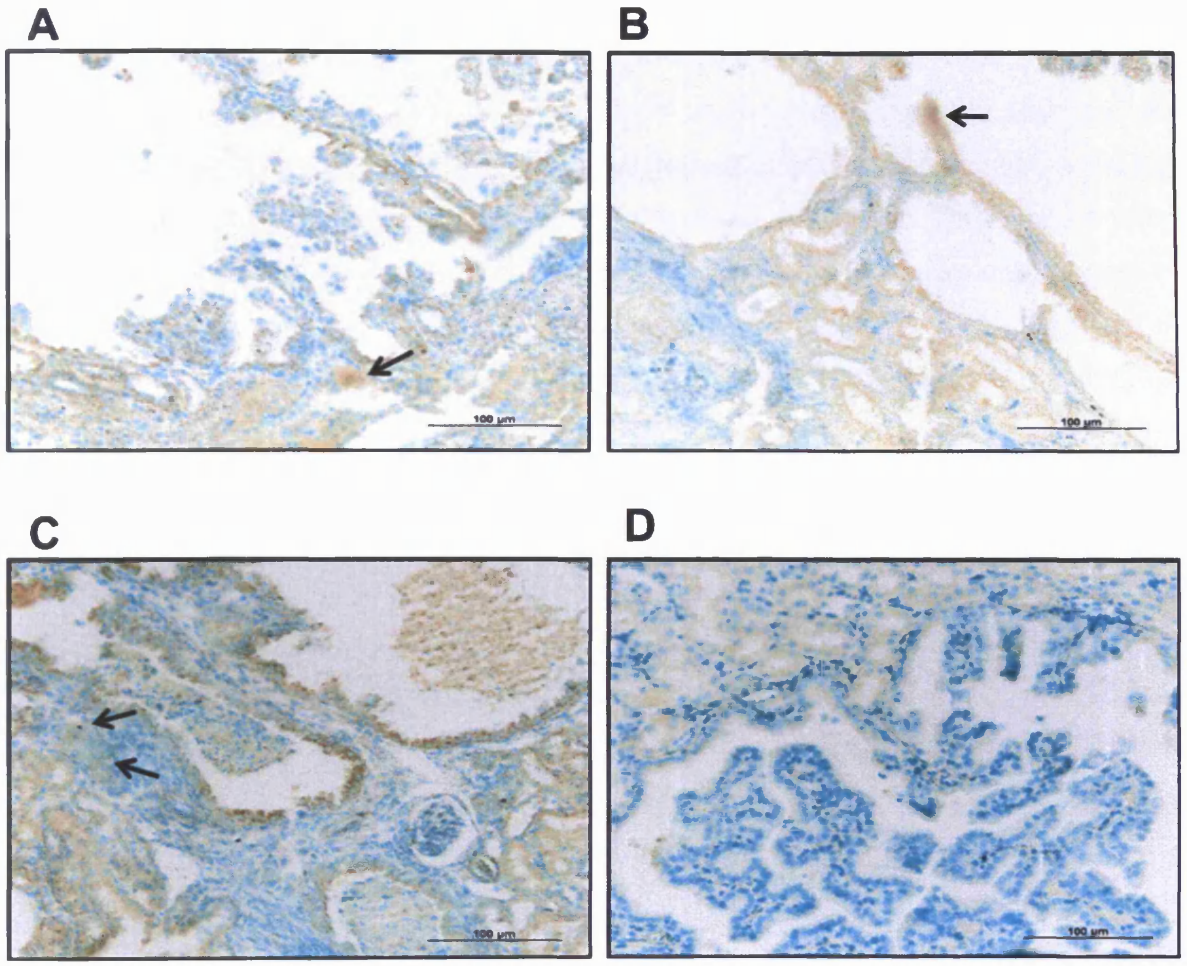


Figure 4.9 Immunohistochemical analysis of HIF-1α targets: Paraffin embedded 4 µm kidney sections were cut from *Tsc1*$^{+/-}$ (**'A'** and **'B'**) and *Tsc2*$^{+/-}$ mice (**'C'** and **'D'**). Immunohistochemistry analysis was carried out by Cardiff University Central Biotechnology Services and kidney sections from each genotype were stained for VEGF-A as described in section 2.3.22. Slides were viewed and photographed under a light microscope. Arrows indicate VEGF 'hotspots'.

4.4 Discussion

This study provides evidence of a secondary pathway by which TSC2 is able to suppress HIF-1α activity. In the comparison of HIF-1α gene targets between the cell lines (figure 4.2) it is clear that rapamycin exerts more of an inhibitory effect on the TSC1-/- cells rather than the TSC2-/- cells. Crucially, in TSC1-/- MEFs, rapamycin treatment normalises VEGF-A and BNIP3 expression to the same level as is seen in the TSC1+/+ cells indicating that the elevation in HIF-1α activity is wholly a result of enhanced mTORC1 activity in these cell lines. This is not the case in the TSC2-/- cells whereby the inhibition of mTORC1 results in less than 50% inhibition of VEGF-A expression, inferring a role for TSC2 in the inhibition of HIF-1α which does not involve mTORC1 signalling.

This has implications in the development of treatments for TSC patients particularly as the expression of HIF-1α target genes such as VEGF can contribute significantly to the manifestations of the disease.

This is not the first time that TSC2 has been implicated in the regulation of HIF-1α gene targets. It was noted in 2003 by Brugarolus *et al.* that VEGF-A expression was only partially reduced in TSC2-/- MEFs upon rapamycin treatment. It was found that under these conditions, HIF-1α levels protein levels were not elevated, leading to the conclusion that VEGF-A could be regulated by TSC2 independently of mTORC1 and HIF-1α. However, only HIF-1α protein levels were investigated. Previous investigations have demonstrated that HIF-1α transcriptional activity can be enhanced without affecting protein levels and this can be modulated by ERK kinase activity [373-375]. Figure 4.1 shows that although HIF-1α protein levels are undetectable by western blotting in the rapamycin treated TSC2-/- MEFs ('4.1'B'), HIF-1α transcriptional activity was still significantly higher than in cells transfected with TSC2. So while Brugarolus *et al.* concluded that TSC2 could modulate VEGF-A independently from mTOR and HIF-1α [365], this study provides evidence that in fact HIF-1α is being modulated by TSC2 and this in turn modulates VEGF-A rather than a direct interaction between TSC2 and VEGF-A. This also highlights the benefits of using transcriptional assays as a more sensitive indication of gene activity compared with western blotting, particularly in the case of HIF-1α which given it's short half life during normoxia, is notoriously difficult to purify and detect by western blotting.

The cell lines used in this chapter were a kind donation from Prof. David Kwiatkowski. The TSC2-/- MEFs are also p53 deficient to confer viability and studies have demonstrated that p53 and its downstream substrate MDM2 can act to inhibit HIF-1α when activated or over-expressed [376]. Therefore, differences in HIF-1α activity between the cell lines could be as a result of differences in p53 status [376]. Brugarolus *et al.,* however, demonstrated that TSC2 knockdown in p53+/+ U20S sarcoma cell lines also caused an elevation in HIF-1α activity [365], furthermore, TSC2+/+ cells exhibited very low levels of HIF-1α activity despite the lack of p53 so it is unlikely that this effect is caused by p53 deletion.

Figure 4.3 also demonstrates that S6K1 kinase activity can be abolished equally in both TSC1 and TSC2 deficient cell lines, yet the TSC2-/- cells are still less sensitive to rapamycin inhibition of HIF-1α. This indicates that mTORC1 activity is similar between the two cell lines and that they are equally sensitive to rapamycin induced downregulation of mTORC1. This supports the hypothesis that the elevation of HIF-1α transcriptional activity observed upon rapamycin treatment is mTORC1 independent.

Functional analysis of TSC2 mutations in terms of their ability to inhibit HIF-1α also provided an interesting insight into the pathogenicity of TSC. Figure 4.5 'B' demonstrates that pathogenic mutations of TSC2 do not result in a 'switch-off' of inhibition like you may expect, in fact the range of inhibition was quite varied. The HIF-1α assay of the mutants provided by Dr. M. Nellist produced variable results however quite a dramatic level of inhibition was seen in all cases (figure 4.4'B'). This was unexpected but as postulated earlier, may be a result of the much higher expression levels seen with the TSC2 mutant constructs. Since the HIF-1α assay comparing the TSC2 mutant constructs generated in the pcDNA3.1 vector produced results much more comparable to the empty vector sample (lane 2 of figure 4,5 'B'), it is likely that the differing effects observed were a result of increased expression of the constructs in the original pcDNA3 vector provided by Dr. M. Nellist in comparison to the expression constructs previously used. The higher level of protein expression within the cells may have interfered with the protein translational machinery and could have induced ER stress within the cell. This could cause an indirect reduction in HIF-1α activity as was seen in chapter 3, whereby inhibition of mTORC1 mediated cap-dependent translation suppressed elevated HIF-1α activity within TSC2-/- MEFs (see figure 3.5'E'). Alternatively the increased protein levels within the cell may have

interfered with DNA binding of HIF-1α within the nucleus to prevent its association with HIF response elements on target genes. It is also plausible however, that the mutants do retain some residual GAP activity towards Rheb and may only inhibit mTORC1 when grossly overexpressed.

The HIF-1α assay of the mutants generated in the lower expressing pcDNA3.1 vector proved to be far more informative with a much higher degree of variations between the mutant constructs. For instance the E92V mutant of TSC2 was able to induce complete inhibition of HIF-1α and was indistinguishable from wild-type in this assay.

Further work by Dunlop *et al.* showed the E92V mutant to be fully functional with regards to inhibition of S6K1 and 4E-BP1 and also demonstrated GAP activity towards Rheb [326]. Clinical details revealed that the patient carrying this mutation was suffering from seizures, a cortical tuber and had three or more hypomelanotic macules, fulfilling two of the major criteria considered when diagnosing TSC which is sufficient evidence to give a definitive diagnosis (in accordance with the TSC alliance). Further genetic analysis however revealed that the E92V mutation was also present in the parents of two unrelated patients presenting with this mutation. The parents did not meet any of the criteria for a diagnosis of TSC therefore it is likely that the E92V mutation is a non-pathogenic genetic polymorphism and thus is not the cause of TSC in this patient.

The R505Q mutation produced a moderate level of inhibition of HIF-1α, similar to that of rapamycin treatment. Patient information revealed that this individual suffered several neuropsychological manifestations similar to those seen in TSC patients, however they did not meet the full diagnostic criteria for TSC. The functional analysis carried out by Dunlop e*t al.* indicated that TSC2 mutated at this site did not have GAP activity towards Rheb and was unable to inhibit 4E-BP1 phosphorylation. It was, however, able to inhibit Rheb induced S6K1 activity. This is unusual and implies a partial loss of function of TSC2. Interestingly only the HIF-1α activity assay indicated partial function for this mutation. This suggests that the HIF-1α transcriptional assay could be a useful tool for examining subtle changes to function, and could be utilised as an indicator of TSC2 protein functionality in the analysis of uncharacterised mutations. Whilst other functional assays tend to result in a 'functional' or 'non-functional' assessment of TSC2, the quantitative nature of the

HIF-1α assay could indicate TSC2 mutations which result in partial loss of function and therefore has potential for use as a prognostic tool.

The most significant finding from mutational analysis of TSC2 was the highlighted differences between apparently non-functional pathogenic mutations and the R1743G/Q/W mutants. Both H597R and L1460P mutations were shown to be non-functional in all aspects analysed by Dunlop *et al.*, SIFT and Polyphen analysis indicated that both mutations would affect protein functionality and were likely to be damaging. In addition to this, clinical information of patients presenting with these mutations was obtained and both fulfilled the criteria for TSC diagnosis. Yet the functional HIF-1α assay revealed that both of these were able to cause significant inhibition of HIF-1α whereas the R1743Q mutant was not. This provides further evidence that TSC2 can modulate HIF-1α independently of mTORC1. It also indicates the potential significance of the CaM-binding domain/TAD within TSC2 in potentially modulating the transcriptional activity of HIF-1α, however further research is required for determining the mechanisms governing this.

There are reports in the literature indicating that calcium influx during hypoxia can cause an increase in the transcriptional activity of HIF-1α without affecting protein levels [375]. This is similar to the effect seen in figure 4.1, whereby transcriptional activity of HIF-1α was still elevated when mTORC1 was inhibited yet protein levels were normalised. It has also been demonstrated that this effect can be inhibited with the use of ERK inhibitors [374, 375]. Further studies investigating independent functions of TSC2 demonstrated that TSC2 mediated inhibition of the oestrogen receptor is dependent upon attenuation of ERK1-2 MAP Kinase [362]. Since ERK1-2 MAP Kinase inhibition can also prevent calcium dependent enhancement of HIF-1α activity it is possible that a similar mechanism occurs in TSC2 mediation of HIF-1α. It could suggest a potential model for HIF-1α inhibition whereby calcium induced binding of calmodulin to TSC2 results in a displacement of HIF-1α, prompting its activation in an ERK1-2 MAP kinase dependent manner. However at this stage of the investigation it is only possible to speculate upon potential mechanisms. Further research into whether TSC2 is required for calcium dependent increases in HIF-1α activity could establish whether this mechanism is plausible. Furthermore, it would be useful to determine whether TSC2 can in fact interact directly with HIF-1α itself.

Work by Kim *et al.* has also been carried out investigating how mutations to the Arg1743 site of TSC2 effect its localisation within the cell. Schluter *et al.* used *in silico* analysis to identify proteins containing PTS sequences, (peroxisomal targeting sequences) and demonstrated that TSC2 contains a PTS1 sequence in its carboxy-terminus. PTS sequences are located on proteins which contribute to peroxisome mediated metabolic processes. Peroxisome mediated processes include β-oxidation of long and very long chain fatty acids, prostaglandins and leukotrines. They are also involved in the synthesis of bile acids and cholesterol. The cell relies on specific peroxisomal import receptors (PEX's) to identify proteins required for these processes and they do so via recognition of PTS sequences. This is the first time TSC2 has been implicated in peroxisome mediated processes.

Kim *et al.* showed that TSC2 could localise to the peroxisome and that mutation to the Arg1743 site prevented TSC2 interaction with the peroxisome import receptor PEX5. The work also indicated that TSC2 was able to modulate ROS (reactive O_2 species) induced suppression of mTORC1 signalling, it was revealed that none of the CaM mutants analysed in this study (R1743G/W/Q) were able to mediate this suppression. The study suggests that TSC1, TSC2 and Rheb are able to translocate to the peroxisome where TSC1 and TSC2 function together as a heterodimer to inhibit the small G-protein Rheb in response to ROS.

Interestingly, ROS has been shown to propagate HIF-1α activity through very loosely defined mechanisms [377]. Furthermore it has been shown that mutant forms of TSC2 can increase ROS production through Rac-1 [378], this may therefore indicate that the elevation of HIF-1α activity seen with rapamycin treatment is a result of increased ROS production due to non-functional TSC2. The Arg1743 mutants which are unable to translocate to the peroxisome may be completely ineffective at suppressing ROS or inhibiting mTOR, explaining the high levels of HIF-1α activity. Further work is required to determine the exact relationship between ROS, TSC2 and HIF-1α.

There is also some evidence in the literature indicating that HIF-1α and its associated hydroxylases can locate to the peroxisome in hepatocytes [379], it is thought that this may be an additional mechanism of HIF-1α regulation. This has only been demonstrated in hepatocytes but if this mechanism does extend to other cell types, it is possible that TSC2 causes localisation of HIF-1α to the peroxisome as a way of mediating its inhibition. This would explain why the R1743G/Q/W mutants of

TSC2 which are incapable of peroxisome localisation are unable to inhibit HIF-1α. This may also explain the elevated HIF-1α protein levels seen in TSC2-/- MEFs.

There are reports in the literature implicating the Arg1743 site with Ca^{2+}-dependent calmodulin binding of TSC2, modulation of steroid receptor transcription and to peroxisomal localisation. Disruption of any of these processes could be responsible for the inability of the R1743G/W/Q mutants to inhibit HIF-1α and may hold the key to understanding TSC2 mediated mTORC1 independent regulation of HIF-1α, however further experimentation is required to clarify the mechanism.

Importantly however, this data indicates that rapamycin may not be sufficient to normalise VEGF expression in TSC patients with specific TSC2 mutations to the CaM-binding domain or exhibiting c-terminal truncations. It may therefore be appropriate to trial a combinational therapy approach for these patients,who may respond more favourably to the use of mTORC1 inhibitors in conjunction with specific VEGF of HIF-1α inhibitors.

The focus of research in TSC has now switched towards clarifying TSC1 and TSC2 independent functions which may also become dysregulated in TSC patients but would not be treatable with rapalogues. It is important to determine these functions particularly in the light of the current clinical trials in order to assess the usefulness of these therapeutics effectively and to improve our understanding of the pathology of the disease.

CHAPTER 5: CHARACTERISATION OF mTORC1 DIRECTED REGULATION OF STAT3

5.1 Introduction

It was initially thought that each STAT protein had a specific receptor and therefore a specific regulatory role within the cell. Knockout studies have indicated that this is true for the most part, with one exception being STAT3 [188]. STAT3 is activated by a wide range of stimuli and influences a multitude of biological processes including the acute phase immune response, angiogenesis, cell growth, survival and migration, cell cycle progression and tumorigenesis. It has also been demonstrated to play a seemingly contradictory role in the regulation of apoptosis [188, 380-382].

STAT3 directed responses are mediated primarily via activation of the gp130 receptor subunit by cytokines [380]. This in turn activates the JAK/STAT signalling pathway, where STAT3 plays a central role in mediating signals to the nucleus to regulate gene expression [188, 380]. When STAT proteins are activated they dimerise and translocate to the nucleus where they bind to promoter regions of target genes in order to activate their transcription. (See section 1.5.4).

As described in section 1.5.4, STAT3 dimerisation and activation is primarily modulated via phosphorylation of Tyr705. A serine phosphorylation site has been identified on STAT1, 3, 4, 5a and 5b which appears to influence STAT transcriptional activity. There is clear evidence within the literature that phosphorylation of the Ser727 residue of STAT1 increases its transcriptional activity [383-385]. However there are mixed reports defining the role of Ser727 phosphorylation in STAT3 activation.

The majority of studies in this area are based around S727A mutants of STAT3. Wen *et al.* demonstrated an approximate 50% reduction in the activation of a transfected IRF-1 promoter (a specific STAT3 target) in cells expressing the S727A mutant in comparison to wild-type, suggesting that Ser727 phosphorylation is required in addition to Tyr705 phosphorylation for maximal activation of STAT3 [383].

However a similar study saw no difference between the S727A mutant and the wild-type in the activation of the haptoglobin acute phase reporter [386], indicating that its activity is not dependent upon phosphorylation of the Ser727 site. This may suggest that the phosphorylation status of these critical residues may effect what genes are transcribed. Consistent with this Ser727 phosphorylation of

STAT3 in the absence of Tyr705 phosphorylation appears to promote prostate tumourigenesis which may suggest that the Ser727 site is more influential in the over-proliferation phenotype produced with constitutive STAT3 activation [382]. It has also been suggested that Ser727 phosphorylation may function to negatively regulate Tyr705 phosphorylation [201] [387]. Furthermore, in contrast to traditional thinking, recent research has indicated that Ser727 phosphorylation alone is sufficient to activate STAT3 in the absence of Tyr705 phosphorylation [382]. This is supported by more recent studies revealing that STAT3 can translocate to the nucleus in the absence of Tyr705 phosphorylation via interaction with importin-α3, indicating a mechanism by which Ser727 phosphorylation may be sufficient to induce STAT3 dimerisation [388, 389]. Furthermore, it has also been demonstrated that Ser727 phosphorylation of STAT3 is essential for Ras mediated oncogenic transformation. Intriguingly Gough *et al.* demonstrated that STAT3 was actually targeted to the mitochondria and oncogenic transformation was dependent upon Ser727 phosphorylation but *not* Tyr705 phosphorylation, the SH2 domain or its DNA binding. STAT3 therefore has functions outside of it's role as a transcription factor governed by Ser727 which appear to increase the chance of malignant transformation [113].

There are mixed reports within the literature concerning the identity of the kinase(s) responsible for Ser727 phosphorylation. Tyr705 phosphorylation is known to be modulated primarily by JAK1/2 however several kinases have been linked with Ser727 phosphorylation including JNK-1 [390], MEKK1 [391] ERK [392], Protein Kinase C [393, 394] and mTOR [202, 395]. A study by Yokogami *et al.* demonstrated that CNTF (ciliary neurotrophic factor) stimulation could activate both Tyr705 and Ser727 phosphorylation causing maximal STAT3 activation. They provided evidence that mTOR was able to phosphorylate a STAT3 peptide corresponding to residues 720-731 in an *in vitro* kinase assay, indicating the first evidence for mTOR as a direct regulator of STAT3. This study aims to verify this and clarify the role of mTOR in the regulation of STAT3. Furthermore, there are several studies in the literature linking HIF-1α and STAT3. It has been suggested that STAT3 is required for full activation of HIF-1α [209]. Other studies have indicated that inhibition of STAT3 results in inhibition of HIF-1α mediated gene expression and that STAT3 is required for HIF-1α activation [211]. Activation of STAT3 has also been demonstrated to result in

stabilisation of HIF-1α protein [381]. This study also aimed to establish whether mTOR is involved in regulating this interaction.

STAT3 has been described as an oncogene and its inappropriate activation is associated with pathogenesis, aberrant Ser727 phosphorylation has been linked to both chronic lymphocytic leukaemia [396] and oncogenic transformation by v-src [397]. Elevation of mTORC1, HIF-1α and STAT3 are common in many cancers and may contribute to cancer pathogenesis through dysregulation of cellular growth, survival, proliferation and angiogenesis. Establishing the regulatory mechanisms behind this could provide potential new pharmacological targets and also facilitate our understanding behind hamartoma disorders resulting from inappropriate mTORC1 activation. This work utilised a STAT3-inducible luciferase reporter (see section 2.3.19) which contains multiple copies of the STAT3 response element upstream of a firely-luciferase reporter in order to observe effects of mTORC1 upon STAT3 mediated gene-expression.

5.2 Methods

5.2.1 Plasmids

Gateway recombination cloning technology (Invitrogen) was utilised to generate GST-STAT3 from an I.M.A.G.E. clone (purchased from ATCC), in accordance with manufacturers guidelines.

5.2.2 STAT3 luciferase assays

The STAT3 luciferase reporter construct was transfected into HEK293 cells cultivated in 12 well plates. Cells were grown under serum starved conditions and treated with 25ng/ml CNTF and 50mM rapamycin overnight prior to lysis. Each luciferase assay was carried out in triplicate at least three times. Cells were transfected using lipofectamine 2000 and lysed with Blenis lysis buffer as described in section 2.3.4.3. Half the lysate was retained for western blotting analysis of over-expressed proteins (see below). Cells were analysed for luciferase assay as described in section 2.3.19, with three measurements being taken per lysate to check the consistency of the readings. Samples were also analysed for total protein using a NanoDrop spectrophotometer and standardised accordingly. Results are representative of at least three independent experiments.

5.2.3 Wetern blotting

Western blotting was carried out as previously described in section 2.3.10. Lysates analysed for the phosphorylation of STAT3 (P-STAT3 Ser727/Tyr705) were either subjected to three 5 second sonication cycles (30 microns) with incubation upon ice between cycles. Or, where indicated lysates were passed through a QiaShredder (Qiagen) three times in quick succession via centrifugation in order to visualise nuclear proteins. Where indicated, western blotting was subjected to densitometry analysis using Image J software to help provide a clearer picture of differential protein expression or phosphorylation.

5.2.4 Far westerns

Recombinant GST-STAT3 was generated from HEK293 cells transfected with the GST-STAT3 vector using calcium chloride transfection reagent (as described in section 2.3.5) from rapamycin treated and untreated cells (rapamycin treatment was for 1hour prior to lysis at 50nM). Cells were lysed in Rheb lysis buffer containing protease inhibitors (excluding DTT) before being subjected to 3x5 second sonication cycles as described above. Lysates were then subjected to high centrifugation for removal of cellular debris before being incubated with glutathione-Sepharose beads for 2 h at 4°C. IP's were then washed as described in section 2.3.13, GST-purification. Recombinant proteins were then dotted onto membranes and the far western was carried out as described in section 2.3.11.

5.2.5 mTORC1 kinase assay

Recombinant GST-STAT3 was purified as described above from HEK293s. Recombinant GST-4E-BP1 was purified from serum starved cells in the same manner. mTORC1 kinase assays were carried out as described in section 2.3.15 at 30°C for 1 h with gentle agitation.

5.2.6 Radiolabelling

Cold assay: HEK293 cells were transfected using lipofectamine 2000 transfection reagent as described in section 2.3.5. Cells were cultured under serum starved conditions, 2 h prior to lysis cells were stimulated with 25ng/ml of CNTF, 1 h prior to lysis cells were treated with 50nM of rapamycin and 5nM okadaic acid for 30 min prior to lysis. Cells were then stimulated with 10µg/ml insulin where indicated. Cells

were lysed in Nuclear Proteins Lysis buffer (see section 2.2) supplemented with protease inhibitors (excluding DTT), 20nM Okadaic acid and a phosphatase inhibitor cocktail (GE Healthcare). Lysates were homogenised using QIAShredders (Qiagen) and were passed through the column three times using high speed centrifugation. 50µl of total lysate was retained for analysis via SDS-PAGE and western blotting. The remainder of lysate was then incubated with glutathione-Sepharose beads for 2 hs at 4°C with rotation. Beads were washed 3 times with lysis buffer (supplements as above) before a final 10 min wash with rotation. GST-STAT3 was then eluted in Rheb storage buffer supplemented with 30mM glutathione plus protease and phosphatase inhibitors and analysed for P-STAT3 Ser727 and Tyr705 using SDS-PAGE and western blotting.

Hot assay: HEK293 cells were cultured in phosphate free media for 4 h, cells were then treated (as above) and pulsed with 5 mCi of [^{32}P]-orthophosphate for 2 h. A GST-purification was carried out as described, eluted proteins were analysed for incorporation of radiolabel using SDS-PAGE, with autoradiography for visualisation.

5.3 Results

5.3.1 Effects of rapamycin treatment upon Ser727 phosphorylation of STAT3.

It was reported in the literature that Ser727 phosphorylation was required for maximal activation of STAT3 during CNTF signalling, Yokogami *et al.* showed evidence that Ser727 phosphorylation may be mediated by mTORC1 under CNTF stimulation, I therefore decided to look at STAT3 phosphorylation in this context.. To do this, the effects of rapamycin treatment upon CNTF mediated phosphorylation of STAT3 were investigated *in vivo*. Untransfected HEK293 cells were analysed for STAT3 phosphorylation under conditions of CNTF stimulation in combination with rapamycin pre-treatment. As reported in the literature, just 15min of CNTF stimulation robustly induced Tyr705 phosphorylation (compare lanes 1 and 2) of STAT3. This phosphorylation was unaffected by rapamycin treatment. CNTF appeared to weakly increase Ser727 phosphorylation of STAT3 (compare lanes 1 and 2) after 15 min of treatment, supporting the notion that Tyr705 may act as a priming phosphorylation site.

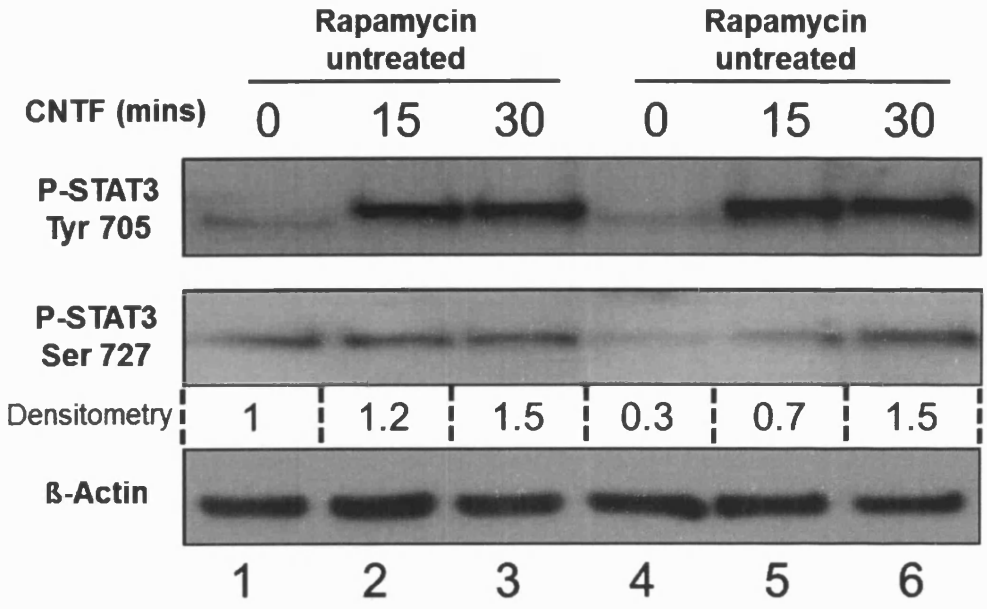


Figure 5.1: Effects of rapamycin treatment upon P-STAT3 Ser727 phosphorylation: Untransfected HEK293 cells were cultured under serum starved conditions. Cells treated with rapamycin were pre-treated for 1h prior to stimulation with CNTF. Cells were stimulated with CNTF and lysed at the indicated time points. Lysates were then analysed for phosphorylation of STAT3 at Tyr705 and Ser727 with β-actin implemented as a loading control using SDS-PAGE and western blotting with phospho-specific antibodies. Image-J software was used to determine densitometry of Ser727 phosphorylation, with results being standardised to unstimulated (lane 1).

Rapamycin reduced the level of phosphorylation of STAT3 at Ser727 in unstimulated cells and at the first time point. After 15min of CNTF stimulation, the level of Ser727 phosphorylation in the rapamycin pre-treated samples was significantly reduced (compare lanes 2 and 5) in agreement with Yokogami's study.

In contrast to Yokogami's work however, there appears to be a reduction in the basal level of Ser727 phosphorylation in the rapamycin treated cells (compare lanes 1 and 4) which may suggest that mTOR is able to phosphorylate STAT3 in the absence of CNTF stimulation.

This difference may be occurring due to the difference in cell lines as Yokogami's study utilised human neuroblastoma NBFL cells whereas this experiment was carried out in HEK293 cells. This data supports the hypothesis that mTORC1 is involved in the fine tuning of STAT3 activation.

5.3.2 STAT3 Ser727 phosphorylation shows sensitivity to rapamycin inhibition

There has been much debate within the literature regarding how the phosphorylation status of the Tyr705 and Ser727 relates to the transcriptional activity of STAT3. Dogma dictates that like other STAT proteins, Tyr705 phosphorylation is necessary for translocation to the nucleus and hence activation. However, several studies have shown evidence that STAT3 can translocate to the nucleus through direct interaction with importin-α3, this appears to be modulated via the Ser727 site independently of Tyrosine phosphorylation [388, 389]. I therefore wanted to determine how the phosphorylation status of both sites corresponded to STAT3 transcriptional activity.

To do this I obtained a STAT3 transcriptional luciferase reporter construct from Panomics and transfected it into HEK293 cells to observe the effects of CNTF and rapamycin treatment upon STAT3 transcriptional activity. Cells were treated overnight with both rapamycin and CNTF to allow an accumulation of luciferase and analysed the following day. CNTF induced a substantial increase in STAT3 transcriptional activity – compare bars 1 and 3, however, this is in contrast with Yokogami's results that showed a 40 fold increase in STAT3 transcriptional activity upon CNTF stimulation. In the case of unstimulated cells, Yokogami saw virtually no STAT3 transcriptional activity within the NBFL cell lines, this could indicate therefore that the HEK293s used within this study have a significantly higher basal level of

STAT3 activity than the NBFL cell line. This could explain why they were only able to detect mTORC1 input in the context of CNTF signalling.

The luciferase assay also revealed that STAT3 transcriptional activity was sensitive to inhibition from rapamycin, particularly in the context of CNTF signalling. Rapamycin caused an average 55.8% reduction in the transcriptional activity of STAT3 in CNTF treated cells, giving a highly significant p-value of less than 0.001. In the case of unstimulated cells, rapamycin caused an average 30% reduction in STAT3 activity but this result was not significant. This supports Yokogami's work which demonstrated a similar level of inhibition by rapamycin in CNTF stimulated cells.

Western blot analysis was carried out upon lysates to assess the phosphorylation levels. Tyr705 phosphorylation was robustly induced by CNTF stimulation and unaffected by rapamycin treatment, as is consistent with the literature. I hypothesised that the Ser727 phosphorylation would mirror the transcriptional assay results, this however did not prove to be the case.

Ser727 phosphorylation was fairly weak in the absence of CNTF, although retained its sensitivity to rapamycin inhibition as was seen in figure 1. Interestingly, there was an increase in Ser727 phosphorylation with CNTF stimulation which was not seen in figure 5.1. This may be a result of the prolonged overnight CNTF treatment as opposed to the shorter lysis time points utilised in figure 1. Furthermore, this increase did not appear to be effected by rapamycin, although the transcriptional assay did indicate a reduction in STAT3 transcriptional activity. There are several possible explanations for this discrepancy which are discussed later. I concluded that the sonication of the sample prior to running SDS-PAGE was not sufficient to break down the nuclear membranes within the sample and therefore may not represent all of the activated STAT3 within the cells. This would explain the weak phosphorylation levels seen at Ser727 and why phosphorylation levels did not correspond with STAT3 transcriptional activation levels. In order to address this, a different approach was taken to extract nuclear STAT3, described later.

It was reported in Yokogami's paper that CNTF treatment was able to activate JAK/STAT signalling leading to Tyr705 phosphorylation of STAT3 mediated by JAK1. In addition to this, Yokogami's study demonstrated that CNTF treatment of HEK293T cells also caused upregulation of the PI3K pathway, resulting in mTOR activation.

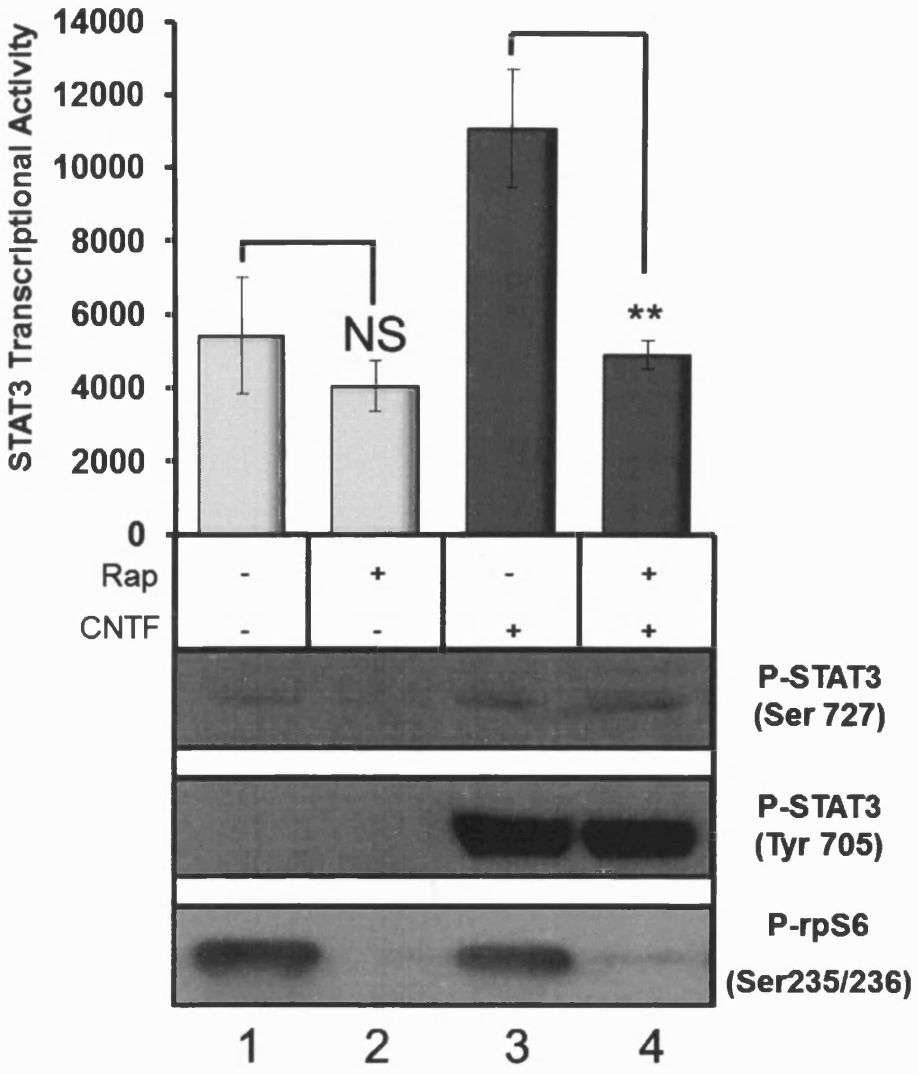


Figure 5.2 Basal STAT3 Ser727 phosphorylation shows sensitivity to rapamycin inhibition: HEK293 cells were transfected with the STAT3 inducible luciferase reporter construct (Panomics), cells were cultured under serum starved conditions but treated with CNTF/Rapamycin where indicated for 12 h prior to lysis. Cells were then harvested in Blenis lysis buffer supplemented with protease inhibitors and sonicated as described in section 2.3.4.3. Total lysate was analysed for STAT3 transcriptional activity. Results are representative of three independent experiments, and standardised to total protein levels as determined by a Bradford assay. Error bars indicative of standard deviation from three independent experiments. * indicates p-value significance <0.05, ** indicates significance <0.001. The remainder of the lysate was analysed for phosphorylation of STAT3 at Tyr705 and Ser727 as well as rpS6 to observe the effects of rapamycin using SDS-PAGE in conjunction with western blotting using phospho-specific antibodies.

To confirm whether this was the case in the HEK293E cells utilised in this study, and also to check the efficacy of the rapamycin treatment, the lysates were also analysed for phosphorylation of rpS6 as readout of mTORC1 activity within the cells. Unexpectedly, CNTF treatment was not able to significantly upregulate phospho-rpS6 levels, although phospho-rpS6 levels did appear to be less sensitive to inhibition from rapamycin in CNTF treated cells compared to untreated. (See lanes 2 and 4). This may be a difference between the HEK293 subtypes of cell line, or it could indicate that CNTF stimulation is causing down-regulation of phosphatases acting towards rpS6.

5.3.3 Cells expressing an active mTOR mutant demonstrate increased Ser727 phosphorylation

An alternative approach was taken in the preparation of lysates for the following experiment. In previous chapters, nuclear and cytoplasmic fractions were produced utilising hypertonic and hypotonic buffers (see methods section 2.3.6). Since these buffers were not compatible with the luciferase assays or GST purification using glutathione-Sepharose-beads, a new approach had to be devised for nuclear preparations. To address this, QIA shredders were obtained from Qiagen. QIA shredders were used to uniformly homogenize lysates and shred DNA and were utilised for mRNA extraction in chapters 3 and 4. Cells were lysed normally and instead of sonication, were passed three times through the QIA shredder before being subjected to SDS-PAGE. In this experiment, the constitutively active mutant of mTOR, E2419K was utilised in conjunction with the Raptor mutant 4 construct. These mutants proved useful in the analysis of mTORC1 directed substrate phosphorylation in chapter 3, where I was able to demonstrate that E2419K mutant expressed higher kinase activity towards 4E-BP1 and HIF-1α as well as identifying Raptor mutant 4 as a dominant inhibitor of mTORC1 signalling. I therefore decided to examine STAT3 activation within cells expressing either the wild-type or active mutant of mTOR alongside either Raptor/ or Raptor mutant 4 to establish whether mTORC1 activity corresponded with STAT3 activity.

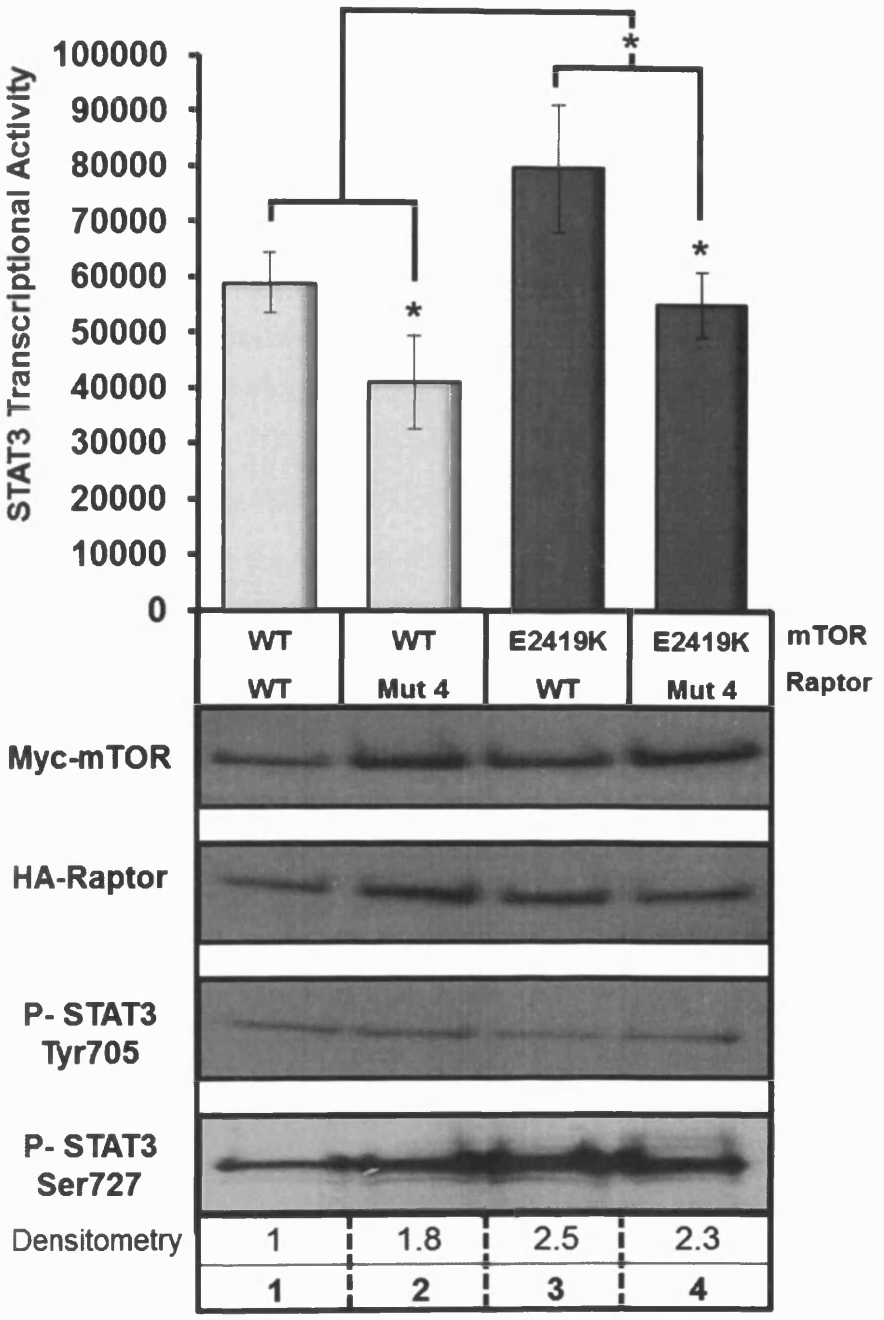


Figure 5.3 Cells expressing an active mTOR mutant demonstrate increased Ser727 phosphorylation: HEK293 cells were transfected with the STAT3 inducible luciferase reporter construct (Panomics) in conjunction with mutants of mTOR/Raptor as indicated. Cells were cultured under serum starved conditions and harvested in Blenis lysis buffer. Lysates were then passed three times through the QIA shredder for homogenisation using high centrifugation. Lysates were then analysed for STAT3 transcriptional activity as described in section 2.3.19 and western blotting with phospho-specific antibodies was used to determine phosphorylation of STAT3 and mTOR/Raptor expression. Image J was used to determine densitometry of Ser727 phosphorylation. Luciferase results are representative of three independent experiments, and standardised to total protein levels as determined by a Bradford assay. Error bars indicative of standard deviation from three independent experiments. * indicates p-value significance <0.05.

As hypothesised, STAT3 activation (as determined by the transcriptional assay) is increased in cells expressing the constitutively active E2419K mutant in comparison to those expressing wild-type (compare lanes 1 and 3, p-value: <0.05). Furthermore this increased activity is inhibited when the active E2419K mTOR mutant is co-expressed with the dominant inhibitor of mTORC1, Raptor mutant 4 (compare lanes 3 and 4, p- value, <0.05). This is consistent with the behaviour of other mTORC1 substrates 4E-BP1 and HIF-1α (see figure 3.1 and 3.2).

When co-expressed with wild-type mTOR, Raptor mutant 4 appeared to cause a reduction in STAT3 transcriptional activity as expected, however, this was coupled with an increase in Ser727 phosphorylation (see densitometry figures, compare lanes 1 and 2). These discrepancies between the phosphorylation status and the transcriptional activity of STAT3 indicates the possibility that STAT3 activation could be modulated via phosphorylation at another and yet undetermined site.

Alternatively, and perhaps more likely is the possibility that prolonged mTORC1 inhibition due to expression of Raptor mutant 4 may have initiated feedback mechanisms from other pathways that further increase STAT3 activation. Since there have been several kinases reported to phosphorylate STAT3 at Ser727 this seems plausible. The transcriptional assay is a result of overnight accumulation of luciferase levels and the readout is a summary of STAT3 activity over time, whereas western blotting measures the phosphorylation level at the time of lysis. It is therefore possible that the long-term suppression of mTORC1 through raptor mutant 4 expression may have triggered upregulation of other kinases functioning to phosphorylate STAT3 by the time of lysis. Feedback pathways may not have been activated in the cells expressing the active mutant alongside Raptor mutant 4 since these cells would have exhibited a higher basal mTORC1 activity due to expression of the E2419K mutant. This theory is supported by the transcriptional assay which shows that Raptor mutant 4 is only able to reduce the activity of STAT3 in these cells to a level similar to those expressing wild-type mTOR and Raptor (Lane 4 compared to lane 1).

5.3.4 4E-BP1 binds avidly to mTORC1 while STAT3 does not

This work so far shows evidence of a role for mTORC1 in STAT3 Ser727 phosphorylation, however, it can be difficult to dissect specific phosphorylation events using *in vivo* experimentation as it is impossible to rule out cross talk between different pathways. There is clear evidence in the literature that Raptor is a key component of the mTORC1 complex and is required for substrate recognition (see section 1.3.4). I demonstrated in chapter 3 how purified recombinant 4E-BP1 protein binds to Raptor using a far western approach (figure 3.5'B').

I wanted to establish whether mTORC1 could interact directly with STAT3 as is seen with the bona fide mTOR substrate 4E-BP1. In order to do this, cDNA of STAT3 was obtained from ATCC as an I.M.A.G.E. clone (integrated molecular analysis of genomes and their expression) and using the Invitrogen gateway system, was cloned into an expression vector containing an N-terminal GST-tag. STAT3 was then transfected into cell lines and purified using GST-purification protocol (see section 2.3.13). Purified recombinant protein was dotted onto PVDF membrane and incubated with lysates containing expressed Raptor.

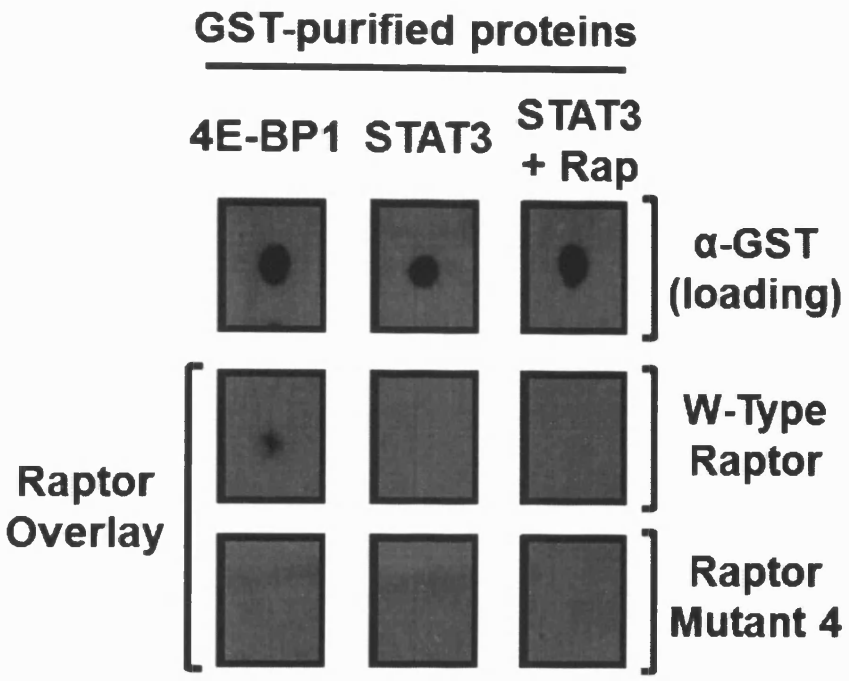


Figure 5.4 4E-BP1 binds avidly to mTORC1 while STAT3 does not: Purified recombinant GST-4E-BP1 and GST-STAT3 were purified using a GST-purification (see section 2.3.13), STAT3 was purified from cells either treated or untreated with rapamycin. 50ng of the purified proteins were dotted onto PVDF membrane before blocking and then overnight incubation with Raptor/Raptor mutant 4 expressing lysates (generated from HEK293 cells) or GST antibody. Western blotting was then utilised to determine Raptor binding and protein levels.

4E-BP1 was utilised as a positive control. Cells were also incubated in lysates expressing Raptor mutant 4 as a negative control. Results demonstrate once again robust binding of mTORC1 to 4E-BP1, however no interaction was seen between Raptor and STAT3. This does not exclude the possibility of mTORC1 directed substrate phosphorylation of STAT3, however it does suggest that the interaction between STAT3 and mTORC1 is certainly not as robust as between 4E-BP1 and mTORC1. The interplay between mTORC1 and STAT3 is likely to be more transient and fragile and may suggest a hierarchy between the substrates of mTORC1 whereby higher binding substrates are more readily phosphorylated.

5.3.5 mTORC1 phosphorylates purified STAT3 protein *in vitro*

Since I was unable to show Raptor interaction, I decided to investigate whether STAT3 could be phosphorylated directly *in vitro* by mTORC1 utilising the mTORC1 kinase assay optimised in chapter 3. GST-STAT3 and GST-Rheb were purified from HEK293 cells separately (as described in section 2.3.13) alongside the active mTOR/Raptor complex. The purified complexes were incubated together in the presence and absence of Rheb, and with ATP. Lysates were then analysed for phosphorylation of substrates using SDS-PAGE and western blotting. Purified 4E-BP1 was also utilised within the assay as a positive control.

As previously shown, 4E-BP1 was robustly phosphorylated by the purified mTORC1 complex at Thr36/45. This was further enhanced by the inclusion of GTP-bound Rheb within the assay as expected, albeit not as strongly as was seen with 4E-BP1. However the fact that phosphorylation of STAT3 was further enhanced by inclusion of GTP-bound Rheb suggests that STAT3 is a direct substrate for mTORC1. This is the first time that the full STAT3 protein has been demonstrated to be phosphorylated *in vitro* using mTORC1 at Ser727. Rheb was able to produce a significant increase in Ser727 phosphorylation confirming that this residue in STAT3 can be directly phosphorylated by mTORC1.

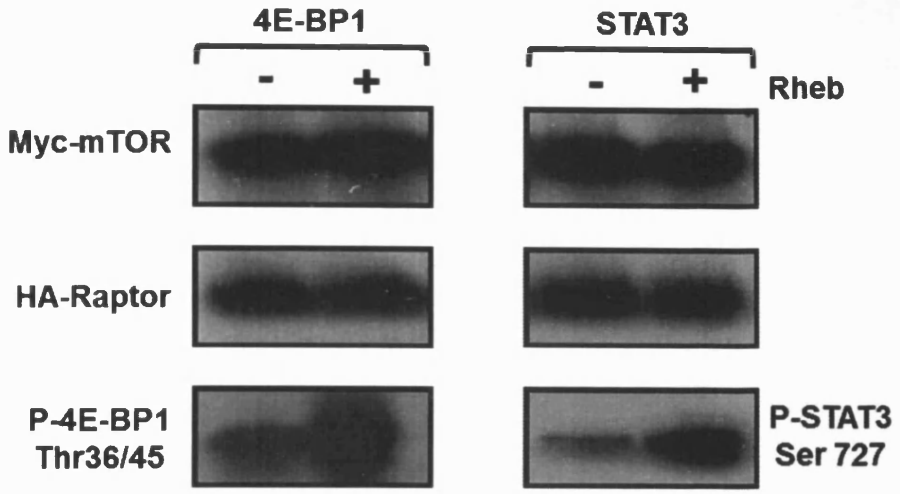


Figure 5.5: mTORC1 phosphorylates purified STAT3 protein *in vitro:* An active mTORC1 complex was purified from insulin stimulated HEK293 cells grown under serum starved conditions (as described in section 2.3.15), GST-STAT3 and GST-4E-BP1 were purified from serum starved HEK293 cells, as was GST-Rheb, which was then loaded with GTPγS. Purified mTORC1 complex was incubated alongside potential substrates with ATP with and without GTPγS-Rheb (as indicated) for 1h at 30°C with gentle agitation, as described in 2.3.15. SDS-PAGE and western blotting with phospho-specific antibodies was utilised to determine specific phosphorylation events as well as mTOR/Raptor purification.

In this assay, greater phosphorylation of 4E-BP1 in comparison to STAT3 was observed. This work supports the hypothesis that a hierarchy exists among mTORC1 substrates, with 4E-BP1 being one of the most readily phosphorylated. This goes some way to explaining why no interaction was observed between Raptor and STAT3 in figure 5.4.

5.3.6 mTORC1 phosphorylates Ser727 in response to insulin stimulation during CNTF signalling

A study by Ceresa *et al.* in 1996 was the first study to establish a link between insulin stimulation and STAT3 activation. Ceresa's study demonstrated that insulin treatment specifically induced Ser727 phosphorylation of STAT3 although did not find evidence for a specific kinase responsible for this [398]. Since insulin also stimulates mTORC1 activity and I have shown that mTORC1 can phosphorylate STAT3 directly, it is not unreasonable to hypothesise that insulin induced STAT3 Ser727 phosphorylation is mediated by mTORC1.

In order to investigate this further, an experiment was set up to view the phosphorylation events of STAT3 in response to insulin and rapamycin treatment. This was carried out in the presence of CNTF to activate Tyr705 phosphorylation. I decided to use [^{32}P]-radiolabelling for this experiment as this enabled me to view the phosphorylation events after short-term stimulation and *in vivo*. Cells expressing myc-mTOR, HA-Raptor and GST-STAT3 were treated with CNTF 2 h prior to lysis, insulin 30 min prior to lysis and rapamycin and okadaic acid 1 h prior to lysis (where indicated). Okadaic acid is a potent inhibitor of PP1 and PP2A phosphatases which have been associated with mTORC1. This ensures that any dephosphorylation seen upon rapamycin treatment is a result of relieved mTORC1 activity specifically towards STAT3. STAT3 was then purified from cells and the phosphorylation status determined by western blotting.

Incorporation of [^{32}P]-radiolabel into the STAT3 protein was visualised using autoradiography whilst phospho-specific antibodies were used to determine the Ser727 phosphorylation levels. Figure 5.6 shows that insulin causes an increase in phosphorylated STAT3 as determined by the incorporation of [^{32}P]-radiolabel, western blotting of cold samples revealed that this was a result of increased Ser727 phosphorylation whilst Tyr705 phosphorylation remained unaffected, in agreement with the work by Ceresa *et al.*

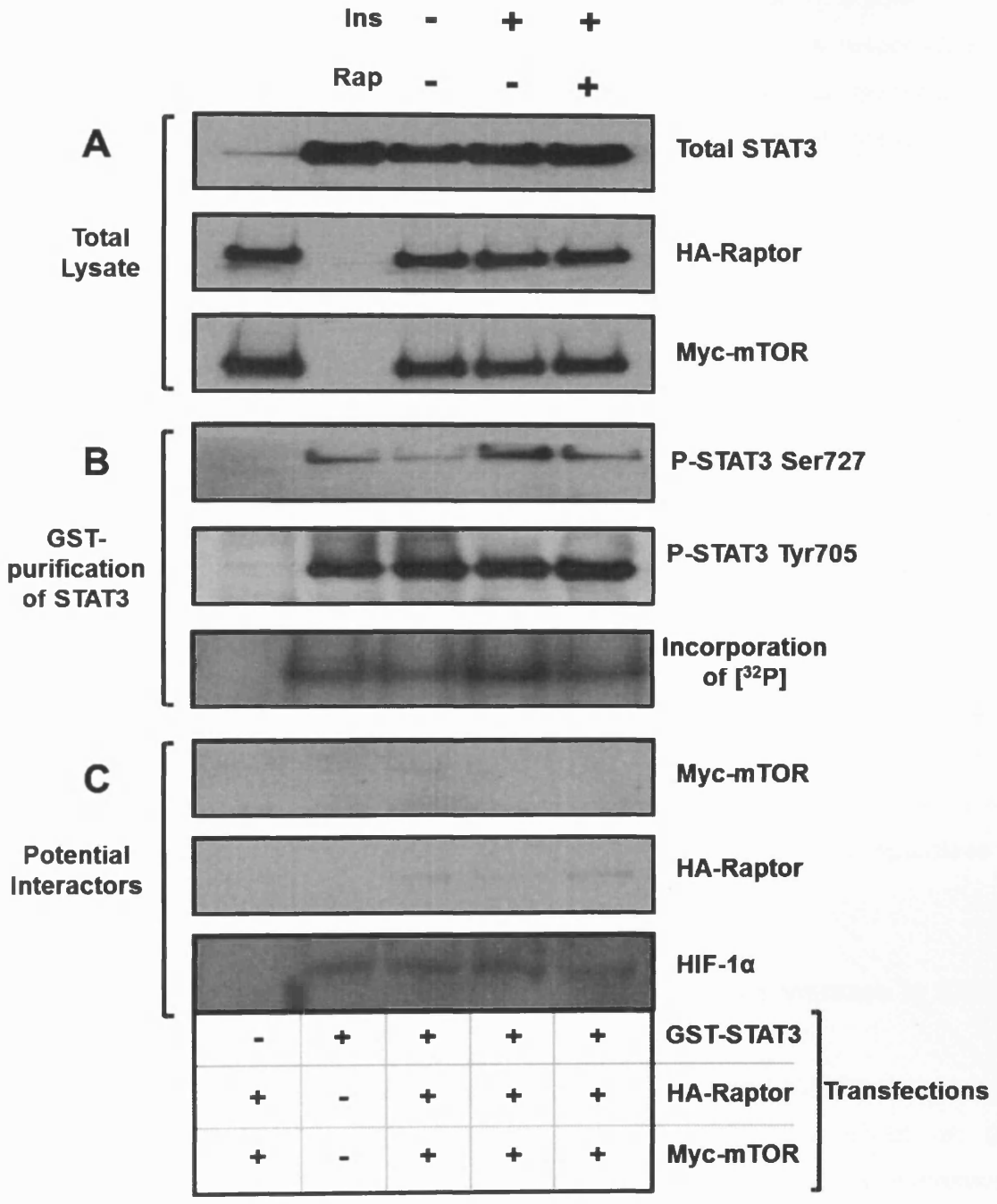


Figure 5.6 mTORC1 phosphorylates Ser727 in response to insulin stimulation during CNTF signalling: Hot assay: HEK293 cells expressing GST-STAT3, HA-Raptor and myc-mTOR were treated with CNTF for 2 h, rapamycin and okadaic acid for 1 h and insulin for 30 min prior to lysis. Total lysate samples were taken to determine expression levels. GST-STAT3 was then purified and examined for phosphorylation status and potential interactors using western blotting. Hot assay, cells were starved of phosphate for 4 h, CNTF, Rapamycin, okadaic acid and insulin were administered as above before re-addition of radiolabelled [^{32}P]-Phosphate for 1h. STAT3 was then purified to determine incorporation of the [^{32}P] radiolabel into STAT3.

Furthermore, as predicted, rapamycin treatment was able to abolish insulin induced Ser727 phosphorylation. This demonstrates that mTORC1 is responsible for insulin induced Ser727 phosphorylation in cells and also indicates that the rapamycin induced dephosphorylation seen with STAT3 is not a result of activating the phosphatase PP2A.

The remaining lysates from the cold assay were analysed by western blotting for possible interactors. Figure 5.6 'C' demonstrates that trace amounts of HA-Raptor co-purified with GST-STAT3, which was seemingly unaffected by rapamycin/insulin treatment. Since rapamycin treatment is thought to inhibit mTORC1 at least in part by interfering with its Raptor binding [69], this may suggest that Raptor is still able to bind to substrates during rapamycin treatment but not mTOR in the presence of rapamycin.

There are several studies implicating STAT3 with HIF-1α and I have so far demonstrated a role for mTORC1 in both HIF-1α and STAT3 regulation, I therefore decided to see if HIF-1α formed part of the mTOR/Raptor/STAT3 complex. Interestingly, HIF-1α was detected under all conditions (excluding the mTOR/Raptor control). There is evidence within the literature that in some cell lines HIF-1α, STAT3 and p300 form a transcriptional complex to modulate the hypoxic response [209]. This experiment supports this theory as HIF-1α co-purified with STAT3 regardless of mTOR expression or activation.

5.3.7 Expression of the TOS mutant of HIF-1α results in an increase in STAT3 transcriptional activity

In order to further explore the relationship between STAT3 and HIF-1α, I examined whether expression of the TOS mutant of HIF-1α had any effect on the transcriptional activity of STAT3. In 2007, Land *et al.* demonstrated that expression of the TOS mutant of HIF-1α dominantly inhibited HIF-1α activity and that the mutant exhibited reduced binding to its transcriptional co-activator p300 [135]. I wanted to see what effect dominant inhibition of HIF-1α had upon STAT3 activity since STAT3 also binds to p300 to form a transcriptional complex. To do this, the STAT3 transcriptional luciferase reporter was utilised and the effects that over-expression of wild-type HIF-1α or the TOS mutant had on STAT3 activity were examined.

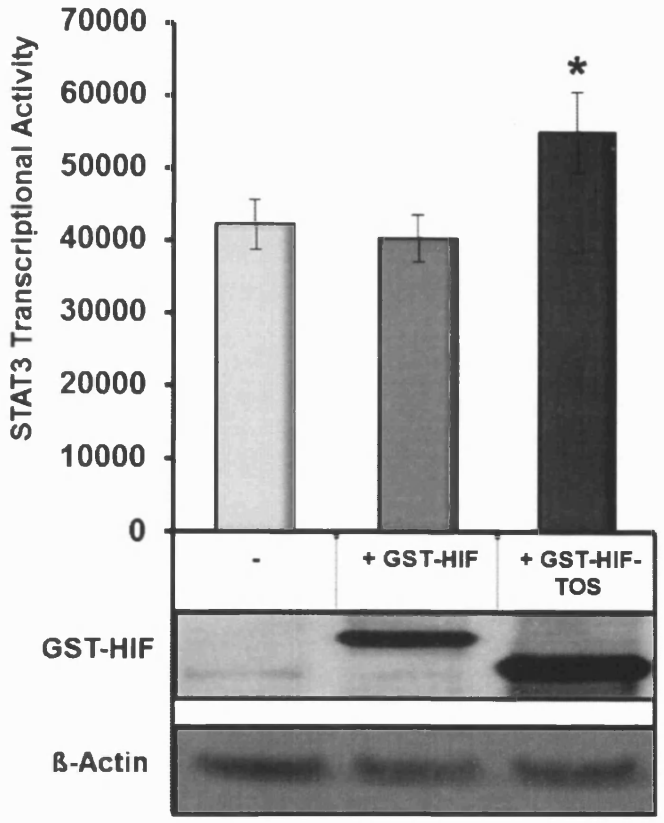


Figure 5.7 Expression of the TOS mutant of HIF-1α results in an increase in STAT3 transcriptional activity: HEK293 cells were transfected with the STAT3 inducible luciferase reporter construct alongside pACATG/GST-HIF/GST-HIF-TOS as indicated. Cells were serum starved and CNTF treated for 12 h prior to lysis. Cells were then harvested in Blenis lysis buffer and analysed for luciferase activity (standardised to total protein levels as determined by a Bradford assay), the remainder of the lysate was analysed using SDS-PAGE and western blotting to determine HIF-1α expression levels with β-actin used as a loading control. Data is representative of three independent experiments, * indicates p-value or 0.05.

Over-expression of wild-type HIF-1α had no significant effects upon STAT3 transcriptional activity in cells (compare lanes 1 and 2). However, expression of the HIF-1α TOS mutant significantly propagated STAT3 transcriptional activity. Since previous work demonstrated that an increase of STAT3 activity increased HIF-1α protein levels and synthesis [210], it is very probable that the cell signal transduction through these two transcription factors are closely linked. This result is consistent with the hypothesis that STAT3 has a regulatory role over HIF-1α, it is likely that reduction of HIF-1α activity by expression of the HIF-1α-TOS caused the cell to compensate by increasing STAT3 activation. It is also a possibility that STAT3 activity is enhanced upon inhibition of HIF-1α transcriptional events as they both share the same transcriptional machinery, i.e., STAT3 is not competing with HIF-1α for p300 hence it's activity is increased.

5.3.8 STAT3 can be inhibited by mutants of TSC2 deficient at inhibiting mTORC1

Kwiatowski *et al.* recently reported upregulation of STAT3 in TSC2-/- cells [399], this is in concordance with the evidence shown here of mTORC1 mediated STAT3 phosphorylation. In chapter 4 I showed evidence of an mTORC1 independent mechanism of regulation towards HIF-1α governed by TSC2. Since I have also shown a functional link between HIF-1α and STAT3, I wanted to establish whether STAT3 was also subject to transcriptional regulation from TSC2. To do this, I repeated the TSC2 rescue experiment utilising the various TSC2 mutant constructs to assess their ability to suppress STAT3 activity. The experiment was carried out as described in chapter 4, however cells were grown in the presence of CNTF as opposed to under hypoxia. As expected, STAT3 transcriptional activity in the TSC2-/- MEFs could be supressed by rapamycin treatment or re-introduction of wild-type TSC2. Interestingly, the effects of rapamycin treatment and over-expression of TSC2 were not significantly different in their ability to repress STAT3 as was seen with HIF-1α. This suggests that in cells lacking TSC2, the main mechanism for STAT3 elevation is via an mTORC1 dependent mechanism.

This data shows that this mTORC1 independent mechanism does not extend to STAT3 activation in TSC2-/- MEFs since the rescued expression of wild-type TSC2 caused a similar level of inhibition as mTORC1 inhibition with rapamycin.

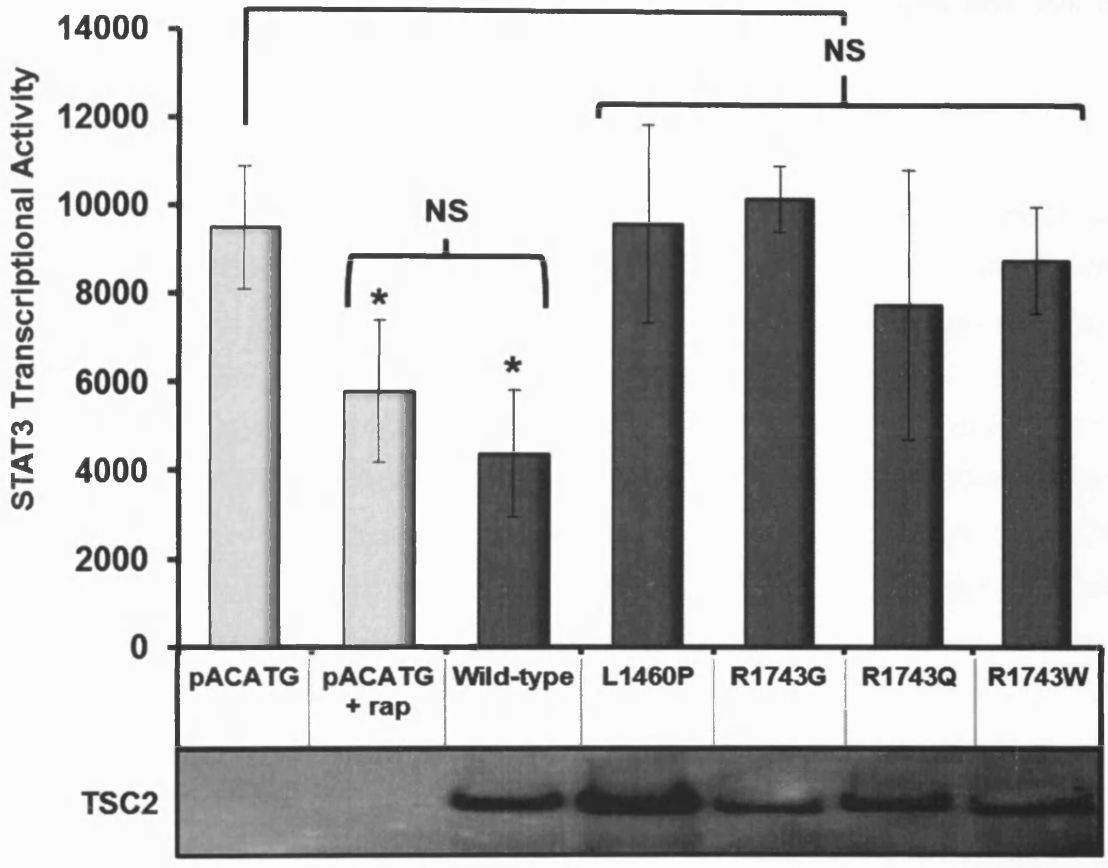


Figure 5.8: STAT3 can be inhibited by mutants of TSC2 deficient at inhibiting mTORC1: TSC2-/- MEFs were transfected with the STAT3 luciferase reporter construct alongside empty vector, wild-type TSC2 or mutant TSC2. Cells were cultured under serum starved conditions in the presence of CNTF for 12 h prior to lysis. Cells were harvested in blenis lysis buffer and analysed for STAT3 transcriptional activity, this was standardised to total protein levels as determined by a Bradford assay. The remainder of the lysate was analysed for TSC2 expression levels by western blotting. Error bars indicative of standard deviation from three independent experiments. NS indicates No Significant inhibition (compared with pACATG). * indicates p-value of 0.05, ** indicates p-value of 0.001.

STAT3 is therefore not subject to the same mTORC1 mediation as HIF-1α Figure 5.8 however does indicate that mTORC1 inhibition can suppress STAT3 activity in the context of TSC1/2 loss, supporting the conclusions of this work and validating STAT3 as a potential therapeutic target for TSC.

5.4 Discussion

Yokogami's study provided the first evidence of a link between mTORC1 signal transduction and STAT3 [202]. Since this study, there has been a significant volume of research in this field however there is conflicting evidence between studies. This study aimed to clarify some of the discrepancies within the literature.

There are several studies providing evidence for upregulation of STAT3 under conditions of mTORC1 activation, particularly in cancer cell lines [400-402]. Each of these studies provides evidence that STAT3 is located downstream of mTORC1. Yokogami's study is so far the only evidence that STAT3 is a direct substrate of mTORC1. Yokogami's evidence however, should be treated with caution as they were only able to show *in vitro* mTORC1 directed phosphorylation of a short STAT3 peptide of just 11 amino acids. Therefore one of the first aims of this study was to confirm that mTORC1 could directly phosphorylate STAT3.

Yokogami's study suggested that CNTF stimulation was required for mTORC1 mediated phosphorylation of STAT3. In figure 5.1, I demonstrated that P-STAT3 Ser727 phosphorylation showed some sensitivity to rapamycin inhibition *in vivo* whilst Tyr705 phosphorylation appeared unaffected. In contrast to Yokogami's study however, the biggest difference between the rapamycin treated and untreated samples occurred in the absence of CNTF stimulation. This may therefore reflect differences between the HEK293E cells utilised in this study and the NBFL cell line used by Yokogami. In addition, it indicates that mTORC1 is able to regulate STAT3 in the absence of CNTF stimulation. This is in agreement with more recently published studies utilising cancer derived cell lines where mTORC1 and STAT3 signalling are inappropriately elevated in the absence of STAT3 stimulation [400, 402]. Interestingly, Yokogami's study saw the greatest suppression of P-STAT3 Ser727 with rapamycin treatment after 15min of CNTF stimulation, Figure 5.1 also shows a substantial decrease in Ser727 phosphorylation at this time point (see densitometry analysis, compare lane 2 with lane 5) indicating that mTORC1 does regulate Ser727 phosphorylation during CNTF signalling. Interestingly though, the 30

min CNTF time point shows no repression of Ser727 phosphorylation in rapamycin treated cells in contrast with Yokogami's study. Again, this may reflect differences within the cell lines, since CNTF is a neuropoietic cytokine and Yokogami was utilising a neuronal cell line for his study, the effects of CNTF may differ between these two cell types.

HEK293E cells were selected for this investigation as they express CNTF receptors, however they enabled me to look at STAT3 expression in a more general context. Yokogami showed evidence that CNTF stimulation activated mTORC1 signalling within the NBFL cell lines, this has also been previously demonstrated within cardiac myocytes [403]. However, an analysis of phospho-rpS6 levels as an indicator of mTORC1 activity in figure 5.2 does not support this, which may indicate that HEK293E cells do not respond in the same manner as NBFL cells to CNTF, although the possibility of very short term mTORC1 activation cannot be ruled out.

Figure 5.2 shows STAT3 transcriptional activity correlates with mTORC1 transcriptional activity, surprisingly however, the transcriptional activity did not appear to correlate with phosphorylation at either of the regulatory sites of STAT3 under CNTF stimulation (see columns 3 and 4 of figure 5.2). I postulated that the lysis protocol implemented was not sufficient to breakdown nuclear membranes and devised an alternative method.

Figure 5.3 once again demonstrates that STAT3 transcriptional activation correlates with mTORC1 activity. In addition to this, much greater levels of P-STAT3 Ser727 phosphorylation were observed when the QIA shredder was utilised to breakdown nuclear membranes (although this may also be a result of over expression of mTOR and Raptor). All cells were stimulated overnight with CNTF therefore Tyr705 phosphorylation was consistent across all conditions, P-STAT3 Ser727 phosphorylation however did show some differentiation. It appeared that expression of the active mutant of mTOR increased phosphorylation as indicated by the densitometry analysis (compare lanes 3 and 4 to 1 and 2) and this was inhibited by co-expression of Raptor mutant 4 as opposed to wild-type. However in the case of wild-type mTOR, Raptor mutant 4 expression appeared to increase the P-STAT3 Ser727 levels. This reason for this discrepancy is not clear, although a likely explanation may be that the luciferase levels are more representative of STAT3 activity over time whereas western blotting indicates the phosphorylation status at time of lysis.

As stated in the introduction, there are several different kinases known to phosphorylate STAT3 at the Ser727 site, it may therefore be likely that when mTORC1 was dominantly inhibited by Raptor mutant 4 that negative feedback loops were activated to increase Ser727 phosphorylation whilst mTORC1 was inhibited. The increased intrinsic activity of the active mutant may have prevented the activation of feedback mechanisms.

Due to the difficulty in dissecting specific mTORC1 mediated signalling events *in vivo*, I decided to investigate whether I could show evidence of mTORC1 activity towards STAT3 *in vitro.* I was unable to demonstrate Raptor interactions with STAT3 using a far western approach, this is likely to indicate that mTORC1 does not interact with STAT3 as readily as it does 4E-BP1 and supports a theory of a hierarchy amongst mTORC1 substrates. It is also a reflection of the lack of sensitivity of this particular methodology. This assay can be utilised to indicate positive interactions, however it is not sensitive enough to rule out interactions if they occur transiently.

The *in vitro* kinase assay was particularly useful in this instance as it allows for much greater control over experimental conditions and prevents cross talk between other signalling pathways. Using the *in vitro* approach, I was able to demonstrate for the first time that the full STAT3 protein can be phosphorylated directly by mTORC1 at Ser727 and that this could be propagated by the presence of Rheb. This confirms STAT3 as a direct substrate specifically for mTORC1 rather than any of its downstream effectors. It also suggests that the lack of Raptor interaction observed using the far western approach was likely to reflect the lack of sensitivity of the methodology as hypothesised.

To reinforce these findings, a radiolabelling experiment was carried out to establish whether rapamycin sensitivity could be replicated within mammalian cells. Over-expression of mTOR/Raptor alongside STAT3 allowed me to look at specific phosphorylation events under different treatments, something which the transcriptional luciferase assay does not permit. The radio-labelling experiment showed that Ser727 phosphorylation could be stimulated in an insulin dependent fashion which was subject to inhibition from rapamycin.

Insulin, as reported by Ceresa *et al.* caused a significant increase in the level of Ser727 phosphorylation whilst Tyr705 remained unaffected. I have demonstrated this and also shown that this can be inhibited by the specific mTORC1 inhibitor rapamycin. This experiment was carried out in the presence of the phosphatase

inhibitor okadaic acid, thus indicating that the rapamycin induced dephosphorylation of STAT3 is not a result of phosphatase activity. Further to this, I was able to co-purify trace amounts of Raptor with STAT3, indicating that mTORC1 specifically interacts with STAT3 *in vivo*. This taken in conjunction with the *in vitro* kinase assay confirms that STAT3 is a direct substrate for mTORC1 and is consistent with several cancer cell line studies indicating that STAT3 is activated downstream of mTORC1 [400, 401].

Interestingly, a series of papers by Prof. David Kwiatowski's research group has investigated STAT3 regulation in the context of TSC. Kwiatowski observed upregulation of both Tyr705 and Ser727 phosphorylation of STAT3 in TSC1/2 deficient null murine neuroepithelial cells. This is unexpected given that I have seen no mTORC1 directed effects towards Tyr705. It could be explained however by a recent paper by Dr Elizabeth Henske, Henske's group showed that Notch signalling could be activated directly by Rheb in an mTORC1 independent fashion (see introduction section 1.7.1.1) [279]. It has previously been identified that expression of Hes 1 and Hes 5 (direct Notch targets) can induce Tyr705 phosphorylation of STAT3 [404]. Therefore, this may explain why Tyr705 phosphorylation of STAT3 was reportedly elevated in TSC1/2-/- cells [399].

Kwiatowski also later demonstrated that rapamycin treatment of TSC1/2-/- cells induced IFN-γ secretion which triggered dephosphorylation of Tyr705-STAT3. I saw no effects upon Tyr705 phosphorylation in this study, suggesting that the rapamycin induced IFN-γ effect is caused by the lack of TSC1/2. Constitutive activation of mTORC1 in these cells may be causing repression of IFN-γ through unknown mTORC1 targets, which is then reversed upon rapamycin treatment.

Alternatively, it may indicate that the IFN-γ or rapamycin treatment is inducing dephosphorylation of Tyr705-STAT3 through induction of a PP1/2A type phosphatase which would have been inhibited by the inclusion of okadaic acid within the radiolabelling experiment. A similar scenario has been described for S6K1, whereby rapamycin treatment induces dephosphorylation of all sites not just those targeted by mTORC1 [157].

Further research is required to elucidate the mechanism behind this regulation, it would be particularly interesting to see if administering IFN-γ directly to TSC1/2-/- MEFs could induce Tyr705 dephosphorylation in the presence of okadaic

acid, or indeed whether rapamycin does affect IFN-γ expression in HEK293s as well as TSC1/2-/- MEFs.

Interestingly, Kwiatowski's group demonstrated that IFN-γ and rapamycin could act in synergy to not only suppress Tyr705 phosphorylation of STAT3 but also to induce apoptosis and reduce proliferation in TSC deficient cells [399]. This data indicates that STAT3 elevation in TSC cells is likely to contribute to the survival of these cells and therefore STAT3 should be considered a target for therapeutic intervention in TSC patients.

The next objective of the study was to examine the relationship between STAT3 and HIF-α. The first report of a functional link between STAT3 and HIF-α was reported in 2005, a study by Gray e*t al.* showed evidence that Src activation was required for hypoxia induced HIF-α expression and activation. They showed that this activation coupled with hypoxia also lead to an increase in STAT3 expression. Furthermore, they isolated an Src induced transcriptional complex consisting of STAT3, HIF-1α and their transcriptional co-activators p300 and Ref-1. This transcriptional complex was able to bind to and activate the VEGF promoter. They suggested that simultaneous binding of both STAT3 and VEGF caused maximal activation of VEGF [209]. An earlier study examining HIF-1α activation demonstrated that Src could activate HIF-1α via increased translation and demonstrated a reliance upon the mTOR signalling pathway [405]. Src activation is associated with oncogenic transformation and its activation is a feature of several cancers, since inappropriate activation of HIF-1α and STAT3 can promote the survival of the tumour cell phenotype, it is important to understand the mechanisms behind their regulation.

This data taken together with the results of this study demonstrating mTORC1 mediated regulation of both STAT3 and HIF-1α may suggest that the STAT3/HIF-α/p300/Ref-1 transcriptional complex is in fact mediated by mTORC1. I therefore decided to investigate whether HIF-1α co-purified with STAT3.

Interestingly, an interaction between HIF-1α and STAT3 was indeed observed. Endogenous HIF-1α co-purified with GST-STAT3 under all conditions. There was no apparent increase with mTOR/Raptor over-expression, although it could be argued that there may be a slight increase in HIF-1α binding in the insulin treated sample compared with the insulin and rapamycin treated cells (compare HIF-1α blot figure 5.6 'C', lanes 4 and 5). As modest differences in binding of HIF-1α to

STAT3 was observed, it is probable that this interaction is not primarily regulated by mTORC1, and may in fact be a result of increased HIF-1α translation in response to insulin rather than increased binding. Even under serum starved non-hypoxic conditions, HIF-1α is constitutively bound to STAT3. Given that I was unable to show direct mTORC1 mediated HIF-1α phosphorylation but did show Raptor interaction, it is possible that mTORC1 directed regulation of HIF-1α might be mediated by STAT3 phosphorylation. Since activated STAT3 translocates to the nucleus, it is possible that STAT3 phosphorylation also increases nuclear accumulation of HIF-1α as well, thus contributing to its activation.

Further studies are required to reveal whether STAT3 plays a regulatory role over HIF-1α or whether they have more of a symbiotic relationship. To try and gain insight into this, I decided to utilise the dominant HIF-1α negative mutant to observe the effects upon STAT3. Interestingly, STAT3 transcriptional activity was increased in response to expression of GST-HIF-TOS, with over-expression of wild-type HIF-1α having no effect. This is consistent with the notion that STAT3 is upstream of HIF-1α, suggesting that the increase in STAT3 activity is the result of a feedback loop initiated due to HIF-1α repression.

Although we are unsure of the mechanisms governing this regulation, it is of particular importance to note that inhibition of HIF-1α triggers an elevation of STAT3. If HIF-1α inhibitors are used in this context therapeutically, they may also function to promote the survival of the targeted cancer cells through activation of STAT3. This provides a case for the use of HIF-1α inhibitors in conjunction with STAT3 inhibitors to reduce the tumour cell phenotype.

Interestingly, HIF-1α appears to be bound to STAT3 in figure 5.6 'C' regardless of mTORC1 activity. This would support the argument for a regulatory role of STAT3 over HIF-1α. Furthermore, western blotting analysis determining expression levels of GST-HIF and GST-HIF-TOS revealed a substantial previously unseen mobility shift between the wild-type and TOS mutant of HIF-1α. (Compare lanes 2 and 3 of figure 5.7). The TOS mutant of HIF-1α showed a significant increase in mobility in comparison to the wild-type construct. This is likely to represent differences in phosphorylation status, whereby the GST-HIF-TOS protein is less phosphorylated as it resolves as a lower band. GST-HIF-1α may therefore be part of a protein complex that is targeted by a kinase or kinase(s), which may include

mTORC1, whereas GST-HIF-TOS is not and may be a direct result of activating STAT3 with CNTF treatment for the transcriptional assay.

To take this study further, I would have liked to be able to investigate whether GST-HIF-TOS was able to form a complex with STAT3. Furthermore, STAT3 contains two potential TOS motifs, FPMEL are amino acids 26-30 and FDMEL at amino acids 756-760. Therefore, it would be of interest to establish whether disruption of these possible TOS motifs could affect both HIF-1α binding and activation, as well as mTORC1 mediated phosphorylation of STAT3 on Ser727.

The final stage of this line of investigation was to look at STAT3 activation under the context of TSC. In chapters 3 and 4, I was able to demonstrate that mTORC1 can mediate HIF-1α regulation, however I also provided evidence for a TSC2 mediated, mTORC1 independent regulation of HIF-1α which may be contributing to the pathogenesis of TSC. Since I have shown evidence of a functional link between STAT3 and HIF-1α, I wanted to determine whether or not STAT3 was regulated in the same manner as HIF-1α in TSC-deficient cells. It seemed that the most logical way to compare the regulation of STAT3 was to establish whether or not re-expression of the TSC2 mutant constructs into TSC2 deficient cell lines produced similar effects upon STAT3 as they did HIF-1α. The transcriptional assay produced a similar looking graph, however there were some key differences indicating that STAT3 is not subject to modulation from TSC2 in the same manner as HIF-1α. Firstly, re-introduction of wild-type TSC2 back into these TSC2 null MEF cell lines caused suppression of STAT3 transcriptional activity as you would expect, however it caused an approximate 50% reduction in activity, whereas HIF-1α transcriptional activity was reduced by around 85%. The rapamycin treated sample also caused a similar level of inhibition towards STAT3. Crucially, there was no significant difference in STAT3 activation between rapamycin treated samples and those where TSC2 was re-expressed. This indicates that the elevation in STAT3 activity I observed is mediated solely by mTORC1 and is not directly modulated by TSC2 as observed with HIF-1α. The inhibition of STAT3 activity observed when mTORC1 was inhibited by rapamycin was significant, although no conditions saw a complete abolishment of STAT3 activity, this is likely to be due to the suppression of IFN-γ in TSC deficient cells as reported by Kwiatowski [399].

Finally, this assay supports the hypothesis that the L1460P 'GAP' mutant of TSC2 does retain some ability to inhibit HIF-1α but not mTORC1 as was

hypothesised in chapter 4, since no significant inhibition of STAT3 was observed with expression of this 'GAP' mutant. This suggests that the quantifiable transcriptional assay is sufficiently sensitive to measure subtle changes in activity, while other methods were unable to detect differences. This supports the work carried out in chapter 4, as I have demonstrated that HIF-1α is subject to differential regulation in the absence of TSC2 to that of STAT3. Furthermore I have identified STAT3 as being a direct downstream for mTORC1 and demonstrated mTORC1 directed phosphorylation of Ser727 both *in vivo* and *in vitro.* This may be contributing to the increased risk of malignancy seen with the hamartoma disorders effecting mTORC1, given that Ser727 phosphorylation has been demonstrated to play a role in Ras-mediated oncogenic transformation [113].

CHAPTER 6: FINAL DISCUSSION

6.1 SELECTION OF mTORC1 TARGETS

The initial aim of this study was to identify and characterise downstream substrates of mTORC1. At the commencement of this study, a report by Land *et al.* was published which showed evidence for mTORC1 involvement in the regulation of HIF-1α, this provided a starting point for my research. I was particularly interested in HIF-1α for a number of reasons. Firstly, it was one of the first examples of evidence that mTORC1 functions as a transcriptional regulator. There is a wealth of evidence concerning the role of mTORC1 in the regulation of translation (detailed within the Introduction – see section 1.2.2), however the role of mTORC1 in regulating gene transcription is much more vague. I therefore wanted to investigate how mTORC1 could manipulate transcriptional events and HIF-1α appeared to be an ideal candidate. Furthermore, the gene targets of HIF-1α are known to contribute significantly to the pathophysiology of a number of human diseases. For example, HIF-1α elevation plays a significant role in the tumourigenesis associated with Von-Hippel Lindau syndrome, whereby shRNA mediated depletion of HIF-1α prevents the formation of tumours in VHL defective renal cell carcinoma cell lines [406].

Furthermore, dysregulation of HIF-1α and its gene target VEGF is now considered to be a unifying feature of the familial hamartoma disorders which include TSC, Peutz-Jehgers syndrome, Cowden's syndrome and Bannayan-Riley-Ruvalcaba syndrome (BRRS). These diseases are characterised by loss of function mutations to tumour suppressors acting upstream of mTOR [285].

mTORC1 dysregulation has been reported in numerous cancer types, cervical, ovarian and pancreatic cancer all exhibit elevation of kinases upstream of mTORC1, including PI3 kinase and Akt, and are therefore likely to exhibit inappropriate mTORC1 activation [407]. In addition, the phosphatase PTEN which is a negative regulator of PI3 kinase/Akt signalling towards mTOR (see figure 1.5) is frequently mutated in a number of different sporadically occurring cancers including prostate cancer, glioblastoma, breast cancer and endometrial tumours [408, 409]. A recent study also carried out a mutational screen of the human cancer genome database and found two incidences of direct point mutations to mTOR which conferred constitutive activation, one was identified in a large intestine adenocarcinoma whilst the other was identified within renal cell carcinoma [301]. It is

likely that mTORC1 is inappropriately activated by point mutations in a variety of cancers as well as through mutations to upstream regulators [301, 410].

The selection of HIF-1α as a target for this study was based upon three premises. Firstly, mTORC1 signalling is elevated in many types of cancer and the familial hamartoma disorders. Secondly, HIF-1α is upregulated downstream of mTORC1 as demonstrated by Land *et al.* [135], and finally, upregulation of HIF-1α is sufficient to induce tumourigenesis in Von-Hippel Lindau disease which makes it a potential therapeutic target in the treatment of cancers where mTORC1 regulation is lost. Elucidating the mechanisms of signal transduction downstream of mTORC1 is paramount to developing suitable therapeutic interventions to target diseases where mTORC1 is upregulated.

STAT3 was also selected as suitable potential mTORC1 signalling target. It was also thought to be acting downstream of mTORC1, although had not been confirmed as a direct substrate. STAT3 also has a prominent role to play in the pathology of many of the diseases associated with mTORC1 activation. STAT3 activation is associated with increased cell growth, increased angiogenesis (VEGF is also a target for STAT3) and metastases [243, 411]. STAT3 is also thought to play a role in tumour induced immunosuppression [412]. Upregulation of STAT3 is seen in pancreatic and breast cancers, melanomas and undoubtedly many other cancer types. Many human tumours produce factors such as IL-6 and IL-10 which also function to activate STAT3, therefore STAT3 is often subject to 'feed-forward' mechanisms which further propagate its expression [412]. Of particular interest in this study however was the reported association between HIF-1α and STAT3.

As described in chapter 5, STAT3 was reported to form a transcriptional complex with HIF-1α and co-factors p300 and Ref-1 [211]. It was also reported that activated STAT3 stabilised HIF-1α protein and increased HIF-1α mRNA levels [413]. This study aimed to elucidate the mechanisms behind mTORC1 mediated regulation of both HIF-1α and STAT3 to determine how they may be disrupted in disease.

6.2 SIGNIFICANT FINDINGS

6.2.1 Relationship between HIF-1α and mTORC1

I initially began to investigate the relationship between HIF-1α and mTORC1. I was able to verify the findings of Land *et al.* to demonstrate that HIF-1α was regulated in

an mTORC1 dependent fashion. The mechanism(s) behind this was unknown, however Land *et al.* previously demonstrated that the TOS-mutant of HIF-1α was degraded at the same rate as wild-type HIF-1α, furthermore they showed that the high levels of HIF-1α activity in VHL-/- MEFs were still highly sensitive to rapamycin [135]. I showed further support for this by demonstrating that rapamycin is unable to influence the degradation of the HIF-1α protein during hypoxia when its synthesis is inhibited. I postulated that mTORC1 must instead regulate the activity of HIF-1α directly and/or enhance the gene-expression of HIF-1α at the transcriptional or translational level (or a combination of all these processes).

Under hypoxia, the cell alters mRNA expression and translation to cause a general suppression of protein synthesis. However, simultaneously it is also able to upregulate proteins which are associated with the hypoxic response, including HIF-1α. The mechanism behind this selection is unclear but there has been speculation within the literature. The S6 kinases were originally thought to selectively enhance the translation of 5'-TOP mRNA's which are a subset of mRNAs associated with cellular growth pathways, for example the ribosomal proteins [414]. HIF-1α mRNA contains large 5'-terminal oligo-polypirimidine tracts (5'-TOP-structures) within the 5'-untranslated region [415]. It was, therefore, speculated that during a general suppression of mTOR and cap-dependent translation (i.e., during hypoxia, see section 1.4.5) the long pyrimidine tracts found within HIF-1α 5'-UTR confer a translational advantage that was mediated by S6K1 activation [416]. However, my results indicated that S6K1 was not involved in mTORC1 directed regulation of HIF-1α. Interestingly, as this thesis was being compiled, a paper was published which demonstrated that shRNA-mediated knockdown of S6K1 suppressed HIF-1α expression in PTEN deficient cells without effecting mRNA levels. Tandon *et al.* concluded that S6K1 regulates the translation of HIF-1α [417]. This is in direct contrast with the results reported in this study demonstrating that 4E-BP1 is able to regulate the translation of HIF-1α independently of S6K1 activity. Conversely, S6K1 is known to phosphorylate proteins involved in regulating cap-dependent translation (see figure 1.8 'Introduction'), this may therefore suggest that S6K1 is required for HIF-1α translation but is not a rate-limiting factor. This is consistent with recent evidence suggesting that the 4E-BP1 component of the mTORC1 signalling pathway is protected from hypoxic induced repression in contrast to S6K1 which is severely repressed during hypoxia [355]. It is likely that 4E-BP phosphorylation is protected

from hypoxia induced suppression of mTORC1 so that some cap-dependent translation may still occur under hypoxia, presumably to allow the cell to respond to the lack of oxygen, at least in part through activation of HIFs.This may therefore explain how mTORC1 is able to continue HIF-1α translation under a hypoxia-induced general repression of protein translation. I therefore propose that the reduction in HIF-1α seen with S6K1 knockdown by Tandon *et al.* [417] is caused by a reduction in the levels of the translational machinery required for protein synthesis. Figure 1.8 (introduction) demonstrates the role S6K1 plays in protein translation and indicates how protein translation may be inhibited through S6K1 knockdown, the results of this study however suggest that the availabillity of eIF4E is a rate limiting step in the specific translation of HIF-1α mRNA.

Recent evidence suggested that S6K1 is not involved in the translation of 5'-TOP mRNAs therefore it was important for this study to determine whether the 5'-TOP motif could confer a translational advantage to HIF-1α during hypoxia. However a comparison of HIF-1α translation with and without the 5'-TOP motif saw no difference between the rate of translation under any condition. This is in concordance with current thinking that HIF-1α is not a genuine 5'-TOP mRNA [355, 357]. This notion is also supported by work from Choo *et al.* who reported that the rate of 5'-UTR driven translation of HIF-1α correlated with the phosphorylation status of 4E-BP1 [418]. Furthermore, a study by Young *et al.* reported that actually the rate of HIF-1α translation was proportional to the abundance of HIF-1α mRNA rather than selective properties of the mRNA enhancing the rate of translation [419].

Analysis of HIF-1α mRNA levels revealed that they are regulated in an mTORC1 dependent fashion that is sensitive to rapamycin inhibition. This indicates a secondary facet by which mTORC1 is able to regulate HIF-1α. I also revealed that the mTORC1 substrate recognition component, Raptor could interact directly with HIF-1α and less well to a mutant of HIF-1α containing a mutated mTORC1 signalling motif. This implies that HIF-1α is a direct substrate for mTORC1 however I was unable to demonstrate direct phosphorylation of HIF-1α by mTORC1 in our *in vitro* kinase assays. These findings are discussed later in relation to STAT3.

6.2.2 TSC2 regulation of HIF-1α

Tuberous sclerosis is a genetic disease arising from genetic mutation to either the TSC1 or TSC2 gene (see section 1.7.1.1). There is evidence to suggest that patients presenting with mutations to the TSC2 gene present with a more severe phenotype compared to those containing a mutation to the TSC1 gene [367]. This is surprising given that TSC1 and TSC2 exert their activity by forming a tumour suppressor complex, therefore loss of function of either TSC1 or TSC2 should exert the same effects downstream. TSC2 may therefore play a more integral role in tumour suppression than TSC1. Clinical trials utilising rapamycin or its derivatives (termed rapalogues) in the treatment of TSC have shown some promising results with rapamycin causing shrinkage of AML's in TSC patients with long term treatment (12 months) [420]. However the results of clinical trials in this field have not been as dramatic as first hoped, causing a switch in the focus of research in the field of TSC. Research is now devoted towards understanding functions which are insensitive to rapamycin inhibition, and uncovering feedback mechanisms which are disrupted upon rapamycin treatment.

As described in chapter 4, there is now evidence to suggest that TSC1 and TSC2 are multi-functioning proteins and characterising these mTORC1 independent interactions is paramount to understanding the mechanisms leading to the manifestations seen in TSC patients, as well as determining the efficacy of mTORC1 inhibitors in therapeutic strategies.

During my investigations into the regulation of HIF-1α, I observed that re-introduction of TSC2 into TSC2-/- MEFs induced a more robust inhibition of HIF-1α transcriptional activity than rapamycin treatment alone. Given the emphasis upon discovering mTORC1 independent functions of TSC1 and TSC2, I decided to analyse the activation of HIF-1α gene targets in the context of TSC1 and TSC2 loss. My analysis revealed that rapamycin treatment normalised HIF-1α activity in the absence of TSC1 but not TSC2. Rapamycin treatment of TSC1-/- MEFs normalised HIF-1α mediated gene expression to a level which was not significantly different to that of the TSC1+/+ MEFs. Conversely, rapamycin treated TSC2-/-MEFs showed significantly higher levels of HIF-1α mediated gene expression in the presence of rapamycin than the untreated wild-types. This suggests that TSC2 is able to regulate HIF-1α both dependently and independently of mTORC1. This could imply that TSC

sufferers exhibiting mutations to the TSC2 gene may benefit from the use of specific HIF-1α inhibitors in conjunction with mTORC1 inhibitors.

This hypothesis was further supported by the functional analysis of patient derived TSC2 mutations whereby every TSC2 variant exhibited some level of HIF-1α inhibition, with the exception of the Arg1743 variants. This evidence suggests a role for this Arg1743 residue in negative regulation of HIF-1α by TSC2 which occurs independently of mTORC1. The mechanism behind this has yet to be elucidated, however the location of the Arg1743 mutation provides some clues as to the mechanism. This Arg1743 residue is found within a transcriptional activation domain, overlapped by a calmodulin binding domain [421] and a peroxisome targeting sequence-1 motif (Kim *et al.* unpublished data). The potential significance of each of these is described below.

TSC2 has previously been associated with the regulation of transcriptional events. A series of publications by Noonan *et al.* provide intriguing evidence concerning the role of TSC2 in the mediation of transcriptional events relating to steroid hormone receptors [350-352, 362, 369, 422]. TSC2 mediated regulation of the oestrogen receptor is the most well defined mechanism of TSC2 independent function, This finding of TSC2 independent funciton is particularly significant when you consider that a particularly severe manifestation of TSC is LAM (see introduction section 1.7.2.1) which appears almost exclusively in women [423] and also shows a higher incidence of TSC2 mutations [424]. York *et al.* describes how calmodulin and the oestrogen receptor compete for binding to the carboxyl terminal of TSC2 [350]. It appears that calmodulin and TSC2 function in opposition to co-ordinate the activity of the oestrogen receptor, i.e., when the oestrogen receptor is bound to calcium loaded calmodulin, its transcriptional activity is enhanced. Conversely, when the oestrogen receptor binds to TSC2, DNA binding is inhibited. York *et al.* identified a CaM/oestrogen receptor binding domain which also functioned as a nuclear localisation sequence, the first residue of which is Arg1743. York reported that the nuclear localisation of TSC2 was induced by phosphorylation of Ser1798 by p90-RSK and was also required for TSC2 mediated inhibition of the oestrogen receptor [369]. Due to the similarities seen between the regulation of the oestrogen receptor and HIF-1α, I hypothesised that the nuclear localisation of TSC2 may be required for its mTORC1 independent HIF-1α regulation. I predicted that the Arg1743 mutants may be defective in their nuclear translocation since Arg1743 is the first residue in

the nuclear localisation sequence and this may prevent interactions with nuclear HIF-1α. However as described in chapter 4 the Arg1743 mutation does not impair the ability of TSC2 to translocate to the nucleus.

Interestingly, Motet *et al.* demonstrated that calcium induced calmodulin activity promoted phosphorylation of ERK1/2 and activation of HIF-1α [375]. p90-RSK is a known downstream substrate of ERK1/2 which phosphorylates TSC2 at Ser1798 and or Ser664 which is inhibitory of the tuberous sclerosis tumour suppressor complex [425]. Therefore, it is possible that Ca2+/CaM/ERK1/2 may up-regulate HIF-1α by two mechanisms, firstly via mTOR activation and secondly by relieving TSC2 mediated inhibition of HIF-1α. In order to determine this, the effects of TSC2 phosphorylation upon HIF-1α would have to be examined outside of the capacity to activate mTORC1. It would be of interest to repeat the analysis of TSC2 mutations upon HIF-1α activity in the context of shRNA mediated Raptor knockdown (or Raptor mutant 4 expression) to eliminate mTORC1 from the equation. This would give insight into the role TSC2 plays outside of its ability to downregulate mTORC1 signalling.

The work by Kim *et al.* in Prof. Cheryl Walker's lab (unpublished data and personal communication) indicated that the R1743G/Q/W mutations are defective at inhibiting mTORC1 due to mislocalisation of TSC2. They show localisation of mTORC1, Rheb and the TSC to the peroxisomes and argue that the R1743G/Q/W mutants of TSC2 lose their ability to inhibit mTORC1 because they are unable to translocate to the peroxisomes and interact with TSC1. They show evidence that the Arg1743 mutation prevented interaction of TSC2 with peroxisomal import receptors.

This may suggest that the loss of TSC2 mediated inhibition of HIF-1α is a product of its mislocalisation away from the peroxisomes. Intriguingly, Kim *et al.* reported that by ataxia telangiectasia mutated (ATM)-dependent kinase also contained a PTS sequence and thus also localised to the peroxisome. ATM is a kinase from the phosphatidylinositol 3'-kinase (PI3K)-related kinase (PIKK) family of which mTOR also belongs to. mTOR and ATM share significant sequence homology suggesting similar mechanisms of regulation [72]. When exposed to DNA damage, cells respond by activating repair pathways as well as suppressing cellular proliferation. ATM is responsible for this regulation and mediates it's effects by phosphorylation of multiple downstream substrates, for example, p53, Chk2 and MDM2 are all regulated downstream of ATM and modulate cell cycle arrest [426].

Interestingly, as this thesis was being compiled, a paper was published revealing that ATM kinase activity was activated in response to hypoxia independently of the DNA damage response [427]. Furthermore, it was demonstrated that ATM could directly phosphorylate HIF-1α at Ser696 in an *in vitro* kinase assay resulting in HIF-1α stabilisation. Cells deficient of ATM failed to accumulate the HIF-1α protein and also failed to suppress mTORC1 signalling in response to hypoxia indicating that the HIF-1α target REDD1 was not expressed (see section 1.4.5.2) [427]. This may imply an association between localisation of ATM and TSC2 to the peroxisome and HIF-1α stability. It may be of interest to compare the localisation of ATM in TSC1 and TSC2 deficient cells to see if it differs. Equally it may also be of significance that ATM is an upstream regulator of p53, the TSC2-/-MEFs utilised for this study are p53 deficient to confer viability, this however may cause disturbances in negative feedback signalling to ATM which could affect HIF-1α. This is one of the disadvantages of utilising cell lines as a model for disease as inevitably there are physiological differences. In chapter 4, I also attempted to use immunohistochemistry to assess the levels of HIF-1α gene target expression in TSC1 and TSC2 heterozygous mice however VEGF expression was too variable to make any reasonable comparison between genotypes and this line of enquiry was not pursued.

Peroxisomes are heavily involved in the regulation of metabolic cellular processes and have been demonstrated to co-ordinate the metabolism of lipids and reactive oxygen species (ROS) in conjunction with the mitochondria [26]. Interestingly ROS have been demonstrated to activate the transcription of HIF-1α in a mechanism dependent upon NF-κB (nuclear factor kappa B) [428].

It may therefore be possible that TSC2 is involved in the metabolism of ROS at the peroxisome and that the elevation of HIF-1α in TSC2-/- MEFs is a product of increased ROS levels as well as unrestrained mTORC1 signalling. Interestingly TSC2 has been previously linked to ROS production, Suzuki *et al.* demonstrated how mutant forms of TSC2 caused activation of Rac1 stimulating NAD(P)H oxidase which results in the generation of ROS [378].

This may explain why the Arg1743 mutants of TSC2 were ineffective at inhibiting HIF-1α as they are unable to translocate to the peroxisome to assist in ROS metabolism or to inhibit mTORC1 signal transduction in concert with TSC1 this potential mechanism (see figure 6.1 for potential mechanism). Other mutant forms of

TSC2 may retain some partial inhibitory activity towards mTORC1 or Rac1 allowing some suppression of HIF-1α. This theory is demonstrated in figure 6.1 overleaf, along with other proposed mechanisms of regulation.

It may therefore be of interest to investigate the levels of ROS production in the TSC1 and TSC2-/- MEFs, if indeed TSC2 is involved in ROS metabolism then this may be contributing to the pathology of TSC and may be a factor in the more severe pathology seen with TSC2 mutations compared with TSC1. Further work is required to determine the exact mechanism governing TSC2 mediated regulation of HIF-1α.

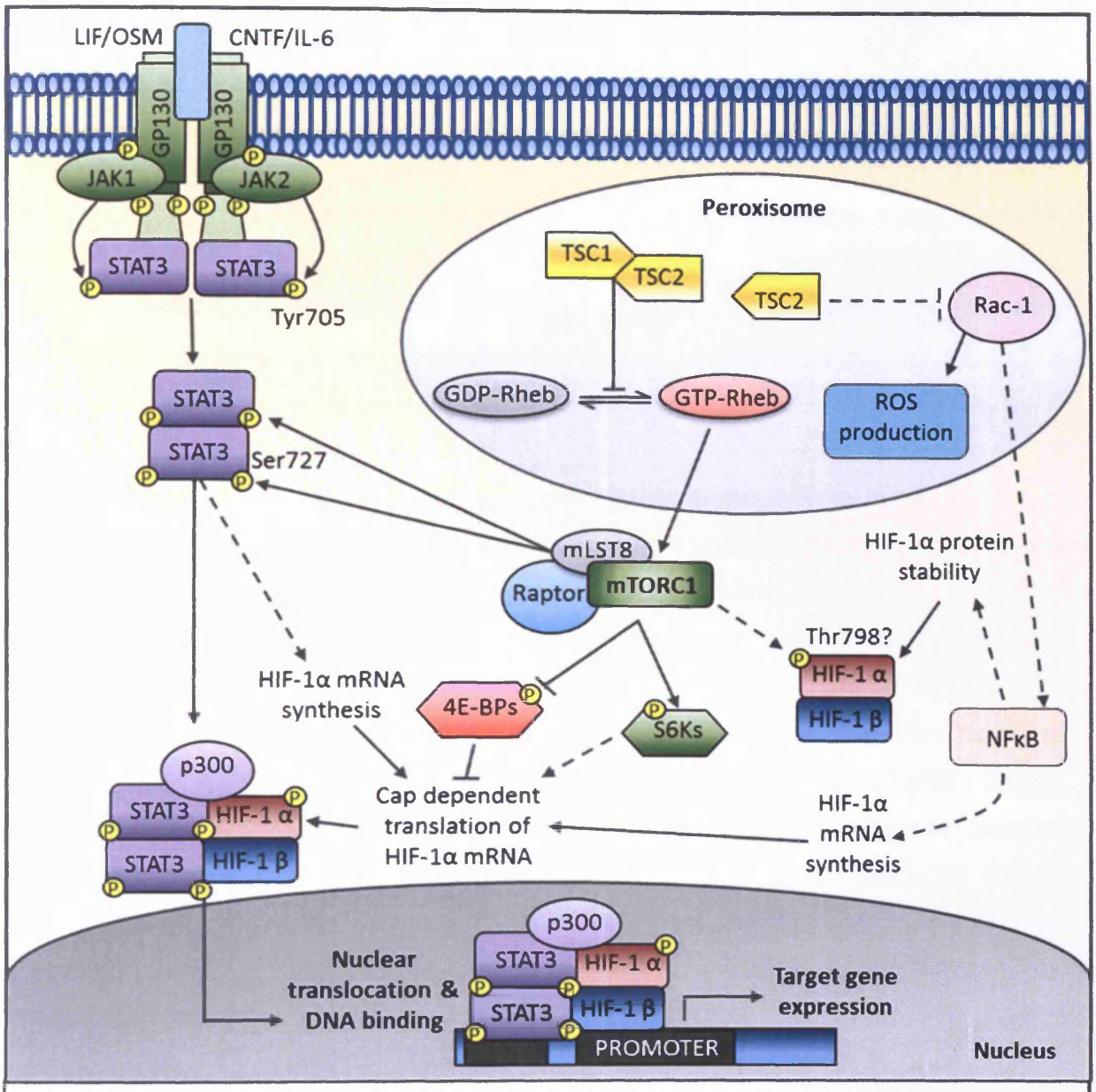


Figure 6.1 Potential mechanisms governing HIF-1α and STAT3: GP130 receptor is activated by several different cytokines causing activation of JAKs, JAKs then phosphorylate the receptor and STAT3 at Tyr705 promoting dimerisation and nuclear translocation. Activated STAT3 can promote HIF-1α mRNA expression through undefined mechanisms. HIF-1 and STAT3 appear to form a transcriptional complex for maximal activation of target genes. mTORC1 can also phosphorylate STAT3 at Ser727 to promote transcriptional activity. mTORC1 can promote translation of HIF-1α mRNA to increase its synthesis through 4E-BP1 phosphorylation, S6K1 activity may be required but is not rate limiting. mTORC1 may also be able to phosphorylate HIF-1 directly to propagate its transcriptional activity (Thr798). TSC2 can suppress HIF-1α through mTORC1 inhibition but also through suppression of Rac1. Rac1 activates NAD(P)H to promote ROS production. ROS is able to influence the transcription of HIF-1 as well as the protein stability, this is dependent upon NFκB.

6.2.3 STAT3, mTOR and HIF-1α

In chapter 5, I analysed the relationship between mTORC1 and STAT3. I demonstrated that Tyr705 was constitutively activated upon CNTF stimulation. Under these conditions, mTORC1 regulated the transcriptional activity of STAT3, most likely via it's direct phosphorylation of STAT3 at Ser727. Intriguingly, I also observed co-purification of both HA-Raptor and endogenous HIF-1α with STAT3, despite a lack of hypoxia to stabilise HIF-1α. This may suggest that both HIF-1α and STAT3 are part of a larger regulatory complex which may be regulated by mTORC1. The hypothesis is supported by the fact that p300 functions as a transcriptional co-factor for both HIF-1α and STAT3.

Furthermore, Land and Tee demonstrated that the inactive TOS mutant of HIF-1α showed reduced binding to p300 [135], which suggests that the TOS motif in HIF-1α facilitates protein interactions. Other groups have also reported STAT3 mediated regulation of HIF-1α. One group suggested that it was modulated by IL-6 stimulation [429], whilst another showed a dependency upon hypoxia induced Src activation [209]. While both studies demonstrated that Tyr705 phosphorylation of STAT3 was required for this, neither studies investigated STAT3 Ser727 phosphorylation levels. I propose that cytokine stimulation via the gp-130 receptor (or hypoxic induction of Src), induces robust Tyr705 phosphorylation of STAT3 allowing complex formation with HIF-1α. Tyr705 phosphorylation was rapidly induced by CNTF treatment and unaffected by mTORC1 activity in all experiments, additionally the level of HIF-1α co-purified with STAT3 correlated with Tyr705 phosphorylation not Ser727 phosphorylation or mTORC1 activity.

I also propose that mTORC1 propagates the transcriptional activity of both HIF-1α and STAT3 at least in part by phosphorylating STAT3 at Ser727. This notion is supported by my findings, where I saw that mTORC1 interacted with but did not phosphorylate HIF-1α, whereas STAT3 co-purifies with both HIF-1α and Raptor, and is phosphorylated at Ser727 by mTORC1 to achieve maximal transcriptional activation. This mechanism is in concordance with current thinking that STAT3 and HIF-1α form a transcriptional complex in response to activation by IL-6 [429], since both IL-6 and CNTF activate STAT3 via the GP-130 receptor [67].

In addition, Gray *et al.* demonstrated that Src inhibition prevented nuclear translocation of the STAT3/HIF-1α complex and hence it's activation [209]. Src is a protein tyrosine kinase which is activated by growth factors and hypoxia, it is also

upstream of PI3 kinase and promotes mTORC1 signalling. In addition, it has been demonstrated to activate STAT3 via Tyr705 phosphorylation [106], likely through direct phosphorylation of STAT3 and through activation of JAKs. This is consistent with my proposed mechanism of regulation as it provides a mechanism by which hypoxia could upregulate both branches of the pathway. Consequently, it is likely that mTORC1 activates a STAT3/HIF/p300 transcriptional complex and this is regulated in part by mTORC1 directed phosphorylation of STAT3 at Ser727 and maybe at other unidentified phosphorylation sites. This possible mechanism may explain why I was unable to demonstrate direct phosphorylation of HIF-1α by mTORC1 despite showing Raptor interaction. It may be that crucial components of the HIF transcriptional complex were lost during purification, i.e. STAT3/p300 which prevented it's *in vitro* phosphorylation. This mechanism is also consistent with the fact that a large mobility shift was observed upon expression of the TOS mutant in comparison to the wild-type GST-HIF-1α and is indicative of differential phosphorylation events between the two HIF-1α constructs. In my previous analysis of the TOS mutant of HIF-1α and the wild-type I had not observed a shift in mobility. However, this experiment was carried out under the context of CNTF stimulation (to activate the STAT3 transcriptional assay) and is likely to be a result of upregulation of STAT3 and or mTORC1.

Further work is required to confirm this mechanism, however time and financial constraints prohibited this within the scope of the PhD project. To test whether this hypothesis is correct, one would have to address several questions that are raised. For instance, the nature of the p300 interaction with both HIF-1α and STAT3 needs to be further elucidated. Land and Tee reported that the TOS mutant of HIF-1α bound less well to p300 [135], suggesting that p300 may play a role in regulating the transcriptional activity of HIF-1α, it also highlights the possibility that p300 may be a direct substrate for mTORC1 itself. I demonstrated that the TOS mutant of HIF-1α bound less well to Raptor, therefore it is likely that the TOS motif within HIF-1α is necessary for optimal protein interactions. Interestingly, the HIF-1α TOS mutant is still able to translocate to the nucleus [135] so the lack of activity within this HIF-1α TOS mutant is likely due to absent phosphorylation events or a result of co-factor dissociation. It is still unclear whether this HIF-1α TOS mutant is able to interact with the HIF response elements on DNA, I attempted EMSA (enhanced mobility shift assays) to examine interactions of HIF-1α to [^{32}P]-radio-

labelled dsDNA possessing a HRE, but these EMSA assays where inconsistent for both HIF-1α and STAT3. Robust EMSA assays could prove useful to further characterise the interactions of HIF-1α with DNA as well as protein interactions such as Raptor and STAT3 could be of use for future work in this field.

It would also be of interest to determine whether the TOS mutant of HIF-1α could also bind with STAT3 and if so what affects this has upon STAT3 phosphorylation sites and transcriptional activity. Furthermore, STAT3 has two potential TOS motifs, the significance of these motifs remains unknown. It would therefore be informative to establish what effect mutations to one or both of these motifs has upon STAT3/HIF-1α activity and to determine the role of mTORC1 interactions in governing this complex.

In addition, Jung *et al.* reported in 2005 that over-expression of STAT3 resulted in increased levels of HIF-1α mRNA [413]. In chapter 3, I observed that HIF-1α mRNA levels are regulated in an mTORC1 dependent fashion, this taken with the evidence in chapter 5 indicating that STAT3 activity and Ser727 phosphorylation is regulated by mTORC1 could indicate that HIF-1α mRNA levels are being regulated downstream of mTORC1 and STAT3. STAT3 may therefore be responsible for regulating the expression or stability of HIF-1α mRNA.. It would be interesting to see whether STAT3 inhibitors could suppress HIF-1α mRNA levels. The use of specific STAT3 inhibitors or shRNA mediated knockdown of STAT3 may have a similar effect to rapamycin treatment upon HIF-1α mRNA levels if STAT3 functioned as a feedforward mechanism to drive HIF-1α gene expression.

Recent publications have also highlighted a potential role of heat-shock protein 90(Hsp90) in the modulation of HIF-1α and STAT3. It was demonstrated that IL-6 induced both STAT3 activation (as measured by Tyr705 phosphorylation) as well as HIF-1α nuclear accumulation in a human pancreatic cancer cell line in agreement with the results of this study. Interestingly, they also demonstrated that IL-6 mediated upregulation of STAT3 and HIF-1α could be repressed with the use of Hsp90 inhibitors. Lang reported that this was independent of PI3K signalling since wortmannin did not suppress STAT3 activation, however this was only assessed in the context of Tyr705 phosphorylation which I have shown to be independent of mTORC1 signalling. Furthermore, they revealed that activation of signalling via IL-6/STAT3/HIF-1α also increased levels of IL-6. This represents an autocrine loop

whereby STAT3 and HIF-1α are able to propagate their own activation, although this may be unique to the cancer cell line utilised [429].

Hsp90 inhibitors may therefore provide an exciting new branch of anti-cancer therapeutics in the future with their ability to target multiple signalling pathways which contribute significantly to the pathogenesis of many different cancers.

In addition to the link between Hsp90 and STAT3/HIF-1α, there is also a reported link between STAT3 and ATM, as indicated earlier, ATM phosphorylates HIF-1α to promote its stability. This is interesting when you consider that ATM signalling is suppressed in STAT3 deficient cells, suggesting that STAT3 is a modulator of ATM [33]. Cam *et al.* showed that ATM phosphorylated HIF-1α directly to promote its stability [427]. If STAT3 can modulate ATM signalling in response to DNA damage, it is plausible that STAT3 can also modulate ATM signalling in response to hypoxia. Induction of ATM by STAT3 could represent a mechanism by which STAT3 promotes the accumulation of HIF-1α protein. Conversely, a paper published in 2003 claimed that STAT3 Ser727 phosphorylation could be induced by UV light and that ATM was required for this [430]. This is consistent with the report associating STAT3 with the DNA damage response since UV light is known to cause damage to DNA. However, Zhang *et al.* reported that ATM deficiency abolished Ser727 phosphorylation of STAT3 [430], whereas the more recent publication reported that STAT3 deficiency suppressed ATM signalling [431]. The reason for this discrepancy is not clear although it may be related to ATM activation by hypoxia, which appears to differ from ATM activation by DNA damage. Nevertheless, these studies clearly provide evidence for an association between ATM and STAT3, which importantly implicates ATM in the regulation of STAT3/HIF-1α. It would be particularly interesting to establish whether ATM was responsible for activating STAT3 under hypoxia, or if indeed STAT3 mediates ATM under hypoxia. Further work would have to be carried out to determine ATM's involvement in STAT3/HIF-1α regulation.

6.3 AIMS

I initiated this project with the following aims:

1. To characterise mTORC1 dependent regulation of HIF-1α.
2. To examine HIF-1α regulation in the context of the disease Tuberous sclerosis.
3. To determine whether STAT3 is regulated by mTOR
4. To determine whether mTOR regulates STAT3 directly or via a downstream effector.
5. To investigate the relationship between mTOR, STAT3 and HIF-1α.

I investigated several avenues of potential mTORC1 directed HIF-1α regulation and was able to examine the translation of HIF-1α, the stability of the HIF-1α protein and mRNA transcript as well as showing evidence for TSC2-mTORC1 independent mechanisms of HIF-1α regulation. Utilising TSC2 mutant constructs I have been able to demonstrate how variable HIF-1α activity may be from patient to patient in the context of TSC, as well identifying the TAD domain of TSC2 as a potential regulatory motif for HIF-1α activity. Analysis of downstream gene targets of HIF-1α revealed previously unreported potential differences in HIF-1α regulation. If these differences translate from cell line models to patients then it is possible that patients with specific TSC2 mutations may exhibit a more severe phenotype due to differential regulation of HIF-1α activity (discussed later).

Furthermore I have clearly demonstrated that mTORC1 regulates STAT3, through direct phosphorylation at Ser727 in both *in vitro* and *in vivo* assays. I also observed that STAT3 transcriptional activity correlates with mTORC1 activity, suggesting that the Ser727 phosphorylation site is key at determining downstream gene expression of STAT3.

Further work is required to fully clarify the relationship between mTORC1, STAT3 and HIF-1α. My *in vivo* radiolabelling experiment demonstrated that both HIF-1α and to a lesser extent Raptor co-purify with STAT3. From this we can hypothesise that mTORC1 may regulate a transcriptional complex consisting of STAT3 and HIF-1α. Furthermore I demonstrated that expression of the dominant negative TOS mutant of HIF-1α can influence STAT3 transcriptional activity, suggesting an

interdependent relationship between HIF-1α and STAT3 however the mechanisms governing the activity of this complex however remain unknown.

6.3.4 Significance

The significance of these findings can best be described in the context of the disease TSC which is characterised on a molecular level by aberrant mTORC1 signalling. My findings explain in part how constitutive mTORC1 activation in TSC patients can increase the levels of HIF-1α activity in cells by enhanced expression/stability of HIF-1α mRNA, translation of HIF-1α protein, as well as potential upregulation of HIF-1α activity, promoting angiogenesis, glucose transport, and erythropoeisis.

HIF-1α upregulates the expression of genes required for tumour expansion and there is no doubt that this significantly facilitates the growth of hamartomas characteristic of the TSC disease. Furthermore, I have provided evidence that HIF-1α is also subject to negative regulation downstream of TSC2 which is independent of mTORC1. I demonstrated that in cell lines deficient of TSC2, rapamycin was unable to normalise HIF-1α gene targets expression. This may in part contribute to the limited success of rapamycin observed in the ongoing clinical trials and suggests the requirement for combinational therapy for patients exhibiting TSC2 mutations, whereby a specific HIF-1α inhibitor is administered in conjunction with rapamycin to target both pathways. However the results of this study also indicate that targeting HIF-1α activity therapeutically may result in an upregulation of STAT3 transcriptional activity by feedback mechanisms, given that expression of the dominant TOS-mutant of HIF-1α caused an increase in STAT3 activity as demonstrated by the transcriptional reporter assay. This indicates that STAT3 plays more of a regulatory role over HIF-1α indicating that STAT3 may be the more appropriate therapeutic target.

This novel mTORC1 independent function of TSC2 towards HIF-1α may also partially account for the increased severity of TSC in patients who possess *TSC2* mutations as opposed to *TSC1* mutations, HIF-1α mediated gene expression could promotes vascularisation and growth of hamartomas, increasing the likelihood of complications in affected organ systems.

I have also demonstrated that STAT3 is regulated at Ser727 by mTORC1 directly, therefore elevation of STAT3 transcriptional activity will also contribute to the pathogenesis of the disease. Kwaitowski's group observed an increase of STAT3

expression in TSC deficient cells lines. Interestingly, they reported upregulation of STAT3 at Tyr705 caused by a suppression of IFN-γ.

IFN-γ is a pleiotropic cytokine which is involved in tumour suppression, therefore it's suppression in the absence of TSC1/2 is likely to contribute to the formation of hamartomas in TSC. Kwaitowski's group did not speculate as to the mechanism behind IFN-γ suppression but it maybe a result of feedback mechansims instigated as a result of upregulated STAT3 activity (through increased Ser727 as mediated by mTORC1). Alternatively it may be a previously uncharacterised downstream signalling effect of mTORC1 signalling.

There are extensive reports within the literature demonstrating persistent STAT3 upregulation in numerous human cancers and transformed cell lines [432-439]. This has lead to the classification of STAT3 as an oncogene [243]. STAT3 activation is thought to contribute to the growth of tumours. STAT3 increases gene expression of cyclin-D1 and c-Myc, which promote cell cycle progression, as well as promoting the survival of tumour cells by inhibiting apoptosis through Bcl_{xL} expression [432, 434].

Furthermore, STAT3 is able to promote angiogenesis in tumours through direct activation of VEGF as well as probable activation of HIF-1α mediated gene expression as seen in this study and others [209, 413, 440].

STAT3 mediated gene expression effectively functions as the 'tumour cell survival kit', facilitating cellular growth, inhibiting cellular death, whilst promoting the formation of blood vessels to increase nutrient supply and facilitating metastases by promoting cell migration and invasion. It is thought that constitutive STAT3 activation is required to maintain the tumour cell phenotype in certain malignancies therefore understanding mechanisms contributing to its regulation is of significant importance.

One example where STAT3 upregulation in cancer has been characterised is in the case of multiple myeloma. Multiple myeloma cells exhibit inappropriate elevation of IL-6 and expression of the IL-6 receptor resulting in constitutive activation of STAT3 through phosphorylation of Tyr705 [192, 441]. Furthermore, IL-6 not only promotes activation of STAT3 through homodimerisation of the gp-130 receptor, but also activates PI3 kinase and Akt signalling through receptor tyrosine kinase activity [442, 443], as is seen with the IL-6 related cytokine, CNTF, which was utilised in this study.

Therefore, in the case of multiple myeloma, inappropriate IL-6 production results in upregulation of STAT3 Tyr705 phosphorylation through the JAK/STAT signalling pathway, but also promotes mTORC1 signalling through PI3 kinase and Akt activation (see figure 1.5). As demonstrated in this study, mTORC1 activation serves to increase the transcriptional activity of STAT3 by increased Ser727 phosphorylation, further propagating the tumour phenotype. Activation of STAT3 can also function to promote HIF-1α activity and may augment HIF-1α mRNA levels, further promoting cell survival and vascularisation of malignant cells, as well as upregulating cell growth pathways mediated downstream of mTORC1.

This study has demonstrated how dual activation of STAT3 and pathways leading to mTORC1 activation might function to further accentuate the tumour cell phenotype.

Furthermore, mTORC1 activation is also associated with suppression of p53 mediated apoptosis [444], whilst STAT3 activation protects against apoptosis through increased Bcl_{xL} expression [445]. It is plausible that combined STAT3 and mTORC1 inhibition could be sufficient to induce apoptosis in tumour cells exhibiting upregulation of these pathways. In support of this, both mTORC1 inhibitors [446] and STAT3 inhibitors [447] have been demonstrated to sensitise tumour cells to DNA-damage induced apoptosis in separate settings. Further research is required to determine exactly how effective these treatments may be in combination.

It is also important to consider that IL-6 is likely to activate other cellular signalling pathways which may impact upon these signalling pathways. For instance, there are reports that IL-6 also functions to upregulate Ras-dependent ERK signalling [448], which also contributes to the tumour cell phenotype. Ras itself is also an oncogene and it has been demonstrated recently that Ser727 phosphorylation of STAT3 is necessary for Ras-mediated oncogenic transformation. With Ser727 phosphorylated STAT3 localising to the mitochondria, independently of Tyr705 phosphorylation and its DNA binding capabilities [113]. This indicates that STAT3 has functions beyond that of a transcription factor which are seemingly governed by Ser727 phosphorylation. It is therefore likely that diseases demonstrating elevation of mTORC1 signalling also exhibit increased mitochondrial STAT3, the significance of which remains unknown.

It may also be the case that this effect is contributing to the increased rate of malignancies observed in TSC patients since Ser727 phosphorylated STAT3 can contribute to Ras-mediated oncogenic transformation.

There is significant evidence demonstrating how STAT3 and mTORC1 signalling can be elevated in cancer as well as the cancer-like syndromes such as TSC. This study has demonstrated crosstalk between the signalling pathways which may be functioning to aggravate the tumour cell phenotype by increasing the transcriptional activity of both STAT3 and HIF-1α. However, further research is required to determine the full implications that these over-lapping cell signalling pathways can evoke. Utilising cell line models not only increases our knowledge base of how cell signalling pathways work but also allows us to predict what happens when they become dysregulated in human disease. This is indispensible in the process of developing new and innovative therapeutic interventions to assist in the ongoing battle against disease.

PUBLICATIONS ARISING FROM THIS THESIS

Dunlop, E. A., K. M. Dodd, et al. (2009). "Mammalian target of rapamycin complex 1-mediated phosphorylation of eukaryotic initiation factor 4E-binding protein 1 requires multiple protein-protein interactions for substrate recognition." Cellular Signalling **21**(7): 1073-1084.

Dunlop, E. A., K. M. Dodd, et al. (2011). "Determining the pathogenicity of patient-derived TSC2 mutations by functional characterization and clinical evidence." Eur J Hum Genet.

In submission:

J. Kim, A. Alexander, S. Cai, J. Zhang, A.R. Tee, J. Tait-Mulder, J.M. Han, E.A. Dunlop, K.M. Dodd, R.D. Folkerth, M.B. Kastan, M. Sahin and C.L. Walker "Identification of a TSC2-mTORC1 Signaling Node at the Peroxisome Disrupted in Tuberous Sclerosis Complex and Zellweger Peroxisome Biogenesis Disorder" (Cell).

In progress:

K.M. Dodd and A.R. Tee. "Characterisation of mTORC1 directed regulation of HIF-1α".

BIBLIOGRAPHY

1. Taylor, C.T. and J.C. McElwain, *Ancient Atmospheres and the Evolution of Oxygen Sensing Via the Hypoxia-Inducible Factor in Metazoans.* Physiology, 2010. **25**(5): p. 272-279.
2. Semenza, G.L., *Life with Oxygen.* Science, 2007. **318**(5847): p. 62-64.
3. Adam-Vizi, V. and C. Chinopoulos, *Bioenergetics and the formation of mitochondrial reactive oxygen species.* Trends in Pharmacological Sciences, 2006. **27**(12): p. 639-645.
4. Murphy, M.P., *How mitochondria produce reactive oxygen species.* Biochem J, 2009. **417**(1): p. 1-13.
5. Droge, W., *Oxidative stress and aging.* Advances in experimental medicine and biology, 2003. **543**: p. 191-200.
6. Barnham, K.J., C.L. Masters, and A.I. Bush, *Neurodegenerative diseases and oxidative stress.* Nat Rev Drug Discov, 2004. **3**(3): p. 205-214.
7. Aragonés, J., et al., *Oxygen Sensors at the Crossroad of Metabolism.* Cell metabolism, 2009. **9**(1): p. 11-22.
8. Mukherjee, S., et al., *Rapid analysis of the DNA-binding specificities of transcription factors with DNA microarrays.* Nat Genet, 2004. **36**(12): p. 1331-1339.
9. Latchman, D.S., *Eukaryotic Transcription Factors* 4E-BPsBiochem J, 1990. **270**(2): p. 281-289.
10. Saha, A., J. Wittmeyer, and B.R. Cairns, *Chromatin remodeling by RSC involves ATP-dependent DNA translocation.* Genes & Development, 2002. **16**(16): p. 2120-2134.
11. Robert G, R., *The complexities of eukaryotic transcription initiation: regulation of preinitiation complex assembly.* Trends in Biochemical Sciences, 1991. **16**(0): p. 402-408.
12. Smale, S.T. and J.T. Kadonaga, *The RNA polymerase II core promoter.* Annual review of biochemistry, 2003. **72**: p. 449-79.
13. Robert G, R., *The role of general initiation factors in transcription by RNA polymerase II.* Trends in Biochemical Sciences, 1996. **21**(9): p. 327-335.

14. Komarnitsky, P., E.-J. Cho, and S. Buratowski, *Different phosphorylated forms of RNA polymerase II and associated mRNA processing factors during transcription.* Genes & Development, 2000. **14**(19): p. 2452-2460.
15. Goodrich, J.A. and R. Tjian, *Transcription factors IIE and IIH and ATP hydrolysis direct promoter clearance by RNA polymerase II.* Cell, 1994. **77**(1): p. 145-156.
16. Proudfoot, N.J., A. Furger, and M.J. Dye, *Integrating mRNA Processing with Transcription.* Cell, 2002. **108**(4): p. 501-512.
17. Moerke, N.J., et al., *Small-Molecule Inhibition of the Interaction between the Translation Initiation Factors eIF4E and eIF4G.* Cell, 2007. **128**(2): p. 257-267.
18. Hernández, G., *On the origin of the cap-dependent initiation of translation in eukaryotes.* Trends in Biochemical Sciences, 2009. **34**(4): p. 166-175.
19. Richter, J.D. and N. Sonenberg, *Regulation of cap-dependent translation by eIF4E inhibitory proteins.* Nature, 2005. **433**(7025): p. 477-480.
20. Rau, M., et al., *A Reevaluation of the Cap-binding Protein, eIF4E, as a Rate-limiting Factor for Initiation of Translation in Reticulocyte Lysate.* Journal of Biological Chemistry, 1996. **271**(15): p. 8983-8990.
21. Marintchev, A., et al., *Topology and Regulation of the Human eIF4A/4G/4H Helicase Complex in Translation Initiation.* Cell, 2009. **136**(3): p. 447-460.
22. Jackson, R.J., C.U.T. Hellen, and T.V. Pestova, *The mechanism of eukaryotic translation initiation and principles of its regulation.* Nat Rev Mol Cell Biol. **11**(2): p. 113-127.
23. Borman, A.M., Y.M. Michel, and K.M. Kean, *Biochemical characterisation of cap–poly(A) synergy in rabbit reticulocyte lysates: the eIF4G–PABP interaction increases the functional affinity of eIF4E for the capped mRNA 5'-end.* Nucleic Acids Research, 2000. **28**(21): p. 4068-4075.
24. Mazumder, B., et al., *Translational Silencing of Ceruloplasmin Requires the Essential Elements of mRNA Circularization: Poly(A) Tail, Poly(A)-Binding Protein, and Eukaryotic Translation Initiation Factor 4G.* Mol. Cell. Biol., 2001. **21**(19): p. 6440-6449.
25. Agirrezabala, X. and J. Frank, *Elongation in translation as a dynamic interaction among the ribosome, tRNA, and elongation factors EF-G and EF-Tu.* Quarterly Reviews of Biophysics, 2009. **42**(03): p. 159-200.

26. Dixit, E., et al., *Peroxisomes Are Signaling Platforms for Antiviral Innate Immunity.* Cell, 2010. **141**(4): p. 668-681.
27. Schmeing, T.M. and V. Ramakrishnan, *What recent ribosome structures have revealed about the mechanism of translation.* Nature, 2009. **461**(7268): p. 1234-1242.
28. Kindler, S., et al., *RNA transport and local control of translation.* Annual review of cell and developmental biology, 2005. **21**: p. 223-245.
29. Holcik, M. and N. Sonenberg, *Translational control in stress and apoptosis.* Nat Rev Mol Cell Biol, 2005. **6**(4): p. 318-327.
30. Spriggs, K.A., et al., *Re-programming of translation following cell stress allows IRES-mediated translation to predominate.* Biology of the Cell, 2008. **100**(1): p. 27-38.
31. Lang, S.A., et al., *Implication of RICTOR in the mTOR inhibitor-mediated induction of insulin-like growth factor-I receptor (IGF-IR) and human epidermal growth factor receptor-2 (Her2) expression in gastrointestinal cancer cells.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2010. **1803**(4): p. 435-442.
32. Pearce, L., Huang, XU., Boudeau, J., Pawlowski, R., Wullschleger, S., Deak, M., Ibrahim, AFM., Gourlay, R., Magnuson, MA., Alessi, DR. , *Identification of Protor as a novel Rictor-binding component of mTOR complex-2.* Biochem J, 2007. **405 (pt3)**: p. 513-522.
33. Yang, Q., et al., *Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity.* Genes & Development, 2006. **20**(20): p. 2820-2832.
34. Wullschleger, S., R. Loewith, and M.N. Hall, *TOR Signaling in Growth and Metabolism.* Cell, 2006. **124**(3): p. 471-484.
35. Dunlop, E.A. and A.R. Tee, *Mammalian target of rapamycin complex 1: Signalling inputs, substrates and feedback mechanisms.* Cellular Signalling, 2009. **21**(6): p. 827-835.
36. Polak, P. and M.N. Hall, *mTOR and the control of whole body metabolism.* Current Opinion in Cell Biology, 2009. **21**(2): p. 209-218.

37. García-martínez, J.M. and D.R. Alessi, *mTOR complex 2 (mTORC2) controls hydrophobic motif phosphorylation and activation of serum- and glucocorticoid-induced protein kinase 1 (SGK1).* Biochem J, 2008. **416**(3): p. 375-385.
38. Huang, J., et al., *The TSC1-TSC2 Complex Is Required for Proper Activation of mTOR Complex 2.* Mol. Cell. Biol., 2008. **28**(12): p. 4104-4115.
39. Pearce, et al., *Identification of protor as a novel rictor-binding component of mTOR complex-2.* Biochem J, 2007. **405**: p. 10.
40. Sarbassov, D.D., et al., *Phosphorylation and Regulation of Akt/PKB by the Rictor-mTOR Complex.* Science, 2005. **307**(5712): p. 1098-1101.
41. Hresko, R.C. and M. Mueckler, *mTOR·RICTOR Is the Ser473 Kinase for Akt/Protein Kinase B in 3T3-L1 Adipocytes.* Journal of Biological Chemistry, 2005. **280**(49): p. 40406-40416.
42. Brazil, D.P., Z.-Z. Yang, and B.A. Hemmings, *Advances in protein kinase B signalling: AKTion on multiple fronts.* Trends in Biochemical Sciences, 2004. **29**(5): p. 233-242.
43. Shiota, C., et al., *Multiallelic Disruption of the rictor Gene in Mice Reveals that mTOR Complex 2 Is Essential for Fetal Growth and Viability.* Developmental Cell, 2006. **11**(4): p. 583-589.
44. Jacinto, E., et al., *Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive.* Nat Cell Biol, 2004. **6**(11): p. 1122-1128.
45. Loewith, R., et al., *Two TOR Complexes, Only One of which Is Rapamycin Sensitive, Have Distinct Roles in Cell Growth Control.* Molecular Cell, 2002. **10**(3): p. 457-468.
46. Sarbassov, D.D., et al., *Prolonged Rapamycin Treatment Inhibits mTORC2 Assembly and Akt/PKB.* Molecular Cell, 2006. **22**(2): p. 159-168.
47. Tee, A.R., et al., *Tuberous sclerosis complex-1 and -2 gene products function together to inhibit mammalian target of rapamycin (mTOR)-mediated downstream signaling.* Proceedings of the National Academy of Sciences of the United States of America, 2003. **99**(21): p. 13571-13576.
48. Saucedo, L.J., et al., *Rheb promotes cell growth as a component of the insulin/TOR signalling network.* Nat Cell Biol, 2003. **5**(6): p. 566-571.

49. Castro, A.F., et al., *Rheb Binds Tuberous Sclerosis Complex 2 (TSC2) and Promotes S6 Kinase Activation in a Rapamycin- and Farnesylation-dependent Manner.* Journal of Biological Chemistry, 2003. **278**(35): p. 32493-32496.
50. Inoki, K., et al., *Rheb GTPase is a direct target of TSC2 GAP activity and regulates mTOR signaling.* Genes & Development, 2003. **17**(15): p. 1829-1834.
51. Manning, B.D. and L.C. Cantley, *Rheb fills a GAP between TSC and TOR.* Trends in Biochemical Sciences, 2003. **28**(11): p. 573-576.
52. Hsu, Y.-C., et al., *Drosophila TCTP is essential for growth and proliferation through regulation of dRheb GTPase.* Nature, 2007. **445**(7129): p. 785-788.
53. Rehmann, H., et al., *Biochemical characterisation of TCTP questions its function as a guanine nucleotide exchange factor for Rheb.* FEBS Letters, 2008. **582**(20): p. 3005-3010.
54. Wang, X., et al., *Re-evaluating the Roles of Proposed Modulators of Mammalian Target of Rapamycin Complex 1 (mTORC1) Signaling.* Journal of Biological Chemistry, 2008. **283**(45): p. 30482-30492.
55. Kluk, M.J. and T. Hla, *Role of the Sphingosine 1-Phosphate Receptor EDG-1 in Vascular Smooth Muscle Cell Proliferation and Migration.* Circ Res, 2001. **89**(6): p. 496-502.
56. Grey, A., et al., *The Phospholipids Sphingosine-1-Phosphate and Lysophosphatidic Acid Prevent Apoptosis in Osteoblastic Cells via a Signaling Pathway Involving Gi Proteins and Phosphatidylinositol-3 Kinase.* Endocrinology, 2002. **143**(12): p. 4755-4763.
57. Maeurer, C., et al., *Sphingosine-1-phosphate induced mTOR-activation is mediated by the E3-ubiquitin ligase PAM.* Cellular Signalling, 2009. **21**(2): p. 293-300.
58. Yang, Q., et al., *TSC1/TSC2 and Rheb have different effects on TORC1 and TORC2 activity.* Proceedings of the National Academy of Sciences, 2006. **103**(18): p. 6811-6816.
59. Sato, T., et al., *Specific Activation of mTORC1 by Rheb G-protein in Vitro Involves Enhanced Recruitment of Its Substrate Protein.* Journal of Biological Chemistry, 2009. **284**(19): p. 12783-12791.
60. Long, X., et al., *Rheb Binds and Regulates the mTOR Kinase.* Current Biology, 2005. **15**(8): p. 702-713.

61. Mavrakis, K.J., et al., *Tumorigenic activity and therapeutic inhibition of Rheb GTPase.* Genes & Development, 2008. **22**(16): p. 2178-2188.
62. Tee, A.R., J. Blenis, and C.G. Proud, *Analysis of mTOR signaling by the small G-proteins, Rheb and RhebL1.* FEBS Letters, 2005. **579**(21): p. 4763-4768.
63. Yu, Y., et al., *Structural Basis for the Unique Biological Function of Small GTPase RHEB.* Journal of Biological Chemistry, 2005. **280**(17): p. 17093-17100.
64. Keith, C.T. and S.L. Schreiber, *PIK-Related Kinases: DNA Repair, Recombination, and Cell Cycle Checkpoints.* Science, 1995. **270**(5233): p. 50.
65. Hay, N. and N. Sonenberg, *Upstream and downstream of mTOR.* Genes & Development, 2004. **18**(16): p. 1926-1945.
66. Andrade, M.A. and P. Bork, *HEAT repeats in the Huntington's disease protein.* Nat Genet, 1995. **11**(2): p. 115-116.
67. Hirano, T., K. Nakajima, and M. Hibi, *Signaling mechanisms through gp130: A model of the cytokine system.* Cytokine & growth factor reviews, 1997. **8**(4): p. 241-252.
68. Vilella-Bach, M., et al., *The FKBP12-Rapamycin-binding Domain Is Required for FKBP12-Rapamycin-associated Protein Kinase Activity and G1 Progression.* Journal of Biological Chemistry, 1999. **274**(7): p. 4266-4272.
69. Oshiro, N., et al., *Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function.* Genes to Cells, 2004. **9**(4): p. 359-366.
70. Takahashi, T., et al., *Carboxyl-terminal region conserved among phosphoinositide-kinase-related kinases is indispensable for mTOR function in vivo and in vitro.* Genes to Cells, 2000. **5**(9): p. 765-775.
71. Dames, S.A., et al., *The Solution Structure of the FATC Domain of the Protein Kinase Target of Rapamycin Suggests a Role for Redox-dependent Structural and Cellular Stability.* Journal of Biological Chemistry, 2005. **280**(21): p. 20558-20564.
72. Perry, J. and N. Kleckner, *The ATRs, ATMs, and TORs Are Giant HEAT Repeat Proteins.* Cell, 2003. **112**(2): p. 151-155.
73. Gingras, A.-C., B. Raught, and N. Sonenberg, *Regulation of translation initiation by FRAP/mTOR.* Genes & Development, 2001. **15**(7): p. 807-826.

74. Kim, D.-H., et al., *mTOR Interacts with Raptor to Form a Nutrient-Sensitive Complex that Signals to the Cell Growth Machinery.* Cell, 2002. **110**(2): p. 163-175.
75. Hara, K., et al., *Raptor, a Binding Partner of Target of Rapamycin (TOR), Mediates TOR Action.* Cell, 2002. **110**(2): p. 177-189.
76. Dunlop, E.A., et al., *Mammalian target of rapamycin complex 1-mediated phosphorylation of eukaryotic initiation factor 4E-binding protein 1 requires multiple protein-protein interactions for substrate recognition.* Cellular Signalling, 2009. **21**(7): p. 1073-1084.
77. Schalm, S.S. and J. Blenis, *Identification of a Conserved Motif Required for mTOR Signaling.* Current Biology, 2002. **12**(8): p. 632-639.
78. Schalm, S.S., et al., *TOS Motif-Mediated Raptor Binding Regulates 4E-BP1 Multisite Phosphorylation and Function.* Current Biology, 2003. **13**(10): p. 797-806.
79. Nojima, H., et al., *The Mammalian Target of Rapamycin (mTOR) Partner, Raptor, Binds the mTOR Substrates p70 S6 Kinase and 4E-BP1 through Their TOR Signaling (TOS) Motif.* Journal of Biological Chemistry, 2003. **278**(18): p. 15461-15464.
80. Lee, V.H.Y., et al., *Analysis of the regulatory motifs in eukaryotic initiation factor 4E-binding protein 1.* FEBS Journal, 2008. **275**(9): p. 2185-2199.
81. Csibi, A., et al., *The Translation Regulatory Subunit eIF3f Controls the Kinase-Dependent mTOR Signaling Required for Muscle Differentiation and Hypertrophy in Mouse.* PLoS ONE, 2010. **5**(2): p. e8994.
82. Oshiro, N., et al., *The Proline-rich Akt Substrate of 40 kDa (PRAS40) Is a Physiological Substrate of Mammalian Target of Rapamycin Complex 1.* Journal of Biological Chemistry, 2007. **282**(28): p. 20329-20339.
83. Foster, K.G., et al., *Regulation of mTOR Complex 1 (mTORC1) by Raptor Ser863 and Multisite Phosphorylation.* Journal of Biological Chemistry, 2010. **285**(1): p. 80-94.
84. Wang, L., et al., *Mammalian Target of Rapamycin Complex 1 (mTORC1) Activity Is Associated with Phosphorylation of Raptor by mTOR.* Journal of Biological Chemistry, 2009. **284**(22): p. 14693-14697.

85. Kim, D.-H., et al., *G[beta]L, a Positive Regulator of the Rapamycin-Sensitive Pathway Required for the Nutrient-Sensitive Interaction between Raptor and mTOR.* Molecular Cell, 2003. **11**(4): p. 895-904.
86. Guertin, D.A., et al., *Ablation in Mice of the mTORC Components raptor, rictor, or mLST8 Reveals that mTORC2 Is Required for Signaling to Akt-FOXO and PKC[alpha], but Not S6K1.* Developmental Cell, 2006. **11**(6): p. 859-871.
87. Yang, Q. and K.-L. Guan, *Expanding mTOR signaling.* Cell Res, 2007. **17**(8): p. 666-681.
88. Haar, E.V., et al., *Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40.* Nat Cell Biol, 2007. **9**(3): p. 316-323.
89. Sancak, Y., et al., *PRAS40 Is an Insulin-Regulated Inhibitor of the mTORC1 Protein Kinase.* Molecular Cell, 2007. **25**(6): p. 903-915.
90. Wang, L., et al., *PRAS40 Regulates mTORC1 Kinase Activity by Functioning as a Direct Inhibitor of Substrate Binding.* Journal of Biological Chemistry, 2007. **282**(27): p. 20036-20044.
91. Fonseca, B.D., et al., *PRAS40 Is a Target for Mammalian Target of Rapamycin Complex 1 and Is Required for Signaling Downstream of This Complex.* Journal of Biological Chemistry, 2007. **282**(34): p. 24514-24524.
92. Pozuelo-Rubio, M., *Regulation of autophagic activity by 14-3-3[zeta] proteins associated with class III phosphatidylinositol-3-kinase.* Cell Death Differ, 2010.
93. Peterson, T.R., et al., *DEPTOR Is an mTOR Inhibitor Frequently Overexpressed in Multiple Myeloma Cells and Required for Their Survival.* Cell, 2009. **137**(5): p. 873-886.
94. Edlich, F., et al., *Bcl-2 regulator FKBP38 is activated by Ca2+/calmodulin.* EMBO J, 2005. **24**(14): p. 2688-2699.
95. Bai, X. and Y. Jiang, *Key factors in mTOR regulation.* Cellular and Molecular Life Sciences, 2010. **67**: p. 239-253.
96. Ma, D., et al., *The Switch I Region of Rheb Is Critical for Its Interaction with FKBP38.* Journal of Biological Chemistry, 2008. **283**(38): p. 25963-25970.
97. Bai, X., et al., *Rheb Activates mTOR by Antagonizing Its Endogenous Inhibitor, FKBP38.* Science, 2007. **318**(5852): p. 977-980.

98. Ramanathan, A. and S.L. Schreiber, *Direct control of mitochondrial function by mTOR.* Proceedings of the National Academy of Sciences, 2009. **106**(52): p. 22229-22232.
99. Desai, B.N., B.R. Myers, and S.L. Schreiber, *FKBP12-rapamycin-associated protein associates with mitochondria and senses osmotic stress via mitochondrial dysfunction.* Proceedings of the National Academy of Sciences, 2002. **99**(7): p. 4319-4324.
100. Maehama, T., et al., *RalA Functions as an Indispensable Signal Mediator for the Nutrient-sensing System.* Journal of Biological Chemistry, 2008. **283**(50): p. 35053-35059.
101. Oldham, S. and E. Hafen, *Insulin/IGF and target of rapamycin signaling: a TOR de force in growth control.* Trends in Cell Biology, 2003. **13**(2): p. 79-85.
102. Vogt, P.K., *PI 3-kinase, mTOR, protein synthesis and cancer.* Trends in Molecular Medicine, 2001. **7**(11): p. 482-484.
103. Ma, L., et al., *Phosphorylation and Functional Inactivation of TSC2 by Erk: Implications for Tuberous Sclerosisand Cancer Pathogenesis.* Cell, 2005. **121**(2): p. 179-193.
104. Richards, S.A., et al., *Ribosomal S6 kinase 1 (RSK1) activation requires signals dependent on and independent of the MAP kinase ERK.* Current Biology, 1999. **9**(15): p. 810-820, S1.
105. Roux, P.P., et al., *Tumor-promoting phorbol esters and activated Ras inactivate the tuberous sclerosis tumor suppressor complex via p90 ribosomal S6 kinase.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(37): p. 13489-13494.
106. Summy, J.M. and G.E. Gallick, *Treatment for Advanced Tumors: Src Reclaims Center Stage.* Clinical Cancer Research, 2006. **12**(5): p. 1398-1401.
107. Exton, J.H., *Regulation of phospholipase D.* Biochimica et Biophysica Acta (BBA) - Molecular and Cell Biology of Lipids, 1999. **1439**(2): p. 121-133.
108. Toschi, A., et al., *Regulation of mTORC1 and mTORC2 Complex Assembly by Phosphatidic Acid: Competition with Rapamycin.* Mol. Cell. Biol., 2009. **29**(6): p. 1411-1420.
109. SUN, Y. and J. CHEN, *mTOR signaling : PLD takes center stage.* Vol. 7. 2008, Georgetown, TX, ETATS-UNIS: Landes Bioscience. 6.

110. Sun, Y., et al., *Phospholipase D1 is an effector of Rheb in the mTOR pathway.* Proceedings of the National Academy of Sciences, 2008. **105**(24): p. 8286-8291.
111. Winter, J.N., et al., *Phosphatidic Acid Mediates Activation of mTORC1 Through the ERK Signaling Pathway.* Am J Physiol Cell Physiol, 2010: p. ajpcell.00039.2010.
112. Inoki, K., T. Zhu, and K.-L. Guan, *TSC2 Mediates Cellular Energy Response to Control Cell Growth and Survival.* Cell, 2003. **115**(5): p. 577-590.
113. Gough, D.J., et al., *Mitochondrial STAT3 Supports Ras-Dependent Oncogenic Transformation.* Science, 2009. **324**(5935): p. 1713-1716.
114. Inoki, K., et al., *TSC2 Integrates Wnt and Energy Signals via a Coordinated Phosphorylation by AMPK and GSK3 to Regulate Cell Growth.* Cell, 2006. **126**(5): p. 955-968.
115. Gwinn, D.M., et al., *AMPK Phosphorylation of Raptor Mediates a Metabolic Checkpoint.* Molecular Cell, 2008. **30**(2): p. 214-226.
116. Sancak, Y., et al., *The Rag GTPases Bind Raptor and Mediate Amino Acid Signaling to mTORC1.* Science, 2008. **320**(5882): p. 1496-1501.
117. Kim, E., et al., *Regulation of TORC1 by Rag GTPases in nutrient response.* Nat Cell Biol, 2008. **10**(8): p. 935-945.
118. Sancak, Y., et al., *Ragulator-Rag Complex Targets mTORC1 to the Lysosomal Surface and Is Necessary for Its Activation by Amino Acids.* Cell, 2010. **141**(2): p. 290-303.
119. Juhász, G., et al., *The class III PI(3)K Vps34 promotes autophagy and endocytosis but not TOR signaling in Drosophila.* The Journal of Cell Biology, 2008. **181**(4): p. 655-666.
120. Byfield, M.P., J.T. Murray, and J.M. Backer, *hVps34 Is a Nutrient-regulated Lipid Kinase Required for Activation of p70 S6 Kinase.* Journal of Biological Chemistry, 2005. **280**(38): p. 33076-33082.
121. Nobukuni, T., et al., *Amino acids mediate mTOR/raptor signaling through activation of class 3 phosphatidylinositol 3OH-kinase.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(40): p. 14238-14243.

122. Nobukuni, T., S.C. Kozma, and G. Thomas, *hvps34, an ancient player, enters a growing game: mTOR Complex1/S6K1 signaling.* Current Opinion in Cell Biology, 2007. **19**(2): p. 135-141.
123. Gulati, P., et al., *Amino Acids Activate mTOR Complex 1 via Ca2+/CaM Signaling to hVps34.* Cell metabolism, 2008. **7**(5): p. 456-465.
124. Ying Yan, R.J.F., Haiyan Wu, Rachel S. Schnur, and Jonathan M. Backer, *hVps15, but not Ca2+/CaM, is required for the activity and regulation of hVps34 in mammalian cells.* Biochem J, 2008. **417**: p. 747-755.
125. Findlay, G.M., et al., *A MAP4 kinase related to Ste20 is a nutrient-sensitive regulator of mTOR signalling.* Biochem J, 2007. **403**(1): p. 13-20.
126. Yan, L., et al., *PP2AT61[Is an Inhibitor of MAP4K3 in Nutrient Signaling to mTOR.* Molecular Cell. **37**(5): p. 633-642.
127. Eichhorn, P.J.A., M.P. Creyghton, and R. Bernards, *Protein phosphatase 2A regulatory subunits and cancer.* Biochimica et Biophysica Acta (BBA) - Reviews on Cancer, 2009. **1795**(1): p. 1-15.
128. Hara, K., et al., *Amino Acid Sufficiency and mTOR Regulate p70 S6 Kinase and eIF-4E BP1 through a Common Effector Mechanism.* Journal of Biological Chemistry, 1998. **273**(23): p. 14484-14494.
129. Kimball, S.R. and L.S. Jefferson, *Regulation of global and specific mRNA translation by oral administration of branched-chain amino acids.* Biochemical and Biophysical Research Communications, 2004. **313**(2): p. 423-427.
130. Tasaki, T., et al., *A Family of Mammalian E3 Ubiquitin Ligases That Contain the UBR Box Motif and Recognize N-Degrons.* Mol. Cell. Biol., 2005. **25**(16): p. 7120-7136.
131. Kume, K., et al., *Role of N-end rule ubiquitin ligases UBR1 and UBR2 in regulating the leucine-mTOR signaling pathway.* Genes to Cells, 2010. **15**(4): p. 339-349.
132. Neklesa, T.K. and R.W. Davis, *A Genome-Wide Screen for Regulators of TORC1 in Response to Amino Acid Starvation Reveals a Conserved Npr2/3 Complex.* PLoS Genet, 2009. **5**(6): p. e1000515.
133. Spielewoy, N., et al., *Npr2, Yeast Homolog of the Human Tumor Suppressor NPRL2, Is a Target of Grr1 Required for Adaptation to Growth on Diverse Nitrogen Sources.* Eukaryotic Cell, 2010. **9**(4): p. 592-601.

134. Kurata, A., et al., *TUSC4/NPRL2, a novel PDK1-interacting protein, inhibits PDK1 tyrosine phosphorylation and its downstream signaling.* Cancer Science, 2008. **99**(9): p. 1827-1834.
135. Land, S.C. and A.R. Tee, *Hypoxia-inducible Factor 1-alpha Is Regulated by the Mammalian Target of Rapamycin (mTOR) via an mTOR Signaling Motif.* Journal of Biological Chemistry, 2007. **282**(28): p. 20534-20543.
136. Jin, H.-O., et al., *Hypoxic condition- and high cell density-induced expression of Redd1 is regulated by activation of hypoxia-inducible factor-1[alpha] and Sp1 through the phosphatidylinositol 3-kinase/Akt signaling pathway.* Cellular Signalling, 2007. **19**(7): p. 1393-1403.
137. Shumway, S.D., Y. Li, and Y. Xiong, *14-3-3β Binds to and Negatively Regulates the Tuberous Sclerosis Complex 2 (TSC2) Tumor Suppressor Gene Product, Tuberin.* Journal of Biological Chemistry, 2003. **278**(4): p. 2089-2092.
138. Li, Y., et al., *Regulation of TSC2 by 14-3-3 Binding.* Journal of Biological Chemistry, 2002. **277**(47): p. 44593-44596.
139. Nellist, M., M.A. Goedbloed, and D.J.J. Halley, *Regulation of tuberous sclerosis complex (TSC) function by 14-3-3 proteins.* Biochem. Soc. Trans., 2003. **31**(Pt 3): p. 587-591.
140. Liu, M.Y., et al., *14-3-3 Interacts with the Tumor Suppressor Tuberin at Akt Phosphorylation Site(s).* Cancer Research, 2002. **62**(22): p. 6475-6480.
141. DeYoung, M.P., et al., *Hypoxia regulates TSC1/2–mTOR signaling and tumor suppression through REDD1-mediated 14–3–3 shuttling.* Genes & Development, 2008. **22**(2): p. 239-251.
142. Morrison, D.K., *The 14-3-3 proteins: integrators of diverse signaling cues that impact cell fate and cancer development.* Trends in Cell Biology, 2009. **19**(1): p. 16-23.
143. Bernardi, R., et al., *PML inhibits HIF-1[alpha] translation and neoangiogenesis through repression of mTOR.* Nature, 2006. **442**(7104): p. 779-785.
144. Kubasiak, L.A., et al., *Hypoxia and acidosis activate cardiac myocyte death through the Bcl-2 family protein BNIP3.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(20): p. 12825-12830.
145. Guo, K., et al., *Hypoxia induces the expression of the pro-aptotic gene BNIP3.* Cell Death Differ, 2002. **8**(4): p. 367-376.

146. Li, Y., et al., *Bnip3 Mediates the Hypoxia-induced Inhibition on Mammalian Target of Rapamycin by Interacting with Rheb.* Journal of Biological Chemistry, 2007. **282**(49): p. 35803-35813.
147. Zick, Y., *Insulin resistance: a phosphorylation-based uncoupling of insulin signaling.* Trends in Cell Biology, 2001. **11**(11): p. 437-441.
148. Um, S.H., et al., *Absence of S6K1 protects against age- and diet-induced obesity while enhancing insulin sensitivity.* Nature, 2004. **431**(7005): p. 200-205.
149. Zhang, H., et al., *Loss of Tsc1/Tsc2 activates mTOR and disrupts PI3K-Akt signaling through downregulation of PDGFR.* The Journal of Clinical Investigation, 2003. **112**(8): p. 1223-1233.
150. Julien, L.-A., et al., *mTORC1-Activated S6K1 Phosphorylates Rictor on Threonine 1135 and Regulates mTORC2 Signaling.* Mol. Cell. Biol., 2010. **30**(4): p. 908-921.
151. Dibble, C.C., J.M. Asara, and B.D. Manning, *Characterization of Rictor phosphorylation sites reveals direct regulation of mTOR complex 2 by S6K1.* Mol. Cell. Biol., 2009: p. MCB.00735-09.
152. Carracedo, A., et al., *Inhibition of mTORC1 leads to MAPK pathway activation through a PI3K-dependent feedback loop in human cancer.* The Journal of Clinical Investigation, 2008.
153. Fenton, T.R. and I.T. Gout, *Functions and regulation of the 70 kDa ribosomal S6 kinases.* The International Journal of Biochemistry & Cell Biology. **In Press, Uncorrected Proof**.
154. Mora, A., et al., *PDK1, the master regulator of AGC kinase signal transduction.* Seminars in Cell & Developmental Biology, 2004. **15**(2): p. 161-170.
155. Peterson, R.T., et al., *Protein phosphatase 2A interacts with the 70-kDa S6 kinase and is activated by inhibition of FKBP12–rapamycinassociated protein.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(8): p. 4438-4442.
156. Montagne, J., et al., *Drosophila S6 Kinase: A Regulator of Cell Size.* Science, 1999. **285**(5436): p. 2126-2129.

157. Moore, C.E.J., et al., *Identification of cAMP-Dependent Kinase as a Third in Vivo Ribosomal Protein S6 Kinase in Pancreatic [beta]-Cells.* Journal of Molecular Biology, 2009. **389**(3): p. 480-494.
158. Ruvinsky, I., et al., *Mice Deficient in Ribosomal Protein S6 Phosphorylation Suffer from Muscle Weakness that Reflects a Growth Defect and Energy Deficit.* PLoS ONE, 2009. **4**(5): p. e5618.
159. Callis, J., M. Fromm, and V. Walbot, *Introns increase gene expression in cultured maize cells.* Genes & Development, 1987. **1**(10): p. 1183-1200.
160. Palmiter, R.D., et al., *Heterologous introns can enhance expression of transgenes in mice.* Proceedings of the National Academy of Sciences of the United States of America, 1991. **88**(2): p. 478-482.
161. Richardson, C.J., et al., *SKAR Is a Specific Target of S6 Kinase 1 in Cell Growth Control.* Current Biology, 2004. **14**(17): p. 1540-1549.
162. Ma, X.M., et al., *SKAR Links Pre-mRNA Splicing to mTOR/S6K1-Mediated Enhanced Translation Efficiency of Spliced mRNAs.* Cell, 2008. **133**(2): p. 303-313.
163. Yamnik, R.L., et al., *S6 Kinase 1 Regulates Estrogen Receptor Î± in Control of Breast Cancer Cell Proliferation.* Journal of Biological Chemistry, 2009. **284**(10): p. 6361-6369.
164. Lin, T., et al., *PHAS-I as a link between mitogen-activated protein kinase and translation initiation.* Science, 1994. **266**(5185): p. 653-656.
165. Mader, S., et al., *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., 1995. **15**(9): p. 4990-4997.
166. Gingras, A.-C., et al., *Regulation of 4E-BP1 phosphorylation: a novel two-step mechanism.* Genes & Development, 1999. **13**(11): p. 1422-1437.
167. Poulin, F., et al., *4E-BP3, a New Member of the Eukaryotic Initiation Factor 4E-binding Protein Family.* Journal of Biological Chemistry, 1998. **273**(22): p. 14002-14007.
168. Tait, S., et al., *Local control of a disorder-order transition in 4E-BP1 underpins regulation of translation via eIF4E.* Proceedings of the National Academy of Sciences, 2010. **107**(41): p. 17627-17632.

169. Tee, A.R. and C.G. Proud, *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., 2002. **22**(6): p. 1674-1683.
170. Choi, K.M., L.P. McMahon, and J.C. Lawrence, *Two Motifs in the Translational Repressor PHAS-I Required for Efficient Phosphorylation by Mammalian Target of Rapamycin and for Recognition by Raptor.* Journal of Biological Chemistry, 2003. **278**(22): p. 19667-19673.
171. Beugnet, A., X. Wang, and C.G. Proud, *Target of Rapamycin (TOR)-signaling and RAIP Motifs Play Distinct Roles in the Mammalian TOR-dependent Phosphorylation of Initiation Factor 4E-binding Protein 1.* Journal of Biological Chemistry, 2003. **278**(42): p. 40717-40722.
172. Weidemann, A. and R.S. Johnson, *Biology of HIF-1[alpha].* Cell Death Differ, 2008. **15**(4): p. 621-627.
173. Makino, Y., et al., *Inhibitory PAS domain protein is a negative regulator of hypoxia-inducible gene expression.* Nature, 2001. **414**(6863): p. 550-554.
174. Castrop, H. and A. Kurtz, *Functional evidence confirmed by histological localization: overlapping expression of erythropoietin and HIF-2[alpha] in interstitial fibroblasts of the renal cortex.* Kidney Int, 2010. **77**(4): p. 269-271.
175. Wiesener, M.S., et al., *Widespread, hypoxia-inducible expression of HIF-2alpha in distinct cell populations of different organs.* FASEB J., 2002: p. 02-0445fje.
176. Altomare, D.A. and J.R. Testa, *Perturbations of the AKT signaling pathway in human cancer.* Oncogene, 2005. **24**(50): p. 7455-7464.
177. Kaelin, W.G., *The von Hippel-Lindau Tumor Suppressor Protein and Clear Cell Renal Carcinoma.* Clinical Cancer Research, 2007. **13**(2): p. 680s-684s.
178. Chowdhury, R., et al., *Structural Basis for Binding of Hypoxia-Inducible Factor to the Oxygen-Sensing Prolyl Hydroxylases.* 2009. **17**(7): p. 981-989.
179. LOENARZ, et al., *Oxygenases for oxygen sensing.* Vol. 80. 2008, Research Triangle Park, NC, ETATS-UNIS: Pure and applied chemistry. 11.
180. Baek, J.H., et al., *OS-9 Interacts with Hypoxia-Inducible Factor 1α and Prolyl Hydroxylases to Promote Oxygen-Dependent Degradation of HIF-1α.* Molecular Cell, 2005. **17**(4): p. 503-512.

181. Lando, D., et al., *FIH-1 is an asparaginyl hydroxylase enzyme that regulates the transcriptional activity of hypoxia-inducible factor.* Genes & Development, 2002. **16**(12): p. 1466-1471.
182. Brahimi-Horn, C., N. Mazure, and J. Pouysségur, *Signalling via the hypoxia-inducible factor-1α requires multiple posttranslational modifications.* Cellular Signalling, 2005. **17**(1): p. 1-9.
183. Bae, S.-H., et al., *Sumoylation increases HIF-1α stability and its transcriptional activity.* Biochemical and Biophysical Research Communications, 2004. **324**(1): p. 394-400.
184. Sumbayev, V.V., et al., *HIF-1α protein as a target for S-nitrosation.* FEBS Letters, 2003. **535**(1-3): p. 106-112.
185. Carrero, P., et al., *Redox-Regulated Recruitment of the Transcriptional Coactivators CREB-Binding Protein and SRC-1 to Hypoxia-Inducible Factor 1alpha.* Mol. Cell. Biol., 2000. **20**(1): p. 402-415.
186. Zhang, T., et al., *The Coiled-Coil Domain of Stat3 Is Essential for Its SH2 Domain-Mediated Receptor Binding and Subsequent Activation Induced by Epidermal Growth Factor and Interleukin-6.* Mol. Cell. Biol., 2000. **20**(19): p. 7132-7139.
187. Schindler, C., D.E. Levy, and T. Decker, *JAK-STAT Signaling: From Interferons to Cytokines.* Journal of Biological Chemistry, 2007. **282**(28): p. 20059-20063.
188. Levy, D.E. and C.-k. Lee, *What does Stat3 do?* The Journal of Clinical Investigation, 2002. **109**(9): p. 1143-1148.
189. Akira and S., *Roles of STAT3 defined by tissue-specific gene targeting.* 2000, Basingstoke, ROYAUME-UNI: Nature Publishing Group. 193.
190. Bromberg, J. and T.C. Wang, *Inflammation and Cancer: IL-6 and STAT3 Complete the Link.* Cancer cell, 2009. **15**(2): p. 79-80.
191. Shen, Y., et al., *Essential Role of STAT3 in Postnatal Survival and Growth Revealed by Mice Lacking STAT3 Serine 727 Phosphorylation.* Mol. Cell. Biol., 2004. **24**(1): p. 407-419.
192. Catlett-Falcone, R., et al., *Constitutive Activation of Stat3 Signaling Confers Resistance to Apoptosis in Human U266 Myeloma Cells.* Immunity, 1999. **10**(1): p. 105-115.

193. Grandis, J.R., et al., *Constitutive activation of Stat3 signaling abrogates apoptosis in squamous cell carcinogenesis in vivo.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(8): p. 4227-4232.
194. Fukada, T., et al., *Two Signals Are Necessary for Cell Proliferation Induced by a Cytokine Receptor gp130: Involvement of STAT3 in Anti-Apoptosis.* Immunity, 1996. **5**(5): p. 449-460.
195. Snyder, M., X.-Y. Huang, and J.J. Zhang, *Identification of Novel Direct Stat3 Target Genes for Control of Growth and Differentiation.* Journal of Biological Chemistry, 2008. **283**(7): p. 3791-3798.
196. Taga, T. and T. Kishimoto, *gp130 and the IL-6 Family of Cytokines.* Annual Review of Immunology, 1997. **15**(1): p. 797-819.
197. Paonessa, G., et al., *Two distinct and independent sites on IL-6 trigger gp 130 dimer formation and signalling.*
198. Kishimoto, et al., *Interleukin-6 family of cytokines and gp130.* Vol. 86. 1995, Washington, DC, ETATS-UNIS: The Americain Society of Hematology.
199. Zhang, J.J., et al., *Ser727-dependent recruitment of MCM5 by Stat1[alpha] in IFN-[gamma]-induced transcriptional activation.* EMBO J, 1998. **17**(23): p. 6963-6971.
200. Nair, J.S., et al., *Requirement of Ca2+ and CaMKII for Stat1 Ser-727 phosphorylation in response to IFN-Î³.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(9): p. 5971-5976.
201. Chung, J., et al., *STAT3 serine phosphorylation by ERK-dependent and -independent pathways negatively modulates its tyrosine phosphorylation.* Molecular and cellular biology, 1997. **17**(11): p. 6508-16.
202. Yokogami, K., et al., *Serine phosphorylation and maximal activation of STAT3 during CNTF signaling is mediated by the rapamycin target mTOR.* Current biology : CB, 2000. **10**(1): p. 47-50.
203. Kim, J.-H., M.-S. Yoon, and J. Chen, *Signal Transducer and Activator of Transcription 3 (STAT3) Mediates Amino Acid Inhibition of Insulin Signaling through Serine 727 Phosphorylation.* Journal of Biological Chemistry, 2009. **284**(51): p. 35425-35432.

204. Togi, S., et al., *HDAC3 influences phosphorylation of STAT3 at serine 727 by interacting with PP2A.* Biochemical and Biophysical Research Communications, 2009. **379**(2): p. 616-620.
205. Liu, L., et al., *Rapamycin Inhibits IGF-1 Stimulated Cell Motility through PP2A Pathway.* PLoS ONE, 2010. **5**(5): p. e10578.
206. Nui, G., et al., *Constitutive Stat3 activity up-regulates VEGF expression and tumor angiogenesis.* Vol. 21. 2002, Basingstoke, ROYAUME-UNI: Nature Publishing Group.
207. Tischer, E., et al., *The human gene for vascular endothelial growth factor. Multiple protein forms are encoded through alternative exon splicing.* Journal of Biological Chemistry, 1991. **266**(18): p. 11947-11954.
208. Wei, D., et al., *Stat3 activation regulates the expression of vascular endothelial growth factor and human pancreatic cancer angiogenesis and metastasis.* Oncogene, 2003. **22**(3): p. 319-329.
209. Gray, M.J., et al., *HIF-1[alpha], STAT3, CBP//p300 and Ref-1//APE are components of a transcriptional complex that regulates Src-dependent hypoxia-induced expression of VEGF in pancreatic and prostate carcinomas.* 2005. **24**(19): p. 3110-3120.
210. Jung, J.E., et al., *STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells.* The FASEB Journal, 2005. **19**(10): p. 1296-1298.
211. Xu, Q., et al., *Targeting Stat3 blocks both HIF-1 and VEGF expression induced by multiple oncogenic growth signaling pathways.* Oncogene, 2005. **24**(36): p. 5552-5560.
212. Jung, J.E., et al., *STAT3 inhibits the degradation of HIF-1α by pVHL-mediated ubiquitination.* Vol. 40. 2008, Seoul, COREE, REPUBLIQUE DE: Korean Society of Medical Biochemistry and Molecular Biology. 7.
213. Schieke, S.M., et al., *The Mammalian Target of Rapamycin (mTOR) Pathway Regulates Mitochondrial Oxygen Consumption and Oxidative Capacity.* Journal of Biological Chemistry, 2006. **281**(37): p. 27643-27652.
214. Cunningham, J.T., et al., *mTOR controls mitochondrial oxidative function through a YY1-PGC-1[agr] transcriptional complex.* Nature, 2007. **450**(7170): p. 736-740.

215. Mootha, V.K., et al., *Errα and Gabpa/b specify PGC-1α-dependent oxidative phosphorylation gene expression that is altered in diabetic muscle.* Proceedings of the National Academy of Sciences of the United States of America, 2004. **101**(17): p. 6570-6575.
216. Gordon, S., et al., *Transcription factor YY1: structure, function, and therapeutic implications in cancer biology.* Oncogene, 2005. **25**(8): p. 1125-1142.
217. Wilkinson, F.H., K. Park, and M.L. Atchison, *Polycomb recruitment to DNA in vivo by the YY1 REPO domain.* Proceedings of the National Academy of Sciences, 2006. **103**(51): p. 19296-19301.
218. Bonawitz, N.D., et al., *Reduced TOR Signaling Extends Chronological Life Span via Increased Respiration and Upregulation of Mitochondrial Gene Expression.* Cell metabolism, 2007. **5**(4): p. 265-277.
219. Chen, C., et al., *TSC–mTOR maintains quiescence and function of hematopoietic stem cells by repressing mitochondrial biogenesis and reactive oxygen species.* The Journal of Experimental Medicine, 2008. **205**(10): p. 2397-2408.
220. Mayer, C. and I. Grummt, *Ribosome biogenesis and cell growth: mTOR coordinates transcription by all three classes of nuclear RNA polymerases.* Oncogene, 2006. **25**(48): p. 6384-6391.
221. Claypool, J.A., et al., *Tor Pathway Regulates Rrn3p-dependent Recruitment of Yeast RNA Polymerase I to the Promoter but Does Not Participate in Alteration of the Number of Active Genes.* Mol. Biol. Cell, 2004. **15**(2): p. 946-956.
222. Cardenas, M.E., et al., *The TOR signaling cascade regulates gene expression in response to nutrients.* Genes & Development, 1999. **13**(24): p. 3271-3279.
223. Zaragoza, D., et al., *Rapamycin Induces the G0 Program of Transcriptional Repression in Yeast by Interfering with the TOR Signaling Pathway.* Mol. Cell. Biol., 1998. **18**(8): p. 4463-4470.
224. Powers, T. and P. Walter, *Regulation of Ribosome Biogenesis by the Rapamycin-sensitive TOR-signaling Pathway in Saccharomyces cerevisiae.* Mol. Biol. Cell, 1999. **10**(4): p. 987-1000.

225. Wei, Y., C.K. Tsang, and X.F.S. Zheng, *Mechanisms of regulation of RNA polymerase III-dependent transcription by TORC1.* EMBO J, 2009. **28**(15): p. 2220-2230.
226. Shor, B., et al., *Requirement of the mTOR Kinase for the Regulation of Maf1 Phosphorylation and Control of RNA Polymerase III-dependent Transcription in Cancer Cells.* Journal of Biological Chemistry, 2010. **285**(20): p. 15380-15392.
227. Kantidakis, T., et al., *mTOR associates with TFIIIC, is found at tRNA and 5S rRNA genes, and targets their repressor Maf1.* Proceedings of the National Academy of Sciences, 2010. **107**(26): p. 11823-11828.
228. Parekh, D., et al., *Mammalian TOR Controls One of Two Kinase Pathways Acting upon nPKCδ and nPKCε.* Journal of Biological Chemistry, 1999. **274**(49): p. 34758-34764.
229. Heidkamp, M.C., et al., *Differential Activation of Mitogen-Activated Protein Kinase Cascades and Apoptosis by Protein Kinase C {epsilon} and {delta} in Neonatal Rat Ventricular Myocytes.* Circ Res, 2001. **89**(10): p. 882-890.
230. Malumbres, M. and M. Barbacid, *Cell cycle, CDKs and cancer: a changing paradigm.* Nat Rev Cancer, 2009. **9**(3): p. 153-166.
231. Toyoshima, H. and T. Hunter, *p27, a novel inhibitor of G1 cyclin-Cdk protein kinase activity, is related to p21.* Cell, 1994. **78**(1): p. 67-74.
232. Harbour, J.W. and D.C. Dean, *Rb function in cell-cycle regulation and apoptosis.* Nat Cell Biol, 2000. **2**(4): p. E65-E67.
233. Wang, X. and C.G. Proud, *Nutrient control of TORC1, a cell-cycle regulator.* Trends in Cell Biology, 2009. **19**(6): p. 260-267.
234. Luo, Y., et al., *Rapamycin resistance tied to defective regulation of p27Kip1.* Mol. Cell. Biol., 1996. **16**(12): p. 6744-6751.
235. Hong, F., et al., *mTOR-Raptor Binds and Activates SGK1 to Regulate p27 Phosphorylation.* Molecular Cell, 2008. **30**(6): p. 701-711.
236. Dalvai, M., et al., *Oct1 is required for mTOR-induced G1 cell cycle arrest via the control of p27kip1 expression.* Cell cycle, 2010. **9**(19): p. 3933-3944.
237. Perez-Roger, I., et al., *Cyclins D1 and D2 mediate Myc-induced proliferation via sequestration of p27Kip1 and p21Cip1.* EMBO J, 1999. **18**(19): p. 5310-5320.

238. Connell-Crowley, L., J. Harper, and D. Goodrich, *Cyclin D1/Cdk4 regulates retinoblastoma protein-mediated cell cycle arrest by site-specific phosphorylation.* Mol. Biol. Cell, 1997. **8**(2): p. 287-301.
239. Rosenwald, I.B., et al., *Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E.* Mol. Cell. Biol., 1993. **13**(12): p. 7358-7363.
240. Rousseau, D., et al., *Translation initiation of ornithine decarboxylase and nucleocytoplasmic transport of cyclin D1 mRNA are increased in cells overexpressing eukaryotic initiation factor 4E.* Proceedings of the National Academy of Sciences of the United States of America, 1996. **93**(3): p. 1065-1070.
241. Hashemolhosseini, S., et al., *Rapamycin Inhibition of the G1 to S Transition Is Mediated by Effects on Cyclin D1 mRNA and Protein Stability.* Journal of Biological Chemistry, 1998. **273**(23): p. 14424-14429.
242. Koziczak, M. and N.E. Hynes, *Cooperation between Fibroblast Growth Factor Receptor-4 and ErbB2 in Regulation of Cyclin D1 Translation.* Journal of Biological Chemistry, 2004. **279**(48): p. 50004-50011.
243. Bromberg, J.F., et al., *Stat3 as an Oncogene.* Cell, 1999. **98**(3): p. 295-303.
244. Hleb, M., et al., *Evidence for Cyclin D3 as a Novel Target of Rapamycin in Human T Lymphocytes.* Journal of Biological Chemistry, 2004. **279**(30): p. 31948-31955.
245. Decker, T., et al., *Rapamycin-induced G1 arrest in cycling B-CLL cells is associated with reduced expression of cyclin D3, cyclin E, cyclin A, and survivin.* Blood, 2003. **101**(1): p. 278-285.
246. Jung, C.H., et al., *mTOR regulation of autophagy.* FEBS Letters, 2010. **584**(7): p. 1287-1295.
247. Kundu, M. and C.B. Thompson, *Macroautophagy versus mitochondrial autophagy: a question of fate?* Cell Death Differ, 2005. **12**(S2): p. 1484-1489.
248. Mizushima, N., et al., *Autophagy fights disease through cellular self-digestion.* Nature, 2008. **451**(7182): p. 1069-1075.
249. Hayashi-Nishino, M., et al., *A subdomain of the endoplasmic reticulum forms a cradle for autophagosome formation.* Nat Cell Biol, 2009. **11**(12): p. 1433-1437.

250. Axe, E.L., et al., *Autophagosome formation from membrane compartments enriched in phosphatidylinositol 3-phosphate and dynamically connected to the endoplasmic reticulum.* The Journal of Cell Biology, 2008. **182**(4): p. 685-701.
251. Levine, B. and G. Kroemer, *Autophagy in the Pathogenesis of Disease.* Cell, 2008. **132**(1): p. 27-42.
252. Kunz, J.B., H. Schwarz, and A. Mayer, *Determination of four sequential stages during microautophagy in vitro.* 2004.
253. Li, S.C. and P.M. Kane, *The yeast lysosome-like vacuole: Endpoint and crossroads.* Biochimica et Biophysica Acta (BBA) - Molecular Cell Research, 2009. **1793**(4): p. 650-663.
254. Noda, T. and Y. Ohsumi, *Tor, a Phosphatidylinositol Kinase Homologue, Controls Autophagy in Yeast.* Journal of Biological Chemistry, 1998. **273**(7): p. 3963-3966.
255. Kamada, Y., et al., *Tor-Mediated Induction of Autophagy via an Apg1 Protein Kinase Complex.* The Journal of Cell Biology, 2000. **150**(6): p. 1507-1513.
256. Hara, T., et al., *FIP200, a ULK-interacting protein, is required for autophagosome formation in mammalian cells.* The Journal of Cell Biology, 2008. **181**(3): p. 497-510.
257. Kim, J., et al., *AMPK and mTOR regulate autophagy through direct phosphorylation of Ulk1.* Nat Cell Biol, 2011. **13**(2): p. 132-141.
258. Wu, H., et al., *Elongation Factor-2 Kinase Regulates Autophagy in Human Glioblastoma Cells.* Cancer Research, 2006. **66**(6): p. 3015-3023.
259. Cheng, Y., et al., *Cytoprotective Effect of the Elongation Factor-2 Kinase-Mediated Autophagy in Breast Cancer Cells Subjected to Growth Factor Inhibition.* PLoS ONE, 2010. **5**(3): p. e9715.
260. Kyei, G.B., et al., *Autophagy pathway intersects with HIV-1 biosynthesis and regulates viral yields in macrophages.* The Journal of Cell Biology, 2009. **186**(2): p. 255-268.
261. Van Grol, J., et al., *HIV-1 Inhibits Autophagy in Bystander Macrophage/Monocytic Cells through Src-Akt and STAT3.* PLoS ONE, 2010. **5**(7): p. e11733.

262. Caldera, V., et al., *STAT3 Expression and It's Correlation with Proliferation and Apoptosis/Autophagy in Gliomas.* Journal of Oncology, 2008. **2008**: p. 8 pages.
263. Aoki, H., et al., *Telomere 3' overhang-specific DNA oligonucleotides induce autophagy in malignant glioma cells.* The FASEB Journal, 2007. **21**(11): p. 2918-2930.
264. Porstmann, T., et al., *SREBP Activity Is Regulated by mTORC1 and Contributes to Akt-Dependent Cell Growth.* Cell metabolism, 2008. **8**(3): p. 224-236.
265. Li, S., M.S. Brown, and J.L. Goldstein, *Bifurcation of insulin signaling pathway in rat liver: mTORC1 required for stimulation of lipogenesis, but not inhibition of gluconeogenesis.* Proceedings of the National Academy of Sciences, 2010. **107**(8): p. 3441-3446.
266. Ferré, P. and F. Foufelle, *SREBP-1c Transcription Factor and Lipid Homeostasis: Clinical Perspective.* Hormone Research in Paediatrics, 2007. **68**(2): p. 72-82.
267. Laplante, M. and D.M. Sabatini, *mTORC1 activates SREBP-1c and uncouples lipogenesis from gluconeogenesis.* Proceedings of the National Academy of Sciences, 2010. **107**(8): p. 3281-3282.
268. Porstmann, T., et al., *A new player in the orchestra of cell growth: SREBP activity is regulated by mTORC1 and contributes to the regulation of cell and organ size.* Biochemical Society Transactions, 2009. **037**(1): p. 278-283.
269. Brown, M.S. and J.L. Goldstein, *A proteolytic pathway that controls the cholesterol content of membranes, cells, and blood.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(20): p. 11041-11048.
270. Reid, B.N., et al., *Hepatic Overexpression of Hormone-sensitive Lipase and Adipose Triglyceride Lipase Promotes Fatty Acid Oxidation, Stimulates Direct Release of Free Fatty Acids, and Ameliorates Steatosis.* Journal of Biological Chemistry, 2008. **283**(19): p. 13087-13099.
271. Chakrabarti, P., et al., *Mammalian Target of Rapamycin Complex 1 Suppresses Lipolysis, Stimulates Lipogenesis, and Promotes Fat Storage.* Diabetes, 2010. **59**(4): p. 775-781.

272. Kersten, S., *Mechanisms of nutritional and hormonal regulation of lipogenesis.* 2001.
273. Le Bacquer, O., et al., *Elevated sensitivity to diet-induced obesity and insulin resistance in mice lacking 4E-BP1 and 4E-BP2.* The Journal of Clinical Investigation, 2007. **117**(2): p. 387-396.
274. Jacinto, E., et al., *TIP41 Interacts with TAP42 and Negatively Regulates the TOR Signaling Pathway.* Molecular Cell, 2001. **8**(5): p. 1017-1026.
275. Chen, J., R.T. Peterson, and S.L. Schreiber, *[alpha]4 Associates with Protein Phosphatases 2A, 4, and 6.* Biochemical and Biophysical Research Communications, 1998. **247**(3): p. 827-832.
276. Feldman, M.E., et al., *Active-Site Inhibitors of mTOR Target Rapamycin-Resistant Outputs of mTORC1 and mTORC2.* PLoS Biol, 2009. **7**(2): p. e1000038.
277. Bissler, J.J., et al., *Sirolimus for Angiomyolipoma in Tuberous Sclerosis Complex or Lymphangioleiomyomatosis.* New England Journal of Medicine, 2008. **358**(2): p. 140-151.
278. Ma, J., et al., *Mammalian target of rapamycin regulates murine and human cell differentiation through STAT3/p63/Jagged/Notch cascade.* The Journal of Clinical Investigation, 2010. **120**(1): p. 103-114.
279. Karbowniczek, M., et al., *The evolutionarily conserved TSC/Rheb pathway activates Notch in tuberous sclerosis complex and Drosophila external sensory organ development.* The Journal of Clinical Investigation, 2010. **120**(1): p. 93-102.
280. Boyd, K.P., B.R. Korf, and A. Theos, *Neurofibromatosis type 1.* J Am Acad Dermatol, 2009. **61**(1): p. 1-16.
281. Cichowski, K. and T. Jacks, *NF1 Tumor Suppressor Gene Function: Narrowing the GAP.* Cell, 2001. **104**(4): p. 593-604.
282. Johannessen, C.M., et al., *TORC1 Is Essential for NF1-Associated Malignancies.* Current Biology, 2008. **18**(1): p. 56-62.
283. Rosner, M., et al., *The mTOR pathway and its role in human genetic diseases.* Mutation Research/Reviews in Mutation Research. **659**(3): p. 284-292.
284. Shaw, R.J., et al., *The LKB1 tumor suppressor negatively regulates mTOR signaling.* Cancer cell, 2004. **6**(1): p. 91-99.

285. Brugarolas, J. and W.G. Kaelin Jr, *Dysregulation of HIF and VEGF is a unifying feature of the familial hamartoma syndromes.* Cancer cell, 2004. **6**(1): p. 7-10.
286. Shackelford, D.B., et al., *mTOR and HIF-1α-mediated tumor metabolism in an LKB1 mouse model of Peutz-Jeghers syndrome.* Proceedings of the National Academy of Sciences, 2009. **106**(27): p. 11137-11142.
287. Pilarski, R., *Cowden Syndrome: A Critical Review of the Clinical Literature.* Journal of Genetic Counseling, 2009. **18**(1): p. 13-27.
288. Eng, C., *PTEN: One Gene, Many Syndromes.* Human Mutation, 2003. **22**(3): p. 183-198.
289. Martignoni, et al., *Molecular Pathology of Lymphangioleiomyomatosis and Other Perivascular Epithelioid Cell Tumors.* Vol. 134. 2010, Northfield, IL, ETATS-UNIS: College of American Pathologists. 8.
290. Kristof, A.S., et al., *Protein expression of urotensin II, urotensin-related peptide and their receptor in the lungs of patients with lymphangioleiomyomatosis.* Peptides, 2010. **31**(8): p. 1511-1516.
291. Carsillo, T., A. Astrinidis, and E.P. Henske, *Mutations in the tuberous sclerosis complex gene TSC2 are a cause of sporadic pulmonary lymphangioleiomyomatosis.* Proceedings of the National Academy of Sciences of the United States of America, 2000. **97**(11): p. 6085-6090.
292. Rosser, T., A. Panigrahy, and W. McClintock, *The Diverse Clinical Manifestations of Tuberous Sclerosis Complex: A Review.* Seminars in Pediatric Neurology, 2006. **13**(1): p. 27-36.
293. Nauli, S.M., et al., *Polycystins 1 and 2 mediate mechanosensation in the primary cilium of kidney cells.* Nat Genet, 2003. **33**(2): p. 129-137.
294. Tao, Y., et al., *Rapamycin Markedly Slows Disease Progression in a Rat Model of Polycystic Kidney Disease.* Journal of the American Society of Nephrology, 2005. **16**(1): p. 46-51.
295. Dere, R., et al., *Carboxy Terminal Tail of Polycystin-1 Regulates Localization of TSC2 to Repress mTOR.* PLoS ONE, 2010. **5**(2): p. e9239.
296. Boehlke, C., et al., *Primary cilia regulate mTORC1 activity and cell size through Lkb1.* Nat Cell Biol, 2010. **12**(11): p. 1115-1122.

297. Steck, P.A., et al., *Identification of a candidate tumour suppressor gene, MMAC1, at chromosome 10q23.3 that is mutated in multiple advanced cancers.* Nat Genet, 1997. **15**(4): p. 356-362.
298. Doll, A., et al., *Novel molecular profiles of endometrial cancer--new light through old windows.* The Journal of Steroid Biochemistry and Molecular Biology, 2008. **108**(3-5): p. 221-229.
299. Ali, I.U., L.M. Schriml, and M. Dean, *Mutational Spectra of PTEN/MMAC1 Gene: a Tumor Suppressor With Lipid Phosphatase Activity.* Journal of the National Cancer Institute, 1999. **91**(22): p. 1922-1932.
300. Crabbe, T., M.J. Welham, and S.G. Ward, *The PI3K inhibitor arsenal: choose your weapon!* Trends in Biochemical Sciences, 2007. **32**(10): p. 450-456.
301. Sato, T., et al., *Single amino-acid changes that confer constitutive activation of mTOR are discovered in human cancer.* Oncogene, 2010. **29**(18): p. 2746-2752.
302. O'Reilly, K.E., et al., *mTOR Inhibition Induces Upstream Receptor Tyrosine Kinase Signaling and Activates Akt.* Cancer Research, 2006. **66**(3): p. 1500-1508.
303. Manning, B.D. and L.C. Cantley, *AKT/PKB Signaling: Navigating Downstream.* Cell, 2007. **129**(7): p. 1261-1274.
304. Coenen, et al., *Rapamycin, not cyclosporine, permits thymic generation and peripheral preservation of CD4 [+] CD25 [+] FoxP3 [+] T cells.* Vol. 39. 2007, Basingstoke, ROYAUME-UNI: Nature Publishing Group. 9.
305. Adeegbe, D., et al., *Cutting Edge: Allogeneic CD4+CD25+Foxp3+ T Regulatory Cells Suppress Autoimmunity while Establishing Transplantation Tolerance.* The Journal of Immunology, 2006. **176**(12): p. 7149-7153.
306. Battaglia, M., et al., *Induction of Tolerance in Type 1 Diabetes via Both CD4+CD25+ T Regulatory Cells and T Regulatory Type 1 Cells.* Diabetes, 2006. **55**(6): p. 1571-1580.
307. Fernandez, D., et al., *Rapamycin reduces disease activity and normalizes T cell activation–induced calcium fluxing in patients with systemic lupus erythematosus.* Arthritis & Rheumatism, 2006. **54**(9): p. 2983-2988.
308. Teachey, D.T., et al., *Rapamycin improves lymphoproliferative disease in murine autoimmune lymphoproliferative syndrome (ALPS).* Blood, 2006. **108**(6): p. 1965-1971.

309. Martin, D., et al., *Synergistic effect of rapamycin and cyclosporin A in the treatment of experimental autoimmune uveoretinitis.* The Journal of Immunology, 1995. **154**(2): p. 922-927.
310. Wymann, M.P. and R. Schneiter, *Lipid signalling in disease.* Nat Rev Mol Cell Biol, 2008. **9**(2): p. 162-176.
311. Tsang, C.K., et al., *Targeting mammalian target of rapamycin (mTOR) for health and diseases.* Drug Discovery Today, 2007. **12**(3-4): p. 112-124.
312. Zoncu, R., A. Efeyan, and D.M. Sabatini, *mTOR: from growth signal integration to cancer, diabetes and ageing.* Nat Rev Mol Cell Biol, 2011. **12**(1): p. 21-35.
313. Newgard, C.B., et al., *A Branched-Chain Amino Acid-Related Metabolic Signature that Differentiates Obese and Lean Humans and Contributes to Insulin Resistance.* Cell metabolism, 2009. **9**(4): p. 311-326.
314. Ravikumar, B., et al., *Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of Huntington disease.* Nat Genet, 2004. **36**(6): p. 585-595.
315. Pan, T., et al., *The role of autophagy-lysosome pathway in neurodegeneration associated with Parkinson's disease.* Brain, 2008. **131**(8): p. 1969-1978.
316. Webb, J.L., et al., *α-Synuclein Is Degraded by Both Autophagy and the Proteasome.* Journal of Biological Chemistry, 2003. **278**(27): p. 25009-25013.
317. Pickford, F., et al., *The autophagy-related protein beclin 1 shows reduced expression in early Alzheimer disease and regulates amyloid- beta accumulation in mice.* J Clin Invest., 2008. **118**(6): p. 2190-2199.
318. Yu, W.H., et al., *Autophagic vacuoles are enriched in amyloid precursor protein-secretase activities: implications for [beta]-amyloid peptide over-production and localization in Alzheimer's disease.* The International Journal of Biochemistry & Cell Biology, 2004. **36**(12): p. 2531-2540.
319. Butterfield, D.A., et al., *Evidence of oxidative damage in Alzheimer's disease brain: central role for amyloid [beta]-peptide.* Trends in Molecular Medicine, 2001. **7**(12): p. 548-554.
320. SantaCruz, K., et al., *Tau Suppression in a Neurodegenerative Mouse Model Improves Memory Function.* Science, 2005. **309**(5733): p. 476-481.
321. Berger, Z., et al., *Rapamycin alleviates toxicity of different aggregate-prone proteins.* Human Molecular Genetics, 2006. **15**(3): p. 433-442.

322. Ljungberg, M.C., et al., *Activation of mammalian target of rapamycin in cytomegalic neurons of human cortical dysplasia.* Annals of Neurology, 2006. **60**(4): p. 420-429.
323. Tang, S.J., et al., *A rapamycin-sensitive signaling pathway contributes to long-term synaptic plasticity in the hippocampus.* Proceedings of the National Academy of Sciences of the United States of America, 2002. **99**(1): p. 467-472.
324. Hoeffer, C.A. and E. Klann, *mTOR signaling: At the crossroads of plasticity, memory and disease.* Trends in Neurosciences, 2010. **33**(2): p. 67-75.
325. Schalm, S.S., A.R. Tee, and J. Blenis, *Characterization of a Conserved C-terminal Motif (RSPRR) in Ribosomal Protein S6 Kinase 1 Required for Its Mammalian Target of Rapamycin-dependent Regulation.* Journal of Biological Chemistry, 2005. **280**(12): p. 11101-11106.
326. Dunlop, E.A., et al., *Determining the pathogenicity of patient-derived TSC2 mutations by functional characterization and clinical evidence.* Eur J Hum Genet, 2011.
327. Boulahbel, H., R. Duran, and E. Gottlieb, *Prolyl hydroxylases as regulators of cell metabolism.* Biochemical Society Transactions, 2009. **37**(Pt 1): p. 291-294.
328. Korge, P., P. Ping, and J.N. Weiss, *Reactive Oxygen Species Production in Energized Cardiac Mitochondria During Hypoxia/Reoxygenation.* Circulation Research, 2008. **103**(8): p. 873-880.
329. Garcia, C.M., et al., *The function of VEGF-A in lens development: Formation of the hyaloid capillary network and protection against transient nuclear cataracts.* Experimental Eye Research, 2009. **88**(2): p. 270-276.
330. Wilson, C., et al., *A mouse model of tuberous sclerosis 1 showing background specific early post-natal mortality and metastatic renal cell carcinoma.* Human Molecular Genetics, 2005. **14**(13): p. 1839-1850.
331. Onda, H., et al., *Tsc2+/– mice develop tumors in multiple sites that express gelsolin and are influenced by genetic background.* The Journal of Clinical Investigation, 1999. **104**(6): p. 687-695.
332. Ho, C. and J. Strauss, *Activation of the control reporter plasmids pRL-TK and pRL-SV40 by multiple GATA transcription factors can lead to aberrant normalization of transfection efficiency.* BMC Biotechnology, 2004. **4**(1): p. 10.

333. Shifera, et al., *Factors modulating expression of Renilla luciferase from control plasmids used in luciferase reporter gene assays.* Vol. 396. 2010, Kidlington, ROYAUME-UNI: Elsevier. 6.
334. Gregg L, S., *Signal transduction to hypoxia-inducible factor 1.* Biochemical Pharmacology, 2002. **64**(5-6): p. 993-998.
335. Stolovich, M., et al., *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., 2002. **22**(23): p. 8101-8113.
336. Jefferies, H.B., et al., *Rapamycin selectively represses translation of the "polypyrimidine tract" mRNA family.* Proceedings of the National Academy of Sciences of the United States of America, 1994. **91**(10): p. 4441-4445.
337. Abraham, R.T., *PI 3-kinase related kinases: [`]big' players in stress-induced signaling pathways.* DNA Repair. **3**(8-9): p. 883-887.
338. Urano, J., et al., *Point mutations in TOR confer Rheb-independent growth in fission yeast and nutrient-independent mammalian TOR signaling in mammalian cells.* Proceedings of the National Academy of Sciences, 2007. **104**(9): p. 3514-3519.
339. Pagé, E.L., et al., *Induction of Hypoxia-inducible Factor-1α by Transcriptional and Translational Mechanisms.* Journal of Biological Chemistry, 2002. **277**(50): p. 48403-48409.
340. Thomas, G.V., et al., *Hypoxia-inducible factor determines sensitivity to inhibitors of mTOR in kidney cancer.* Nat Med, 2006. **12**(1): p. 122-127.
341. Wouters, B.G., et al., *Control of the hypoxic response through regulation of mRNA translation.* Seminars in Cell & Developmental Biology. **16**(4-5): p. 487-501.
342. Tang, H., et al., *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., 2001. **21**(24): p. 8671-8683.
343. Haghighat, A. and N. Sonenberg, *eIF4G Dramatically Enhances the Binding of eIF4E to the mRNA 5'-Cap Structure.* Journal of Biological Chemistry, 1997. **272**(35): p. 21677-21680.

344. Tee, A.R., J.A. Tee, and J. Blenis, *Characterizing the interaction of the mammalian eIF4E-related protein 4EHP with 4E-BP1.* FEBS Letters, 2004. **564**(1-2): p. 58-62.

345. Sonenberg, N. and A.-C. Gingras, *The mRNA 5' cap-binding protein eIF4E and control of cell growth.* Current Opinion in Cell Biology, 1998. **10**(2): p. 268-275.

346. Fingar, D.C., et al., *mTOR Controls Cell Cycle Progression through Its Cell Growth Effectors S6K1 and 4E-BP1/Eukaryotic Translation Initiation Factor 4E.* Mol. Cell. Biol., 2004. **24**(1): p. 200-216.

347. Maquat, L.E., W.-Y. Tarn, and O. Isken, *The Pioneer Round of Translation: Features and Functions.* 2010. **142**(3): p. 368-374.

348. Hu, C.-J., et al., *Differential Roles of Hypoxia-Inducible Factor 1{alpha} (HIF-1{alpha}) and HIF-2{alpha} in Hypoxic Gene Regulation.* Mol. Cell. Biol., 2003. **23**(24): p. 9361-9374.

349. Murthy, V., et al., *Similarities and differences in the subcellular localization of hamartin and tuberin in the kidney.* Am J Physiol Renal Physiol, 2000. **278**(5): p. F737-746.

350. York, B., et al., *Cross-talk between tuberin, calmodulin, and estrogen signaling pathways.* FASEB J., 2005: p. 04-3142fje.

351. Noonan, D.J., et al., *A Calmodulin Binding Site in the Tuberous Sclerosis 2 Gene Product Is Essential for Regulation of Transcription Events and Is Altered by Mutations Linked to Tuberous Sclerosis and Lymphangioleiomyomatosis.* Archives of Biochemistry and Biophysics, 2002. **398**(1): p. 132-140.

352. Henry, K.W., et al., *Tuberous Sclerosis Gene 2 Product Modulates Transcription Mediated by Steroid Hormone Receptor Family Members*, in *Journal of Biological Chemistry*. 1998. p. 20535-20539.

353. Jefferies, H.B.J., et al., *Rapamycin suppresses 5[prime]TOP mRNA translation through inhibition of p70s6k.* EMBO J, 1997. **16**(12): p. 3693-3704.

354. Pende, M., et al., *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., 2004. **24**(8): p. 3112-3124.

355. Hui, A.S., et al., *Calcium signaling stimulates translation of HIF-α during hypoxia.* The FASEB Journal, 2006. **20**(3): p. 466-475.
356. Kawasome, H., et al., *Targeted disruption of p70s6k defines its role in protein synthesis and rapamycin sensitivity.* Proceedings of the National Academy of Sciences, 1998. **95**(9): p. 5033-5038.
357. Koh, M.Y., T.R. Spivak-Kroizman, and G. Powis, *HIF-1 regulation: not so easy come, easy go.* Trends in Biochemical Sciences, 2008. **33**(11): p. 526-534.
358. Vo, N. and R.H. Goodman, *CREB-binding Protein and p300 in Transcriptional Regulation.* Journal of Biological Chemistry, 2001. **276**(17): p. 13505-13508.
359. Galban, S. and M. Gorospe, *Factors interacting with HIF-1alpha mRNA: novel therapeutic targets.* Curr Pharm Des, 2009. **15**(33): p. 3853-3860.
360. Minet, E., et al., *HIF1A Gene Transcription Is Dependent on a Core Promoter Sequence Encompassing Activating and Inhibiting Sequences Located Upstream from the Transcription Initiation Site and cis Elements Located within the 5'UTR.* Biochemical and Biophysical Research Communications, 1999. **261**(2): p. 534-540.
361. Niu, G., et al., *Signal Transducer and Activator of Transcription 3 Is Required for Hypoxia-Inducible Factor-1α RNA Expression in Both Tumor Cells and Tumor-Associated Myeloid Cells.* Molecular Cancer Research, 2008. **6**(7): p. 1099-1105.
362. Finlay, G.A., et al., *Estrogen-induced Smooth Muscle Cell Growth Is Regulated by Tuberin and Associated with Altered Activation of Platelet-derived Growth Factor Receptor-β and ERK-1/2.* Journal of Biological Chemistry, 2004. **279**(22): p. 23114-23122.
363. Sancak, O., et al., *Mutational analysis of the TSC1 and TSC2 genes in a diagnostic setting: genotype - phenotype correlations and comparison of diagnostic DNA techniques in Tuberous Sclerosis Complex.* Eur J Hum Genet, 2005. **13**(6): p. 731-741.
364. El-Hashemite, N., et al., *Loss of Tsc1 or Tsc2 Induces Vascular Endothelial Growth Factor Production through Mammalian Target of Rapamycin.* Cancer Research, 2003. **63**(17): p. 5173-5177.
365. Brugarolas, J.B., et al., *TSC2 regulates VEGF through mTOR-dependent and -independent pathways.* Cancer cell, 2003. **4**(2): p. 147-158.

366. Burton, T.R. and S.B. Gibson, *The role of Bcl-2 family member BNIP3 in cell death and disease: NIPping at the heels of cell death.* Cell Death Differ, 2009. **16**(4): p. 515-523.
367. Dabora, S.L., et al., *Mutational Analysis in a Cohort of 224 Tuberous Sclerosis Patients Indicates Increased Severity of TSC2, Compared with TSC1, Disease in Multiple Organs.* The American Journal of Human Genetics, 2001. **68**(1): p. 64-80.
368. Coevoets, R., et al., *A reliable cell-based assay for testing unclassified TSC2 gene variants.* Eur J Hum Genet, 2008. **17**(3): p. 301-310.
369. York, B., D. Lou, and D.J. Noonan, *Tuberin Nuclear Localization Can Be Regulated by Phosphorylation of Its Carboxyl Terminus.* Molecular Cancer Research, 2006. **4**(11): p. 885-897.
370. Rosner, M., et al., *The tuberous sclerosis gene products hamartin and tuberin are multifunctional proteins with a wide spectrum of interacting partners.* Mutation Research/Reviews in Mutation Research. **658**(3): p. 234-246.
371. Kwiatkowski, D.J., et al., *A mouse model of TSC1 reveals sex-dependent lethality from liver hemangiomas, and up-regulation of p70S6 kinase activity in Tsc1 null cells.* Human Molecular Genetics, 2002. **11**(5): p. 525-534.
372. Schrijvers, B.F., A. Flyvbjerg, and A.S. De Vriese, *The role of vascular endothelial growth factor (VEGF) in renal pathophysiology.* Kidney Int, 2004. **65**(6): p. 2003-2017.
373. Hur, E., et al., *Mitogen-Activated Protein Kinase Kinase Inhibitor PD98059 Blocks the trans-Activation but Not the Stabilization or DNA Binding Ability of Hypoxia-Inducible Factor-1α.* Molecular Pharmacology, 2001. **59**(5): p. 1216-1224.
374. Minet, E., et al., *ERK activation upon hypoxia: involvement in HIF-1 activation.* FEBS Letters, 2000. **468**(1): p. 53-58.
375. Mottet, D., et al., *Role of ERK and calcium in the hypoxia-induced activation of HIF-1.* Journal of Cellular Physiology, 2003. **194**(1): p. 30-44.
376. Ravi, R., et al., *Regulation of tumor angiogenesis by p53-induced degradation of hypoxia-inducible factor 1α.* Genes & Development, 2000. **14**(1): p. 34-44.
377. Haddad, J.J. and S.C. Land, *A non-hypoxic, ROS-sensitive pathway mediates TNF-[alpha]-dependent regulation of HIF-1[alpha].* FEBS Letters, 2001. **505**(2): p. 269-274.

378. Suzuki, T., et al., *Tuberous sclerosis complex 2 loss-of-function mutation regulates reactive oxygen species production through Rac1 activation.* Biochemical and Biophysical Research Communications, 2008. **368**(1): p. 132-137.

379. Khan, Z., G.K. Michalopoulos, and D.B. Stolz, *Peroxisomal Localization of Hypoxia-Inducible Factors and Hypoxia-Inducible Factor Regulatory Hydroxylases in Primary Rat Hepatocytes Exposed to Hypoxia-Reoxygenation.* Am J Pathol, 2006. **169**(4): p. 1251-1269.

380. Hirano, T., K. Ishihara, and M. Hibi, *Roles of STAT3 in mediating the cell growth, differentiation and survival signals relayed through the IL-6 family of cytokine receptors.* Oncogene, 2000. **19**(21): p. 2548-56.

381. Jung, J., Kim, HS., Lee, CS., Shin, YJ., Kim, YN., Kang, GH., Kim, TY., Juhnn, YS., Kim, SJ., Park, JW., Ye, SK., Chung, MH., *STAT3 inhibits the degradation of HIF-1 alpha by pVHL-mediated ubiquitination.* Exp Mol Med., 2008. **40**(5): p. 479-485.

382. Qin, H.R., et al., *Activation of Signal Transducer and Activator of Transcription 3 through a Phosphomimetic Serine 727 Promotes Prostate Tumorigenesis Independent of Tyrosine 705 Phosphorylation.* Cancer Research, 2008. **68**(19): p. 7736-7741.

383. Wen, Z., Z. Zhong, and J.E. Darnell, *Maximal activation of transcription by statl and stat3 requires both tyrosine and serine phosphorylation.* Cell, 1995. **82**(2): p. 241-250.

384. Goh, K.C., S.J. Haque, and B.R.G. Williams, *p38 MAP kinase is required for STAT1 serine phosphorylation and transcriptional activation induced by interferons.* EMBO J, 1999. **18**(20): p. 5601-5608.

385. Kovarik, P., et al., *Stress-induced phosphorylation of STAT1 at Ser727 requires p38 mitogen-activated protein kinase whereas IFN-γ uses a different signaling pathway.* Proceedings of the National Academy of Sciences of the United States of America, 1999. **96**(24): p. 13956-13961.

386. Kim, H. and H. Baumann, *The Carboxyl-terminal Region of STAT3 Controls Gene Induction by the Mouse Haptoglobin Promoter.* Journal of Biological Chemistry, 1997. **272**(23): p. 14571-14579.

387. Lim, C.P. and X. Cao, *Serine phosphorylation and negative regulation of Stat3 by JNK.* The Journal of biological chemistry, 1999. **274**(43): p. 31055-61.

388. Liu, L., K.M. McBride, and N.C. Reich, *STAT3 nuclear import is independent of tyrosine phosphorylation and mediated by importin-α3.* Proceedings of the National Academy of Sciences of the United States of America, 2005. **102**(23): p. 8150-8155.
389. Huang, S., et al., *ARHI (DIRAS3), an imprinted tumour suppressor gene, binds to importins and blocks nuclear import of cargo proteins.* Bioscience Reports, 2009. **30**(3): p. 159-168.
390. Lim, C.P. and X. Cao, *Serine Phosphorylation and Negative Regulation of Stat3 by JNK.* Journal of Biological Chemistry, 1999. **274**(43): p. 31055-31061.
391. Lim, C.P. and X. Cao, *Regulation of Stat3 Activation by MEK Kinase 1.* Journal of Biological Chemistry, 2001. **276**(24): p. 21004-21011.
392. Kuroki, M. and J.T. O'Flaherty, *Extracellular signal-regulated protein kinase (ERK)-dependent and ERK-independent pathways target STAT3 on serine-727 in human neutrophils stimulated by chemotactic factors and cytokines.* Biochem. J., 1999. **341**(3): p. 691-696.
393. Gartsbein, et al., *The role of protein kinase C; activation and STATt3 Ser727 phosphorylation in insulin-induced keratinocyte proliferation.* Vol. 119. 2006, Cambridge, ROYAUME-UNI: Company of Biologists. 12.
394. Jain, N., et al., *Protein Kinase C δ Associates with and Phosphorylates Stat3 in an Interleukin-6-dependent Manner.* Journal of Biological Chemistry, 1999. **274**(34): p. 24392-24400.
395. Kim, J.-H., et al., *Regulation of Interleukin-6-induced Hepatic Insulin Resistance by Mammalian Target of Rapamycin through the STAT3-SOCS3 Pathway.* Journal of Biological Chemistry, 2008. **283**(2): p. 708-715.
396. Frank, D.A., S. Mahajan, and J. Ritz, *B lymphocytes from patients with chronic lymphocytic leukemia contain signal transducer and activator of transcription (STAT) 1 and STAT3 constitutively phosphorylated on serine residues.* The Journal of Clinical Investigation, 1997. **100**(12): p. 3140-3148.
397. Turkson, J., et al., *Requirement for Ras/Rac1-Mediated p38 and c-Jun N-Terminal Kinase Signaling in Stat3 Transcriptional Activity Induced by the Src Oncoprotein.* Mol. Cell. Biol., 1999. **19**(11): p. 7519-7528.
398. Ceresa, et al., *Insulin stimulates the serine phosphorylation of the signal transducer and activator of transcription (STAT3) isoform.* 1996, American

Society for Biochemistry and Molecular Biology: Bethesda, MD, ETATS-UNIS. p. 4.

399. El-Hashemite, N., et al., *Perturbed IFN-γ-Jak-Signal Transducers and Activators of Transcription Signaling in Tuberous Sclerosis Mouse Models.* Cancer Research, 2004. **64**(10): p. 3436-3443.

400. Zhou, J., et al., *Activation of the PTEN/mTOR/STAT3 pathway in breast cancer stem-like cells is required for viability and maintenance.* Proceedings of the National Academy of Sciences, 2007. **104**(41): p. 16158-16163.

401. Yau, C.Y.F., et al., *Inhibition of Integrin-Linked Kinase by a Selective Small Molecule Inhibitor, QLT0254, Inhibits the PI3K/PKB/mTOR, Stat3, and FKHR Pathways and Tumor Growth, and Enhances Gemcitabine-Induced Apoptosis in Human Orthotopic Primary Pancreatic Cancer Xenografts.* Cancer Research, 2005. **65**(4): p. 1497-1504.

402. Lee, C., et al., *Targeting YB-1 in HER-2 Overexpressing Breast Cancer Cells Induces Apoptosis via the mTOR/STAT3 Pathway and Suppresses Tumor Growth in Mice.* Cancer Research, 2008. **68**(21): p. 8661-8666.

403. Oh, et al., *Activation of Phosphatidylinositol 3-Kinase through Glycoprotein 130 induces Protein Kinase B and P70 S6 Kinase phosphorylation in cardiac myocytes.* Vol. 273. 1998, Bethesda, MD, ETATS-UNIS: American Society for Biochemistry and Molecular Biology. 8.

404. Kamakura, S., et al., *Hes binding to STAT3 mediates crosstalk between Notch and JAK-STAT signalling.* Nat Cell Biol, 2004. **6**(6): p. 547-554.

405. Karni, et al., *Activated pp60c-Src leads to elevated hypoxia-inducible factor (HIF)-alpha; expression under normoxia.* 2002. **277**(45): p. 7.

406. Kondo, K., et al., *Inhibition of HIF is necessary for tumor suppression by the von Hippel-Lindau protein.* Cancer cell, 2002. **1**(3): p. 237-246.

407. Chang, F., et al., *Involvement of PI3K//Akt pathway in cell cycle progression, apoptosis, and neoplastic transformation: a target for cancer chemotherapy.* Leukemia, 2003. **17**(3): p. 590-603.

408. Li, J., et al., *PTEN, a Putative Protein Tyrosine Phosphatase Gene Mutated in Human Brain, Breast, and Prostate Cancer.* Science, 1997. **275**(5308): p. 1943-1947.

409. Neshat, M.S., et al., *Enhanced sensitivity of PTEN-deficient tumors to inhibition of FRAP/mTOR.* Proceedings of the National Academy of Sciences of the United States of America, 2001. **98**(18): p. 10314-10319.
410. Vivanco, I. and C.L. Sawyers, *The phosphatidylinositol 3-Kinase-AKT pathway in human cancer.* Nat Rev Cancer, 2002. **2**(7): p. 489-501.
411. Bromberg, J., *Stat proteins and oncogenesis.* The Journal of Clinical Investigation, 2002. **109**(9): p. 1139-1142.
412. Yu, H., M. Kortylewski, and D. Pardoll, *Crosstalk between cancer and immune cells: role of STAT3 in the tumour microenvironment.* Nat Rev Immunol, 2007. **7**(1): p. 41-51.
413. Jung, J.E., et al., *STAT3 is a potential modulator of HIF-1-mediated VEGF expression in human renal carcinoma cells.* The FASEB Journal.
414. Dufner, A. and G. Thomas, *Ribosomal S6 Kinase Signaling and the Control of Translation.* Experimental Cell Research, 1999. **253**(1): p. 100-109.
415. Iyer, N.V., S.W. Leung, and G.L. Semenza, *The Human Hypoxia-Inducible Factor 1[alpha] Gene:HIF1AStructure and Evolutionary Conservation.* Genomics, 1998. **52**(2): p. 159-165.
416. Laughner, E., et al., *HER2 (neu) Signaling Increases the Rate of Hypoxia-Inducible Factor 1{alpha} (HIF-1{alpha}) Synthesis: Novel Mechanism for HIF-1-Mediated Vascular Endothelial Growth Factor Expression.* Mol. Cell. Biol., 2001. **21**(12): p. 3995-4004.
417. Tandon, P., et al., *Requirement for ribosomal protein S6 kinase 1 to mediate glycolysis and apoptosis resistance induced by Pten deficiency.* Proceedings of the National Academy of Sciences, 2011. **108**(6): p. 2361-2365.
418. Choo, A.Y., et al., *Rapamycin differentially inhibits S6Ks and 4E-BP1 to mediate cell-type-specific repression of mRNA translation.* Proceedings of the National Academy of Sciences, 2008. **105**(45): p. 17414-17419.
419. Young, R.M., et al., *Hypoxia-mediated Selective mRNA Translation by an Internal Ribosome Entry Site-independent Mechanism.* Journal of Biological Chemistry, 2008. **283**(24): p. 16309-16319.
420. Davies, D.M., et al., *Sirolimus Therapy in Tuberous Sclerosis or Sporadic Lymphangioleiomyomatosis.* New England Journal of Medicine, 2008. **358**(2): p. 200-203.

421. Ali, M., et al., *Mutation and polymorphism analysis of TSC1 and TSC2 genes in Indian patients with tuberous sclerosis complex.* Acta Neurologica Scandinavica, 2005. **111**(1): p. 54-63.
422. Lou, D., N. Griffith, and D.J. Noonan, *The Tuberous Sclerosis 2 Gene Product Can Localize to Nuclei in a Phosphorylation-Dependent Manner.* Molecular Cell Biology Research Communications, 2001. **4**(6): p. 374-380.
423. Chorianopoulos, D. and G. Stratakos, *Lymphangioleiomyomatosis and Tuberous Sclerosis Complex.* Lung, 2008. **186**(4): p. 197-207.
424. Sato, T., et al., *Mutation analysis of the <i>TSC1</i> and <i>TSC2</i> genes in Japanese patients with pulmonary lymphangioleiomyomatosis.* Journal of Human Genetics, 2002. **47**(1): p. 20-28.
425. Meloche, S. and J. Pouyssegur, *The ERK1//2 mitogen-activated protein kinase pathway as a master regulator of the G1- to S-phase transition.* Oncogene, 2007. **26**(22): p. 3227-3239.
426. Hickson, I., et al., *Identification and Characterization of a Novel and Specific Inhibitor of the Ataxia-Telangiectasia Mutated Kinase ATM.* Cancer Research, 2004. **64**(24): p. 9152-9159.
427. Cam, H., et al., *mTORC1 Signaling under Hypoxic Conditions Is Controlled by ATM-Dependent Phosphorylation of HIF-1[alpha].* Molecular Cell, 2010. **40**(4): p. 509-520.
428. Bonello, S., et al., *Reactive Oxygen Species Activate the HIF-1{alpha} Promoter Via a Functional NF{kappa}B Site.* Arterioscler Thromb Vasc Biol, 2007. **27**(4): p. 755-761.
429. Lang, S.A., et al., *Targeting Heat Shock Protein 90 in Pancreatic Cancer Impairs Insulin-like Growth Factor-I Receptor Signaling, Disrupts an Interleukin-6/Signal-Transducer and Activator of Transcription 3/Hypoxia-Inducible Factor-1α Autocrine Loop, and Reduces Orthotopic Tumor Growth.* Clinical Cancer Research, 2007. **13**(21): p. 6459-6468.
430. Zhang, Y., et al., *Ataxia telangiectasia mutated proteins, MAPKs, and RSK2 are involved in the phosphorylation of STAT3.* Vol. 278. 2003, Bethesda, MD, ETATS-UNIS: American Society for Biochemistry and Molecular Biology. 10.
431. Barry, S.P., et al., *STAT3 modulates the DNA damage response pathway.* International Journal of Experimental Pathology, 2010. **91**(6): p. 506-514.

432. Turkson, J., et al., *Phosphotyrosyl Peptides Block Stat3-mediated DNA Binding Activity, Gene Regulation, and Cell Transformation.* Journal of Biological Chemistry, 2001. **276**(48): p. 45443-45455.
433. Sen, B., et al., *Sustained Src Inhibition Results in Signal Transducer and Activator of Transcription 3 (STAT3) Activation and Cancer Cell Survival via Altered Janus-Activated Kinase–STAT3 Binding.* Cancer Research, 2009. **69**(5): p. 1958-1965.
434. Yu, H., D. Pardoll, and R. Jove, *STATs in cancer inflammation and immunity: a leading role for STAT3.* Nat Rev Cancer, 2009. **9**(11): p. 798-809.
435. Sergei, I.G. and K. Michael, *Dangerous liaisons: STAT3 and NF-κB collaboration and crosstalk in cancer.* Cytokine & growth factor reviews, 2010. **21**(1): p. 11-19.
436. Leong, P.L., et al., *Targeted inhibition of Stat3 with a decoy oligonucleotide abrogates head and neck cancer cell growth.* PNAS, 2003. **100**(7): p. 4138-4143.
437. Corvinus, F.M., et al., *Persistent STAT3 Activation in Colon Cancer Is Associated with Enhanced Cell Proliferation and Tumor Growth1*, Neoplasia Press Inc.
438. Garcia, et al., *Constitutive activation of Stat3 by the Src and JAK tyrosine kinases participates in growth regulation of human breast carcinoma cells.* Vol. 20. 2001, Basingstoke, ROYAUME-UNI: Nature Publishing Group.
439. Niu, G., et al., *Roles of activated Src and Stat3 in signaling in melanoma tumour cell growth.* Oncogene, 2002. **21**(46): p. 7001-7010.
440. Kim, H.L., et al., *HIF-1alpha and STAT3 client proteins interacting with the cancer chaperone Hsp90: Therapeutic considerations.* Cancer Biology & Therapy, 2008. **7**(1): p. 1-5.
441. Bharti, A.C., et al., *Nuclear factor-{kappa}B and STAT3 are constitutively active in CD138+ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis.* Blood, 2004. **103**(8): p. 3175-3184.
442. Hsu, J.-H., et al., *Role of the Akt kinase in the expansion of multiple myeloma clones: effects on cytokine-dependent proliferative and survival responses.* Oncogene, 2002. **21**(9): p. 1391-1400.

443. Hsu, J.-h., et al., *The AKT kinase is activated in multiple myeloma tumor cells.* Blood, 2001. **98**(9): p. 2853-2855.
444. Feng, Z., et al., *The coordinate regulation of the p53 and mTOR pathways in cells.* PNAS, 2005. **102**(23): p. 8204-8209.
445. Takeda, K., et al., *Stat3 Activation Is Responsible for IL-6-Dependent T Cell Proliferation Through Preventing Apoptosis: Generation and Characterization of T Cell-Specific Stat3-Deficient Mice.* The Journal of Immunology, 1998. **161**(9): p. 4652-4660.
446. Beuvink, I., et al., *The mTOR Inhibitor RAD001 Sensitizes Tumor Cells to DNA-Damaged Induced Apoptosis through Inhibition of p21 Translation.* Cell, 2005. **120**(6): p. 747-759.
447. Barton, B.E., et al., *Signal transducer and activator of transcription 3 (STAT3) activation in prostate cancer: Direct STAT3 inhibition induces apoptosis in prostate cancer lines.* Molecular Cancer Therapeutics, 2004. **3**(1): p. 11-20.
448. Hu, L., et al., *Downstream effectors of oncogenic ras in multiple myeloma cells.* Blood, 2003. **101**(8): p. 3126-3135.