

CHARACTERISING THE SIGNALLING MECHANISM OF THE MTOR-DEPENDENT PHOSPHATASE

LYNDSEY A. SEYMOUR

2011

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

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CONTENTS

<i>Summary</i>	<i>viii</i>
<i>Declaration</i>	<i>ix</i>
<i>Acknowledgements</i>	<i>x</i>
<i>Abbreviations</i>	<i>xi</i>
<i>List of figures</i>	<i>xvi</i>
<i>List of tables</i>	<i>xviii</i>
<i>Amino acid abbreviations</i>	<i>xix</i>
<i>Codon table</i>	<i>xx</i>

1	INTRODUCTION	1
1.1	Phosphatases	1
1.1.1	General introduction	1
1.1.2	The PP2A holoenzyme	1
1.1.2.1	The catalytic subunit	1
1.1.2.2	The A subunit	2
1.1.2.3	The B subunit	2
1.1.3	Regulation of PP2A	4
1.1.3.1	Methylation	4
1.1.3.2	Phosphorylation	5
1.1.3.3	Degradation	6
1.1.3.4	Interaction with regulatory subunits	7
1.1.3.5	Translation	7
1.1.3.6	Oxidation status	8
1.2	Target of Rapamycin	8
1.2.1	TORC1	8
1.2.2	TORC2	9
1.2.3	TORC1 and regulation of transcription	9
1.2.3.1	Nitrogen Catabolite Repression	9
1.2.3.2	Stress Response Element	9
1.2.3.3	Metabolic genes	10
1.2.4	Phosphatases in TORC1 signalling	10
1.2.4.1	Tip41	12
1.3	The mTORC1 complex	13
1.3.1	mTOR	13
1.3.2	Raptor	13
1.3.3	mLST8	15
1.3.4	DEPTOR	16
1.3.5	Tel1 and Tti1	16
1.3.6	PRAS40	16
1.3.7	CUL4-DDB1	17
1.3.7.1	Ubiquitination	17
1.3.7.1.1	Polyubiquitination	17
1.3.7.1.2	Monoubiquitination	19
1.4	Processes regulated by mTORC1	20
1.4.1	The TOS motif	20
1.4.2	Regulation of translation	20

1.4.2.1	Translation in eukaryotes	20
1.4.2.2	4EBP1	25
1.4.2.3	S6K1	25
1.4.2.3.1	Ribosomal protein S6	26
1.4.2.3.2	eIF3-PIC	26
1.4.2.3.3	SKAR	27
1.4.2.3.4	eEF2K	27
1.4.3	Regulation of transcription	28
1.4.3.1	HIF1	28
1.4.3.2	STAT1	29
1.4.3.3	STAT3	29
1.4.3.4	YY1	30
1.4.3.5	SREBP	31
1.4.3.6	GLI3	31
1.4.4	Regulation of proliferation	32
1.4.5	Regulation of autophagy	33
1.4.5.1	ULK1	33
1.4.5.2	Atg9	34
1.4.5.3	LC3	34
1.4.6	Hierarchical phosphorylation of substrates	35
1.5	Signalling inputs to mTORC1	35
1.5.1	The TSC1/2 complex	35
1.5.2	Rheb	35
1.5.3	Rheb-mediated mTORC1 activation	36
1.5.3.1	FKBP38	37
1.5.3.2	PLD1	37
1.5.3.3	TCTP as a Rheb GEF	38
1.5.3.4	An mTORC1-independent function of Rheb	38
1.5.4	Amino acids	38
1.5.4.1	hVps34	39
1.5.4.2	Rag GTPases	40
1.5.4.3	RalA	40
1.5.5	Growth factors	41
1.5.6	Mitogenic stimuli	42
1.5.6.1	PA	42
1.5.6.2	MAPK	43
1.5.6.3	AMPK	44
1.5.6.4	Wnt	44
1.5.7	Hypoxic input	45
1.5.7.1	REDD1	45
1.5.7.2	Bnip3	46
1.5.8	Autophagic input	46
1.5.8.1	ROS	46
1.5.8.2	ULK1 and AMPK	47
1.5.9	Inhibitors of mTORC1	47
1.5.9.1	PI3K Inhibitors	47
1.5.9.2	Rapamycin	48
1.5.9.3	TorinI	48
1.5.10	Feedback inhibition	49
1.5.10.1	Feedback to IRS1	49

1.5.10.2	PDGFR	51
1.5.10.3	S6K1 and GSK3	51
1.5.10.4	eIF4E	51
1.5.10.5	PP2A _{Bβ} and PDK1	51
1.5.10.6	Feedback following chronic inhibition	52
1.6	Phosphatase regulation of mTORC1	52
1.6.1	PP2A	52
1.6.2	Alpha4	53
1.6.2.1	General introduction	53
1.6.2.2	Functions	53
1.6.2.2.1	Regulation of mTORC1	53
1.6.2.2.2	Independent regulation of STAT1	54
1.6.2.2.3	Apoptosis	55
1.6.2.2.4	Memory and Learning	55
1.6.3	Tip41	56
1.6.3.1	General introduction	56
1.6.3.2	Functions	56
1.6.3.2.1	ATM/ATR	56
1.6.3.2.2	mTORC1 and ATM/ATR	58
1.6.3.2.3	Tip41 and ATM/ATR	58
1.6.3.2.4	NF- κ B	59
1.6.3.2.5	mTORC1 and NF- κ B	59
1.6.3.2.6	Tip41 and NF- κ B	60
1.6.4	B α	61
1.6.4.1	Inhibition by E4ORF4	61
1.6.4.2	Functions	61
1.6.4.2.1	Regulation by mTORC1	61
1.6.4.2.2	Cdk1	62
1.6.4.2.3	Wnt signalling	63
1.6.5	B'	63
1.6.6	B β	64
1.6.7	PP6c	64
1.6.7.1	Structure	64
1.6.7.2	Functions	65
1.6.7.2.1	NF- κ B	65
1.6.7.2.2	DNA Repair	65
1.7	mTORC2	66
1.7.1	The mTORC2 complex	66
1.7.1.1	Rictor and mSin1	67
1.7.1.2	mLST8	67
1.7.1.3	Protor	67
1.7.1.4	Hsp70	67
1.7.2	Functions of mTORC2	68
1.7.2.1	Actin remodelling	68
1.7.2.2	Angiogenesis	69
1.7.2.3	Akt	69
1.7.3	Regulation of mTORC2	69
1.7.3.1	Feedback from mTORC1	69
1.7.3.2	TSC1/2	69
1.8	PP2A and disease	70

1.8.1	Genetic disease	70
1.8.1.1	Opitz Syndrome	70
1.8.2	Neurological disease	70
1.8.2.1	Alzheimer's disease	70
1.8.3	Cancer	72
1.9	Diseases and mTORC1	73
1.9.1	Genetic disease	73
1.9.1.1	Tuberous Sclerosis Complex	73
1.9.1.2	Lymphangiomyomatosis	74
1.9.1.3	Von Hippel-Lindau disease	74
1.9.1.4	PTEN syndromes	74
1.9.1.5	Peutz-Jeghers syndrome	74
1.9.2	Neurological disease	75
1.9.2.1	Parkinson's disease	75
1.9.2.2	Huntington Disease	75
1.9.3	Intractable epilepsy	76
1.9.4	Type II diabetes	76
1.9.4.1	Hsp70 and diabetes	77
1.9.5	Cancer	78
1.9.5.1	TSC1/2	78
1.9.5.2	Rheb	78
1.9.5.3	SGK1	79
1.9.5.4	Ras	79
1.9.5.5	PLD1	80
1.9.5.6	Feedback loops and therapeutics	80
1.10	Project aims	81

2	MATERIALS AND METHODS	84
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2.1	Suppliers	84
2.2	Materials	85
2.2.1	Chemicals	85
2.2.2	Plasmid details	85
2.2.3	Primers	86
2.2.4	Antibodies	87
2.2.5	Molecular biology and cloning	87
2.2.6	Cell culture	87
2.2.7	Transfection and cell lysis	89
2.2.8	Protein purification, cell fractionation and associated techniques	89
2.2.9	SDS PAGE	89
2.2.10	Isoelectric Focussing, electrotransfer and western blotting	89
2.2.11	Gel staining and Mass Spectrometry	89
2.2.12	mRNA extraction and quantitative PCR	89
2.3	Equipment	90
2.3.1	Plastics and glassware	90
2.3.2	Molecular biology and cloning	90
2.3.3	Cell culture	90
2.3.4	SDS PAGE and electrotransfer	90
2.3.5	Mass Spectrometry	90

2.3.6	Luciferase assays	91
2.3.7	Q-PCR	91
2.3.8	General equipment	91
2.3.9	Software	91
2.4	Methods	91
2.4.1	General reagents	91
2.4.2	Buffers and solutions	91
2.4.3	Molecular biology	93
2.4.3.1	PCR	93
2.4.3.2	Agarose gel electrophoresis	93
2.4.3.3	Cloning	93
2.4.3.4	Transformation and selection of competent cells	94
2.4.3.5	Plasmid DNA preparation	94
2.4.3.6	Site-directed mutagenesis	94
2.4.4	Cell culture	95
2.4.5	Transfection	95
2.4.5.1	CaCl ₂ precipitation transfection protocol	95
2.4.5.2	Lipofectamine 2000 transfection	95
2.4.6	Cell treatments	96
2.4.7	Standard cell lysis	96
2.4.8	Immunoprecipitation	96
2.4.9	GST purification	96
2.4.10	Catch and Release	97
2.4.11	Cell fractionation	97
2.4.12	Cross-linking with DTBP	97
2.4.13	In vivo radiolabelling	98
2.4.14	S6K1 assay	98
2.4.15	Luciferase reporter assay	98
2.4.16	mTORC1 kinase assay	99
2.4.16.1	Purification of mTOR/Raptor complex	99
2.4.16.2	Purification of GST-Rheb	99
2.4.16.3	Substrate purification	99
2.4.16.4	Assay preparation	100
2.4.17	mTORC2 kinase assay	100
2.4.18	SDS PAGE	101
2.4.19	Electrotransfer	101
2.4.20	Western blot analysis	101
2.4.21	Staining, fixing and drying polyacrylamide gels	101
2.4.22	Mass Spectrometry	102
2.4.23	Far western blotting	103
2.4.24	Isoelectric focussing and SDS PAGE	103
2.4.25	Q-PCR	104

3 CHARACTERISING INTERACTIONS OF TIP41 105

3.1	Introduction	105
3.2	Results	106
3.2.1	Purification of Tip41 using Tip41 polyclonal antibody	106
3.2.2	Tip41 interacts in complex with PP2Ac and Alpha4	108

3.2.3	Tip41 interacts directly with PP2Ac, but not Alpha4	110
3.2.4	PP2Ac interacts with S6K1 in response to mTORC1 inhibition	113
3.2.5	Wortmannin but not staurosporine effectively inhibits mTORC1 in vitro	115
3.2.6	Tip41 is not directly phosphorylated by mTORC1 in vitro	117
3.2.7	Tip41 may downregulate Raptor via proteasomal degradation	119
3.2.8	Raptor interacts with 4EBP1 but not Tip41	121
3.2.9	Tip41 is not involved in mTORC2 signalling	123
3.2.10	Tip41 phosphorylation is negatively regulated by insulin signalling	126
3.3	Discussion	130

4 TIP41 IN THE mTOR PATHWAY 137

4.1	Introduction	137
4.2	Results	138
4.2.1	Over-expression of Tip41 can inhibit or activate mTORC1	138
4.2.2	Tip41 induces an mTORC1 feedback loop, indicative of chronic inhibition, by upregulation of Akt	142
4.2.3	Overexpression of Tip41 leads to inhibition of HIF1 activity	145
4.2.4	Inactive mutants of Tip41 are underexpressed in comparison to wild-type protein	145
4.2.5	Analysis of PP2A _{Tip41} in mTORC1 signalling	149
4.2.6	MG132 partially rescued expression of Tip41(D71L)	151
4.2.7	Tip41 knockdown reduces S6K1 phosphorylation via induction of a negative feedback loop	153
4.2.8	Tip41 knockdown increases activity of HIF1	155
4.2.9	A novel nuclear isoform of Tip41	157
4.2.10	Tip41 acts upstream of mTORC1	157
4.2.11	Tip41 does not interact with TSC2	162
4.2.12	Tip41 does not inhibit mTORC1 via the NF- κ B or ATM/ATR signalling pathways	162
4.3	Discussion	166

5 OTHER PP2A COMPLEXES AND mTOR 174

5.1	Introduction	174
5.2	Results	175
5.2.1	E4ORF4 activates mTORC1 signalling by sequestering the B α regulatory subunit of PP2Ac	175
5.2.2	B α is regulated by ubiquitination in an mTORC1 specific manner	177
5.2.3	Depletion of B α increases S6K1 phosphorylation and activity of HIF1	180
5.2.4	PP2A _{Bα} does not regulate phosphorylation within the PI3K/Akt/TSC2 pathway upstream of mTORC1	185
5.2.5	PP2A _{Bα} inhibits mTORC1 downstream of the TSC1/2 complex	187
5.2.6	PP6c is a PP2A family member with links to mTORC1 signalling	189
5.2.7	Depletion of PP6c inhibits mTORC1 signalling	189
5.2.8	Depletion of PP6c specifically inhibits mTORC1 downstream	

5.3	of the TSC1/2 complex	193
5.3	Discussion	194
6	GENERAL DISCUSSION	202
6.1	Phosphatases and mTORC1 – selection of targets	202
6.2	Principal findings	203
6.2.1	A PP2A _{Tip41} complex regulates mTORC1 activity	203
6.2.2	Tip41 acts in parallel to PI3K/Akt mediated activation of mTORC1	204
6.2.3	Alpha4 may regulate activity of PP2A _{Tip41}	206
6.2.4	PP2A _{Tip41} -substrate binding may be regulated by phosphorylation of Tip41	207
6.2.5	PP2A _{Tip41} may oppose Rheb-mediated activation of mTORC1	208
6.2.6	A specific nuclear isoform of Tip41 may regulate activity of HIF1	208
6.2.7	PP2A _{Bα} attenuates mTORC1 signalling	210
6.2.8	PP2A _{Bα} acts downstream of the TSC1/2 complex	212
6.2.9	PP2A _{Bα} may be downregulated by an mTORC1 feedback mechanism involving the ubiquitin ligase CUL4-DDB1	213
6.2.10	PP6c enhances mTORC1 signalling	214
6.3	Significance in relation to human disease	214
6.3.1	Cancer	214
6.3.2	TS	216
6.3.3	Type II diabetes	217
6.3.4	AD	217
6.4	Future directions	218
6.5	Summary	220
	<i>References</i>	221

Summary

The mechanistic Target of Rapamycin Complex 1 (mTORC1) complex is central in the regulation of many crucial cellular processes including translation, transcription, proliferation and autophagy. Deregulation of the complex is evident in a number of diseases including Tuberous Sclerosis, Alzheimer's Disease and cancer. Whilst the signalling events leading to activation of mTORC1 are well understood, the inhibitory phosphatase activity that prevents aberrant signalling has received comparatively little attention.

In yeast, phosphatases are an integral part of TORC1 signalling. Poor nitrogen supply leads to activation of the phosphatases Pph21/22 and Sit4 and subsequent dephosphorylation of TORC1 substrates. Under these conditions, the phosphatase negative regulatory protein Tap42 is sequestered by Tip41. In good nitrogen supply, TORC1 phosphorylates Tip41 leading to release of Tap42 and subsequent inhibition of Pph21/22 and Sit4. This allows the accumulation of phosphorylated TORC1 substrates.

This thesis investigated the role of Tip41 in mTORC1 signalling. Purification of Tip41 identified direct interaction with PP2Ac (human Pph21/22). As overexpression of Tip41 resulted in inhibition of mTORC1 signalling, Tip41 is proposed as a *bona fide* positive regulatory subunit of PP2Ac. Further investigation indicated that hypophosphorylated PP2A_{Tip41} may directly oppose Rheb-mediated activation of mTORC1 thus promoting Raptor degradation. In addition, a specific nuclear isoform of Tip41 was identified, which may specifically regulate the transcription factor HIF1.

Studies using the adenoviral protein E4ORF4 also identified the PP2A_{Bα} complex in regulation of mTORC1 signalling. The data in this thesis show that PP2A_{Bα} acts downstream of the TSC1/2 complex to inhibit mTORC1. Results also indicate that PP2A_{Bα} may be negatively regulated by ubiquitin-mediated proteasomal degradation of Bα in an mTORC1-specific manner. Therefore PP2A_{Bα} may be subject to an mTORC1 feedback mechanism that is required for activation of downstream substrates. These data indicate that phosphatase activity is critical in regulation of mTORC1, reflecting the mechanism in yeast.

DECLARATION

This work has not previously been accepted in substance for any degree and is not concurrently submitted in candidature for any degree.

Signed L. Seymar (candidate) Date 21st July 2011

STATEMENT 1

This thesis is being submitted in partial fulfillment of the requirements for the degree of PhD

Signed L. Seymar (candidate) Date 21st July 2011

STATEMENT 2

This thesis is the result of my own independent work/investigation, except where otherwise stated.

Other sources are acknowledged by explicit references.

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STATEMENT 3

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Abbreviations

μM	Micromolar
26S	26 Svedberg
4EBPs	eIF4E-Binding Proteins
5' UTR	5' Untranslated Region
Aβ	Amyloid β
AD	Alzheimer's disease
AGC	cAMP-dependent, cGMP-dependent and Protein Kinase C
AML	Acute Myeloid Leukaemia
AMP	Adenosine Monophosphate
AMPK	AMP-activated Protein Kinase
Ankrd	Ankyrin repeat domain
APC	Anaphase promoting complex
Atg9	Autophagy related 9
AT-LD	AT-like disorder
ATM	Ataxia telangiectasia mutated-dependent kinase
ATP	Adenosine triphosphate
ATR	ATM and RAD3 related
ATRIP	ATR-interacting protein
AVO1	Adheres voraciously 1
BAD	Bcl2-associated agonist of cell death
Bnip3	Bcl2/adenovirus E1B 19kDa interacting protein 3
BSA	Bovine serum albumin
CaM	Calmodulin
CaMKII	Calcium/calmodulin-dependent protein kinase II
cAMP	cyclic AMP
Cdc	Cell division cycle
Cdh1	Cadherin 1
Cdk	Cyclin-dependent kinase
Chk1	Checkpoint homologue 1
CUL4	Cullin 4
Da	Dalton
DDB1	DNA damage binding protein 1
DEPTOR	DEP domain-containing mTOR-interacting protein
DMEM	Dulbecco's modified eagle medium
DMOG	Dimethyloxallyl glycine
DNA	Deoxyribonucleic acid
DNA-PK	DNA protein kinase
D-PBS	Dulbecco's phosphate-buffered saline
DSB	DNA double strand break
E4ORF1	Early Region 4 Open Reading Frame 1
ECL	Enhanced chemoluminescence

eEF	eukaryotic Elongation factor
eEF2K	eukaryotic Elongation factor 2 kinase
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
eIF	eukaryotic Initiation factor
EJC	Exon junction complex
ENPP1	Ectonucleotide pyrophosphatase phosphodiesterase 1
ERK	Extracellular signal regulated kinase
ESCRT	Endosomal sorting complexes required for transport
F actin	Filamentous actin
FAT	FRAP, ATM and TRRAP domain
FIP200	Focal adhesion kinase family interacting protein of 200kDa
FKBP	FK506-binding protein
FOXO4	Forkhead box protein O4
FRB	FKBP/Rapamycin binding
GAP	GTPase activating protein
GDP	Guanosine diphosphate
GEF	Guanosine exchange factor
GFP	Green fluorescent protein
GLI3	Glioma-associated oncogene family zinc finger 3
Gln3	Glutamine metabolism 3
GLUT	Glucose transporter
GSK3	Glycogen synthase kinase
GST	Glutathione S transferase
GTP	Guanosine triphosphate
GTPase	Guanine triphosphate hydrolase
H2AX	Histone 2A
HA	Haemagglutinin
HCC	Hepatic cell carcinoma
HD	Huntington Disease
HEAT	Huntingtin, elongation factor 3, A subunit of PP2A and TOR1
HEK293	Human embryonic kidney cell
HERC1	Homologous to E6-AP carboxy terminus and regulator of chromosome condensation 1
HER-II	Human tyrosine kinase-type cell surface receptor, type II
HIF	Hypoxia inducible factor
HRE	HIF response element
Hsp	Heat shock protein
Htc1	Hypertrichosis 1
hVps34	human vacuolar protein sorting 34
IEF	Isoelectric focussing
IFN- γ	Interferon- γ
IGF	Insulin-like growth factor
IKK	IκB kinase

IL-1	Interleukin-1
IL-R	Interleukin receptor
ILVs	Interluminal vesicles
IR	Insulin receptor
IRS-1	Insulin receptor substrate-1
IκB	Inhibitor of NF-κB
JNK	c-Jun N terminal kinase
KOG	Kontroller of growth
KSR	Kinase suppressor of Ras
LAM	Lymphangioleiomyomatosis
LC3	Microtubule associated protein 1 light chain 3
LCMT1	Leucine carboxymethyl transferase 1
LKB1	Liver kinase B1
LST8	Lethal with Sec Thirteen 8
MALDI-TOF	Matrix-assisted laser desorption/ionisation – time of flight
MAPK	Mitogen activated protein kinase
MEF	Mouse embryonic fibroblast
MEK	MAPK/ERK kinase
Met-tRNAi^{Met}	Initiator methionyl transfer RNA
miRNA	micro RNA
MNK	MAPK interacting kinase
MRE11	Meiotic recombination 11
MRN	MRE11-RAD50-NBS1
mRNA	messenger RNA
MSN	Multicopy suppressor of SNF1 mutation
MT	Microtubule
MVB	Multivesicular body
NCR	Nitrogen catabolite repression
NF	Neurofibromatosis
NF-1	Neurofibromin-1
NF-κB	Nuclear factor kappa light chain enhancer of activated B cells
NHEJ	Non-homologous end joining
nM	nanomolar
NRX	Nucleoredoxin
ODD	Oxygen-dependent degradation domain
OGT	O-β-N-acetylglucosaminyltransferase
OS	Opitz Syndrome
PA	Phosphatidic acid
PAS	PER-ARNT-SIM
PC-3	Prostate cancer 3 cell line
PCR	Polymerase chain reaction
PDGFR	Platelet derived growth factor receptor
PDK1	PIP₃-dependent protein kinase 1

PGC1α	P eroxisome proliferator activated receptor coactivator 1 α
PGE2	P rostaglandin E2
PH	P leckstrin homology
PI3K	P hosphatidylinositol-3-Kinase
PIAS1	P rotein inhibitor of activated STAT1
PIC	P reinitiation complex
PIKK	PI3K -related kinase
PIP₂	P hosphatidylinositol-4,5-bisphosphate
PIP₃	P hosphatidylinositol-3,4,5-triphosphate
PJS	P eutz-Jegher's S yndrome
PKC	P rotein kinase C
PLD1	P hospholipase D1
PMA	P horbol 12-myristate 13-acetate
PME1	P hosphatase methyltransferase 1
PP1/2/4/6	P rotein phosphatase 1/2/4/6
PP2A	P rotein phosphatase 2A
PP2A_{Alpha4}	PP2A dimeric complex of C and Alpha4
PP2A_{Ba}	PP2A trimeric complex of A, C and Bα subunits
PP2Ac	PP2A catalytic subunit
PP2A_D	PP2A dimeric complex of A and C subunits
PP2A_{Tip41}	PP2A dimeric complex of C and Tip41
PP6c	P rotein phosphatase 6 catalytic subunit
PP6R1	PP6 regulatory subunit 1
Pph21/22	P rotein phosphatase 21/22
PPlase	P eptidyl-prolyl isomerase
PPM	P rotein phosphatase magnesium/manganium dependent
PPM1	P rotein phosphatase methyltransferase 1
PPP	P rotein phosphatases
PRAS40	P roline enriched Akt substrate of 40kDa
Protor	P rotein observed with Rictor
PTC	P re-termination complex
PTEN	P hosphatase and t ensin homologue
Q-PCR	Q uantitative PCR
Rag	R as-related small GTP binding protein
Ral GDS	Ral guanine nucleotide dissociation stimulator
RalA	V-ral simian leukemia viral oncogene homologue A
Raptor	R egulatory associated protein of mTOR
Ras	R at sarcoma viral oncogene homologue
Rb	R etinoblastoma
REDD1	R egulated in development and DNA damage responses 1
Rheb	R as homologue enriched in brain
Rho	R as homologue
Rictor	R aptor independent companion of mTORC2

RNC	R aptor N terminal conserved domain
ROS	R eactive o xygen s pecies
RPA	R eplication p rotein A
RRM	R NA recognition m otif
RTK	R eceptor t yrosine k inase
S6K1	R ibosomal S 6 k inase 1
SAPs	S it4 associated p roteins
SCAP	S terol cleavage activated p rotein
SDS PAGE	S odium dodecyl sulphate p olyacrylamide g el electrophoresis
shRNA	s mall h airpin r ibonucleic a cid
Sit4	S uppressor of initiation of transcription 4
SKAR	S 6K1 A ly/ R EF-like substrate
SREBP	S terol regulatory e lement b inding p rotein 1
SREs	S terol regulatory e lements
ssDNA	s ingle s trand D N A
ST	S mall T
STAT	S ignal transducer and a ctivator of transcription
STRE	S tress response e lement
TAB1	T AK1 binding p rotein 1
TAD	T ranscriptional a ctivation d omain
TAK1	T ransforming growth factor β -activated k inase 1
Tap42	T wo A phosphatase associated p rotein of 42 kDa
TC	T ernary c omplex
TCF	T cell f actor
TCTP	T ranslationally controlled tumour p rotein
Tel2	T elomere maintenance 2
Tip41	T ap42 intreracting p rotein of 41 kDa
TNF	T umour n ecrosis f actor
TOPBP1	T opoiso m er a se b inding p rotein 1
TOR	T arget of rapamycin
TOS	T OR signalling
Tpd3	t RNA processing deficient 3
TRAF6	T NF receptor associated f actor 6
tRNA	t ransfer r ibonucleic a cid
TSC	T uberous S clerosis C omplex
Tti1	T el2 interacting 1
Ubc13	U biquitin conjugating 13
UIM	U biquitin interacting m otif
ULK1	U nc51-like k inase 1
Ure2	U reidosuccinate transport 2
VEGF	V ascular e ndothelial g rowth f actor
VHL	v on H ippel L indau
WT	W ild t ype
YY1	Y in y ang 1

List of figures

1.1	The PP2A holoenzyme	3
1.2	Control of phosphatase activity by TOR	11
1.3	Signalling inputs and outputs of mTORC1	22
1.4	Insulin signalling to mTORC1	23
1.5	Sequence alignment of mouse, human and yeast Tip41	57
3.1	Purification of endogenous Tip41	108
3.2	Tip41 interacts with PP2Ac, Alpha4 and Hsp70	110
3.3	Tip41 interacts directly with PP2Ac	112
3.4	PP2Ac interaction with S6K1 is increased by rapamycin	115
3.5	Optimisation of the mTORC1 kinase assay	117
3.6	Tip41 is not a direct substrate of mTORC1	119
3.7	Raptor-substrate interaction analysis	121
3.8	Multiple protein-protein interactions occur between Raptor and 4EBP1	123
3.9	Tip41 is not a substrate of mTORC2	126
3.10	Tip41 does not act upstream of mTORC2	128
3.11	Tip41 phosphorylation is sensitive to insulin stimulation	129
4.1	Tip41 overexpression can either enhance or inhibit phosphorylation of S6K1	140
4.2	Tip41 overexpression leads to both inhibition and activation of 4EBP1 phosphorylation	142
4.3	Tip41-mediated inhibition of mTORC1 induces a feedback loop leading to upregulation of Akt	145
4.4	Tip41 negatively regulates HIF1 activity	147
4.5	The Tip41 TOS mutant expressed at lower levels than HA-Tip41 wild-type	149
4.6	A Tip41 mutant deficient in PP2Ac binding is expressed at a lower level than wild-type counterparts	151
4.7	MG132 partially rescues levels of V5-Tip41(D71L) in comparison to wild-type levels	153
4.8	Optimisation of Tip41 knockdown using shRNA	155
4.9	Knockdown of Tip41 reduces S6K1 phosphorylation by induction of the negative feedback loop via IRS-1	157
4.10	Knockdown of Tip41 substantially increases activity of HIF1	159
4.11	Discovery of a distinct nuclear isoform of Tip41	160
4.12	Constitutive activation of Rheb confers resistance to the inhibitory action of Tip41	162
4.13	GST-TSC2 did not co-purify with Tip41	164
4.14	Tip41 does not alter activity of the ATM/ATR kinases or IKKβ	166
5.1	E4ORF4 activates mTORC1 by inhibition of PP2A$_{B\alpha}$	177
5.2	Bα subunit is ubiquitinated upon insulin stimulation, which is inhibited by rapamycin	180
5.3	Optimisation of Bα knockdown	183
5.4	Knockdown of Bα increases mTORC1 activity	184
5.5	PP2A$_{B\alpha}$ inhibits TSC2-induced activation of mTORC1	187
5.6	Optimisation of PP6c knockdown	191
5.7	Knockdown of PP6c reduces activity of mTORC1 substrates	193
5.8	Knockdown of PP6c does not alter growth factor signalling upstream of mTORC1	196

6.1	Tip41 and Bα are PP2Ac regulatory subunits involved in inhibition of mTORC1 signalling
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210

List of tables

1.1	Yeast proteins and human orthologues relating to TOR/mTOR signalling	14
1.2	mTORC1 effectors and substrates	21
2.1	Raptor mutant details	87
2.2	Sequences of shRNA clones	89
2.3	Preparation of DNA for CaCl₂ transfection	96

Amino acid abbreviations

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

Codon table

	U	C	A	G	
U	F	S	Y	C	U
	F	S	Y	C	C
	L	S	STOP	STOP	A
	L	S	STOP	W	G
C	L	P	H	R	U
	L	P	H	R	C
	L	P	Q	R	A
	L	P	Q	R	G
A	I	T	N	S	U
	I	T	N	S	C
	I	T	K	R	A
	M	T	K	R	G
G	V	A	D	G	U
	V	A	D	G	C
	V	A	E	G	A
	V	A	E	G	G

1.1 Phosphatases

1.1.1 General introduction

Phosphorylation events are central to the transduction of signals through the cell. Whilst kinases are responsible for phosphorylation of substrates, their action is opposed by a variety of phosphatase complexes. Phosphatases are divided into two families, based on their ability to remove phosphate groups from serine/threonine or tyrosine residues. Three families of serine/threonine phosphatases exist.

Phosphoprotein phosphatases (PPPs) are oligomeric enzymes including a catalytic subunit with one or more regulatory subunits (Cohen, 2002). The protein phosphatase magnesium/manganese dependent (PPM) family, consisting of PP2C, is monomeric with two structural domains (Lu & Wang, 2008). Thirdly, dephosphorylation of RNA polymerase II is controlled by the transcription factor II F interacting carboxyl terminal domain phosphatase family (Ghosh et al., 2008). The PPP family includes protein phosphatase 1 (PP1), PP2A, PP4, PP6, PP2B (calcineurin), PP5 and PP7. PP2A represents the major Ser/Thr phosphatase in mammalian cells. It is involved in almost every cellular process hence deregulation can result in diseases such as cancer, Opitz syndrome (OS) and Alzheimer's disease (AD). The enzyme evidently has a large number of substrates, and specificity is achieved by interaction with a number of regulatory and scaffold subunits.

1.1.2 The PP2A holoenzyme

1.1.2.1 The catalytic subunit

The core PP2A enzyme consists of the dimeric complex (PP2A_D) containing the 36kDa catalytic subunit (PP2A_C) and the 65kDa structural A subunit. PP2A_C is encoded by two genes, *PPP2CA* and *PPP2CB*, which encode the PP2A_C α - and β -isoforms, respectively. Both PP2A_C isoforms show functional redundancy as there is no difference in activity or substrate binding (Zhou et al., 2003).

1.1.2.2 The A subunit

The A subunit also has two isoforms (α and β), with 87% amino acid sequence identity (Hemmings et al., 1990). The α -isoform is more abundant than the β -isoform and has 6-fold increased affinity for PP2Ac (Ruediger et al., 2001(b)). The A subunit is C shaped with double layered α helices (Xu et al., 2006). It contains 15 tandem Huntingtin-Elongation-A subunit-TOR (HEAT) repeats consisting of 39 amino acids, which mediate interaction with PP2Ac. The A subunit binds PP2Ac via HEAT repeats 11 to 15 that induces structural rearrangement within the A subunit (Xing et al., 2006, Xu et al., 2006). Hydrogen bonds within HEAT repeat 11 lead to pivoting within the A subunit around HEAT repeat 13 resulting in conformational rearrangement. An adjacent proline residue possibly confers the ability to bend (Xu et al., 2006). Flexibility of the A subunit may allow binding to the many regulatory subunits of PP2Ac.

1.1.2.3 The B subunit

The PP2A holoenzyme is formed following interaction of PP2A_D with one of a number of B regulatory subunits (Figure 1.1). Four subfamilies of regulatory subunit exist: B (PR55), B' (B56 or PR61), B'' (PR72) and B''' (PR93/PR110). Although there is no homology between the subfamilies, domains within B' that bind the A subunit contain residues conserved within B and B'' (Li & Virshup, 2002). The B subunits may therefore share a common core structure and mode of interaction with the A subunit. The B (PR55) subfamily has four members, denoted α , β , γ and δ , each encoded by a distinct gene. These contain a high level of sequence similarity, with the B α and B β sharing 86% and the B α and B γ sharing 81% protein sequence homology (Mayer et al., 1991, Zolnierowicz et al., 1994). Although B α and B δ are ubiquitously expressed (Strack et al., 1999), both B β and B γ are found exclusively in the brain (Strack et al., 1998). Studies using subdissected brain tissue uncovered individual tissue and intracellular distribution, as well as differential expression dependent on developmental stage for each isoform (Strack et al., 1998). Whereas the levels of B β decrease sharply after birth, expression of B γ markedly increases. In addition, B β is predominantly cytosolic whereas B γ is associated with the cytoskeleton.

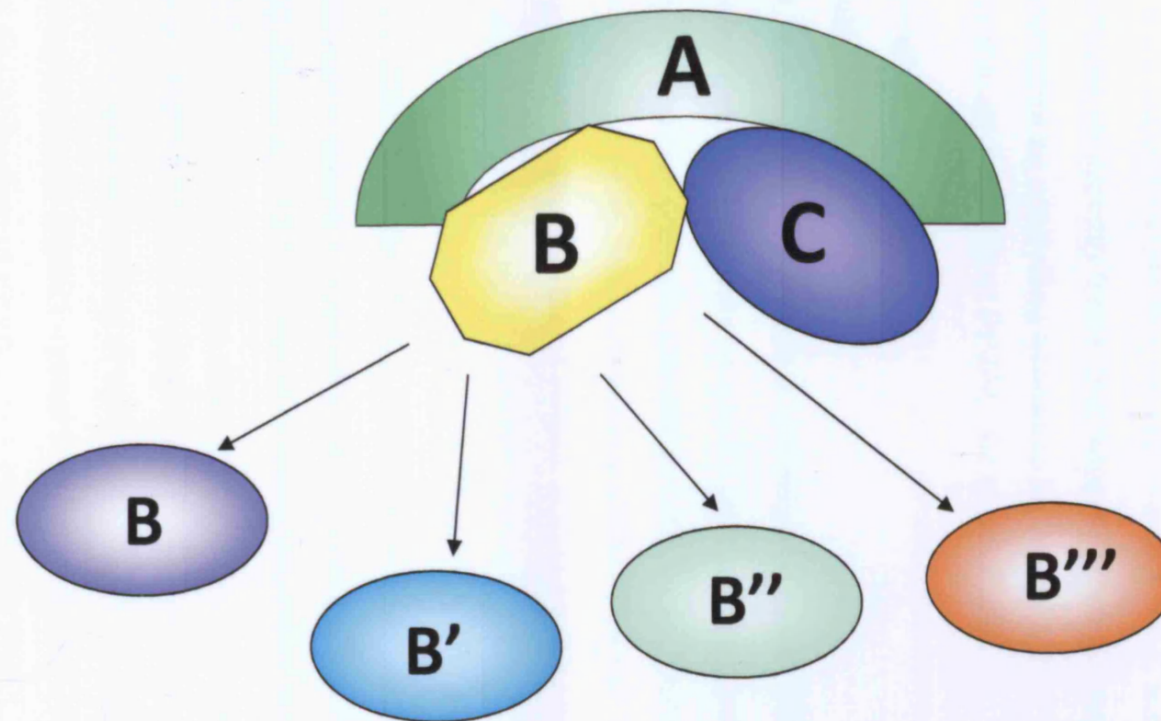


Figure 1.1: The PP2A holoenzyme. The classical PP2A trimeric enzyme consists of a catalytic 'C' subunit, regulatory 'B' subunit and scaffold 'A' subunit. The A subunit bridges interaction between the B and C subunits. Four families of B regulatory subunit exist, namely B, B', B'' and B''''. These are involved in substrate recognition.

Structurally, the B regulatory subunit contains a 7 bladed β propeller, each with 4 anti-parallel strands (Strack et al., 2002, Xu et al., 2008). Interaction with PP2A_D occurs via the A subunit. In addition, electrostatic bonds between residues within the B subunit and HEAT repeats 3 to 7 of the A subunit confer specificity of binding (Xu et al., 2008). Acidic substitution of B γ residues RR¹⁶⁵EE prevents interaction with PP2A_D, which is rescued by RR¹⁰⁰EE substitution within the A subunit (Strack et al., 2002). The B α subunit makes few interactions with the C subunit making the PP2A_{B α} complex structurally loose, and appears to confer substrate specificity to the PP2A holoenzyme by controlling access to the active site of PP2Ac via close proximity to the catalytic site of PP2Ac (Xu et al., 2008).

1.1.3 Regulation of PP2A

1.1.3.1 Methylation

The PP2A holoenzyme is regulated in a number of ways. Firstly, methylation of the catalytic subunit at L309 appears to specifically regulate binding of the B α subunit. Deletion of this residue prevents interaction with B α and demethylation of PP2Ac reduces the amount of co-immunoprecipitated B α (Yu et al., 2001, Wei et al., 2001, Longin et al., 2007). Methylation of PP2Ac is controlled by the activity of 2 enzymes in human cells: Leucine Carboxymethyltransferase 1 (LCMT1) and Protein Phosphatase Methylesterase 1 (PME1), which methylate and demethylate PP2Ac, respectively. Stability of the PP2A_{B α} holoenzyme relies on methylation of PP2Ac, and demethylation results in separation of the complex into PP2A_D and B α (Tolskyth et al., 2000, Wu et al., 2000). Loss of PP2Ac methylation results in destabilisation, where LCMT1 knockdown causes degradation of B α and eventually apoptosis (Longin et al., 2007). Studies in yeast have shown that loss of methyltransferase PPM1 results in reduced binding of cell division cycle 55 (cdc55 - yeast B α) to Protein phosphatase 21 (Pph21 - yeast PP2Ac) (Wei et al., 2001). Demethylation, and hence destabilisation of PP2A_{B α} , is controlled by PME1. Following demethylation, and displacement of B α , PME1 also inactivates PP2A_D by rearrangement of the catalytic site of PP2Ac (Xing et al., 2008). Within PME1, S156 is essential for methylesterase activity and mutation of this residue results in aberrant binding to methylated PP2A_D (Longin et al., 2008).

Cellular localisation of PP2A may also be controlled by PME1, which contains a nuclear localisation signal and therefore accumulates in the nucleus, along with demethylated inactive PP2A_D (Longin et al., 2008). Demethylated PP2A is also associated with the mitotic spindle during cytokinesis (Longin et al., 2008). PME1 may therefore be used to stabilise PP2A_D within the nucleus to ensure successful mitosis.

PME1 also sequesters inactive PP2A_D to prevent the accumulation of premature PP2A holoenzyme. Incubation with LCMT1 does not result in activation of the PP2A_D-PME1 complex (Longin et al., 2004). Instead, reactivation requires an additional protein, Phosphotyrosyl Phosphatase Activator of PP2A (PTPA). Studies in yeast have shown that PTPA binds unmethylated PP2A_D via the C terminus of PP2Ac, but that activation requires binding of PME1 (Hombauer et al., 2007). Thus PME1 may stabilise inactive PP2A_D in preparation for activation by PTPA. Binding of PTPA to PP2A_D-PME1 results in dissociation of PME1 from the complex (Jordens et al., 2006). PTPA then activates PP2A_D via its peptidyl-prolyl isomerase (PPIase) activity and by activation of PP2Ac catalytic activity. PTPA binding to PP2A_D occurs via a highly conserved hydrophobic groove and mutation of this groove yields inactive PTPA (Leulliot et al., 2006). Studies in yeast specifically identified Asp205 (Asp213 in mammalian PTPA) as essential for PP2Ac activation. This lies within the PP2A_D binding site and mutation reduces PPIase activity (Leulliot et al., 2006). Within PP2Ac, P190 is the target for PTPA PPIase activity as mutation of this Proline residue prevents interaction and activation by PTPA (Jordens et al., 2006). Studies in yeast identified the conserved W202 within PTPA as the residue that interacted with Pro190 within PP2Ac (Leulliot et al., 2006). In addition to PPIase activity, PTPA appears to stabilise metal ion binding within the PP2Ac catalytic site. Pph21 mutant H59S that is defective for metal ion binding show increased binding to Ypa1 (yeast PTPA) compared to wild type. In addition, metal ions were not tightly bound to the active site on deletion of Ypa1/Ypa2 in yeast (Fellner et al., 2003).

1.1.3.2 Phosphorylation

Phosphorylation of PP2Ac is also used as a method for regulation of PP2A activity. This occurs at T304 and Y307 and results in an 80% reduction in PP2A activity *in vitro* (Guo & Damuni, 1993). Phosphomimetic T304E prevents B α binding thereby

reducing activity via displacement of the regulatory subunit (Longin et al., 2007). Studies in yeast have shown that Pph21 phosphomimetic mutants reduce cdc55p binding with a subsequent phenotype indicative of reduced Pph21-cdc55 activity (Gentry et al., 2005). In human cells, phosphomimetic mutation Y307D results in reduced binding of the B α subunit (Nunbhakdi-Craig et al., 2007, Wei et al., 2001). This indicates that Y307 phosphorylation prevents B α binding (Chung et al., 1999). Interestingly, mutation of Y307 and L309 resulted in co-purification of Alpha4 with PP2Ac (Chung et al., 1999). This indicates that phosphorylation and methylation of PP2Ac prevents B α binding thus allowing interaction of PP2Ac with Alpha4.

Phosphorylation of PP2Ac at Y307 is implicated in breast cancer incidence. The Human Tyrosine Kinase-Type Cell Surface Receptor, Type II (HER-II) Receptor Tyrosine Kinase (RTK) is constitutively active in 20 to 30% of breast cancers and is the target for the drug Herceptin. HER-II results in activation of Phosphoinositide 3 Kinase (PI3K) and Mitogen-Activated Protein Kinase (MAPK) cascades and mutation is associated with poor prognosis and an aggressive phenotype. Activation of HER-II in breast cancer cell lines results in phosphorylation of Y307, with inhibition resulting in the opposing effect. In HER-II positive tumours Y307 phosphorylation was increased. Thus constitutive activation of HER-II in breast carcinoma results in increased phosphorylation of PP2Ac at Y307 and thus reduced phosphatase activity that may be associated with aggressive phenotype in some patients (Wong et al., 2009).

Phosphorylation of the B regulatory subunit is also a method of PP2A regulation. For example, Extracellular Signal-Regulated Kinase (ERK), a member of the MAPK family, phosphorylates B' following growth factor stimulation, resulting in dissociation of B' from PP2A and inactivation of the phosphatase complex (Letourneux et al., 2006). This prevents PP2A mediated dephosphorylation and inactivation of ERK. Therefore, phosphorylation of B' is critical in the ERK mediated growth factor response.

1.1.3.3 Degradation

Another mode of PP2A regulation may be through proteasomal degradation following ubiquitination of PP2Ac. OS is caused by mutation of *MID1*, which encodes the ubiquitin ligase Midline1. Yeast two hybrid analysis of Midline1

revealed *in vitro* interaction with Alpha4 (Trockenbacher et al., 2001). Further investigation revealed co-localisation of GFP-Alpha4 with Midline1 on microtubules (MTs). Whilst no ubiquitination of Alpha4 itself was detected, proteasome inhibition resulted in the accumulation of ubiquitinated PP2Ac. In addition, OS derived fibroblasts contain hypophosphorylated Microtubule Associated Proteins (MAPs). Therefore it is possible that in order to activate MAPs, Midline1 binds Alpha4 in order to ubiquitinate PP2Ac, resulting in PP2Ac degradation and hyperphosphorylation of MAPs. In the case of OS, this regulation is removed by dysfunctional Midline1 resulting in hypophosphorylation of MAPs which may be key in the pathogenic phenotype of the disease.

1.1.3.4 Interaction with regulatory subunits

Binding of the B regulatory subunit to PP2A_D can also present a method of PP2A regulation. MAPKs transmit mitogenic stimuli from G protein coupled receptors and RTKs in order to promote cell growth. Kinase Suppressor of Ras (KSR) facilitates signal transduction within the MAPK cascade, and is constitutively bound to PP2A_D. Following growth factor stimulation, B α binds PP2A_D thus activating phosphatase activity resulting in dephosphorylation of KSR and promoting MAPK activity (Ory et al., 2003).

1.1.3.5 Translation

PP2A activity may also be regulated via selective degradation of B subunit mRNA. This is associated with both hepatic cell and lung carcinoma. Microarray analysis of miRNA levels within murine lung cancer showed an increase in miRNA31 within these cells (Liu et al., 2010). Knockdown of miRNA31 resulted in reduced growth and tumorigenicity, which was concurrent with an increase in B α expression. The pattern was mirrored in human lung cancer tissue. Therefore, it appears that miRNA31 levels are reduced in lung cancer, resulting in degradation of B α mRNA and reduced B α protein, ultimately leading to hyperphosphorylation of PP2A_{B α} substrates.

Another miRNA involved in the control of B α mRNA levels is miRNA222. Increased levels of miRNA222 are associated with advanced hepatic cell carcinoma (HCC) and reduced patient survival (Wong et al., 2010). Knockdown of miRNA222 in hepatic cells leads to a reduction in Akt signalling and decreased cell motility. In HCC,

increased miRNA222 results in reduced levels of B α , therefore PP2A_{B α} , which may cause a more aggressive phenotype.

1.1.3.6 Oxidation status

The oxidation status of PP2A_D may also regulate activity of the complex (Foley & Kintner, 2005). Nucleoredoxin (NRX) is an oxidoreductase within the nucleus. Immunoprecipitation studies using PP2Ac as substrate showed binding between NRX and PP2A_D and incubation of the proteins doubled the activity of PP2A *in vitro* (Lechward et al., 2006, Foley et al., 2007). Two residues within PP2Ac, C269 and C272, may be targeted by NRX for disulphide bond formation although it is unclear how this may alter PP2A activity as the redox state of PP2Ac does not appear to affect regulatory subunit binding (Foley et al., 2011). However, these data suggest a role of PP2A in redox sensitivity within the cell.

1.2 Target of Rapamycin

A screen in yeast for gene mutations that cause resistance to the drug rapamycin revealed the existence of two proteins, Target of Rapamycin 1 (TOR1) and TOR2 (Heitman et al., 1991). The two proteins share 67% sequence identity and are redundant in response to rapamycin-induced signalling. Two TOR complexes exist in yeast: Target of Rapamycin Complex 1 (TORC1) and TORC2.

1.2.1 TORC1

The rapamycin sensitive TORC1 contains either TOR1 or TOR2 along with Kontroller of Growth 1 (KOG1) and Lethal with Sec Thirteen 8 (LST8) (Leowith et al., 2002, Wedaman et al., 2003). Interaction analysis within TORC1 revealed close proximity between the Raptor N Terminal Conserved (RNC) domain of KOG1 (which recognises TOR substrates) and the catalytic domain of TOR, placing substrates in the ideal location for phosphorylation (Adami et al., 2007). The same study placed the FK506 Binding Protein (FKBP)-Rapamycin binding site within this area of the TORC1 complex. Given that rapamycin does not disrupt interaction between TOR and KOG1, FKBP-Rapamycin likely inhibits TORC1 by either steric hindrance with substrate binding or by inhibiting phosphotransfer activity, or both.

1.2.2 TORC2

TORC2 is composed exclusively of TOR2 with Adheres Voraciously 1 (AVO1), AVO2, AVO 3 and LST8 (Leowith et al., 2002, Wedaman et al., 2003). Regulation of this complex is insensitive to rapamycin and plays an additional role in regulation of the actin cytoskeleton. AVO1 and AVO3 bind TOR2 at the N terminus. Both are phosphorylated by TOR2 and modulate integrity of the TORC2 complex. AVO2 binds TOR2 via AVO1 and AVO3. LST8 was shown to bind independently at the C terminus of TOR2. In addition to modulating integrity of the TORC2 complex, LST8 enhances TOR2 kinase activity (Wullschleger et al., 2005).

1.2.3 TORC1 and regulation of transcription

1.2.3.1 Nitrogen Catabolite Repression

Glutamine Metabolism 3 (Gln3) is a transcription factor that is activated on inhibition of TOR leading to the expression of nitrogen-regulated genes (Beck & Hall 1999, Cardenas et al 1999, Duvel et al 2003). In the presence of good nitrogen supply, such as glutamine, Gln3 is phosphorylated by TOR and sequestered in the cytoplasm by its repressor Ureidosuccinate Transport 2 (Ure2). Ure2 is also phosphorylated by TOR to mediate formation of the complex with Gln3 (Cardenas et al 1999, Hardwick et al., 1999, Bartram et al 2000). Rapamycin treatment or poor nitrogen supply, such as proline, decreases the amount of Gln3-Ure2 causing translocation of Gln3 to the nucleus thus leading to expression of Nitrogen Catabolite Repression (NCR) sensitive genes including permeases and enzymes required for the degradation of poor nitrogen sources (Cardenas et al 1999, Bartram et al 2000, Komeili et al., 2000, Crespo et al., 2002).

1.2.3.2 Stress Response Element

TOR also regulates other nutrient-responsive transcription factors. Multi-copy Suppressor of SNF1 Mutation 2 (MSN2) and MSN4 bind to the Stress Response Element (STRE) of responsive genes following inactivation of PKA (Protein Kinase A) (Görner et al., 1998). In yeast, signal transduction involving both PKA and TORC1 leads to the co-ordinated control of the nutrient stress response. TORC1 indirectly activates PKA by phosphorylating the Akt homologue SCH9 (Soulard et al., 2010). In response to stress including carbon or nitrogen limitation (Crespo et al., 2002), inhibition of PKA (via TOR) results in the nuclear localisation of MSN2 and MSN4.

Genes regulated by the STRE include *HSP12* which is involved in stabilisation of the plasma membrane during cellular stress (Welker et al., 2010).

1.2.3.3 Metabolic genes

The transcription factors Retrograde Regulation 1 (RTG1) and RTG3 are also regulated by TOR to control expression of genes involved in the TCA cycle and other metabolic pathways that promote glutamine synthesis (Liao & Butow, 1993, Hardwick et al., 1999, Liu & Butow, 1999). Again, poor nitrogen supply (Pro) or rapamycin treatment results in inhibition of TOR and the localisation of RTG1 and RTG3 to the nucleus (Hardwick et al., 1999, Komeili et al., 2000, Crespo et al., 2002). Thus in the absence of a rich nitrogen supply, glutamine synthesis is enhanced.

1.2.4 Phosphatases in TORC1 signalling

Phosphatases are an integral component of TOR signalling (Figure 1.2). The PP2A-like phosphatases in yeast include Pph21, Pph22 and Suppressor of Initiation of Transcription 4 (Sit4). Two A Phosphatase-Associated Protein of 42kDa (Tap42) regulates the PP2A catalytic subunits Pph21 and Pph22 and Sit4. Activation of TORC1 signalling leads to phosphorylation and activation of Tap42, which inhibits Pph21/22 and Sit4 (Jiang & Broach 1999) allowing the expression of STRE genes (Schmidt et al 1998, Duvel et al 2003). On inhibition of TOR, a feedback mechanism results whereby Tap42 is dephosphorylated by Pph21/22 (Jiang & Broach 1999). This leads to the dissociation of the inhibitory Tap42-phosphatase complex and in the case of Pph22/21, allows interaction with the regulatory subunits tRNA processing deficient 3 (Tpd3 – the yeast A subunit) and cdc55, leading to formation of an active phosphatase complex (Como & Arndt 1996, Yan et al 2006).

Sit4 activity on repression of TORC1 is regulated by Sit4 associating protein (SAPs) (Luke et al., 1996). Specifically, deletion of SAP190 results in rapamycin resistance, indicating that SAP190 regulates Sit4 activity downstream of TORC1 (Jablonowski et al., 2009). Hyperphosphorylation of TORC1 substrates is also observed in strains lacking SAP185, indicating that this regulatory subunit may also control Sit4 activity downstream of the kinase (Rohde et al., 2004), although this result was later disputed (Jablonowski et al., 2009).

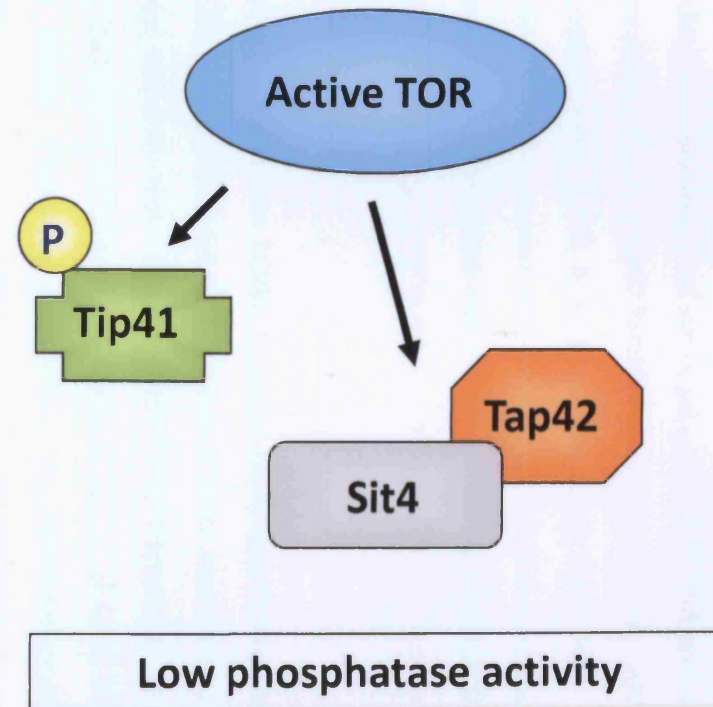
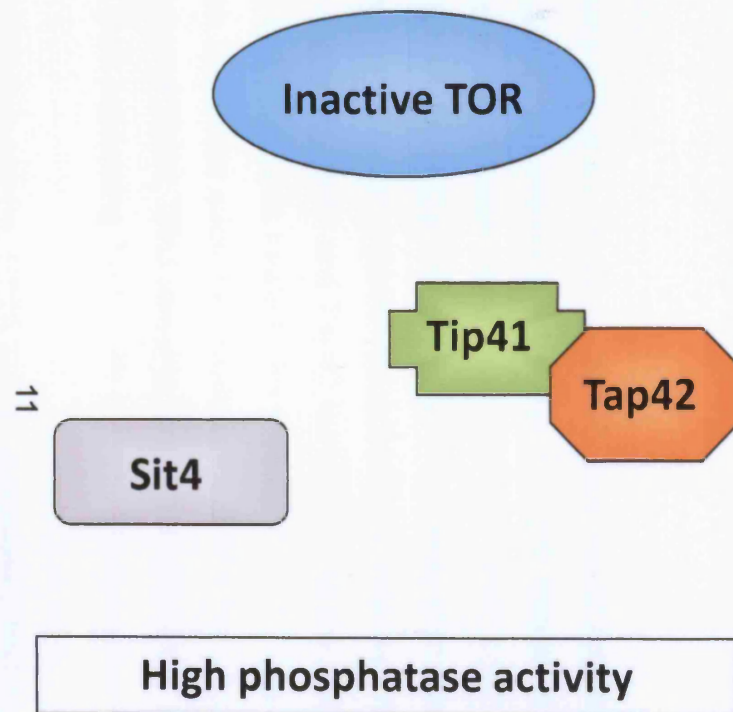


Figure 1.2: Control of phosphatase activity by TOR. When TOR is inactive, Tip41 sequesters the negative regulatory Sit4 subunit Tap42 resulting in high phosphatase activity and dephosphorylation of TOR substrates. Following activation, TOR phosphorylates Tip41 allowing Tap42 interaction with Sit4. This inhibits phosphatase activity and allows accumulation of hyperphosphorylated TOR substrates.

Studies with deletion mutants of Sit4 and rapamycin resistant mutants of Tap42 have implicated Sit4 and PP2A activity as necessary for the expression of Gln3 responsive genes. Wang et al (2003) used a series of Sit4 mutants defective in Tap42 binding and showed that whereas wild type Sit4 was capable of dephosphorylating Gln3, Tap42 binding mutants did not. Although this shows that Sit4 dephosphorylates Gln3, a discrepancy arose as to why Tap42 was necessary when the Sit4-Tap42 complex was previously shown to dissociate on activation of TOR. The authors postulated that Tap42 may therefore direct Sit4 towards substrates but disrupts phosphatase activity until dissociation following TOR activation.

Phosphatase regulation in TORC1 signalling is specific to the pathways inhibiting TORC1. Gln3 dephosphorylation in response to rapamycin, but not amino acid insufficiency, requires Sit4. Sit4 activity is not responsive to nitrogen supply and deletion of Sit4 did not prevent dephosphorylation of Gln3 in proline-grown cells (Tate et al., 2006). Therefore whilst Sit4 may dephosphorylate Gln3 in response to certain TORC1 inhibitory conditions, it is not the principal means of control in response to nitrogen supply. This indicates that distinct phosphatase complexes may act on TORC1 signalling in response to each stimulus.

1.2.4.1 Tip41

The inhibition of Sit4 by Tap42 is controlled by Tap42 interacting protein of 41kDa (Tip41) (Figure 1.2). Initially identified as a Tap42 interaction protein by yeast two hybrid analysis, Tip41 was found to antagonise Tap42 activity under TORC1 inactivating conditions (Jacinto et al., 2001). Rapamycin treatment resulted in decreased Tip41 phosphorylation via a feedback loop involving Sit4, and the interaction between Tip41 and Tap42 increased. As the dissociation of Sit4-Tap42 is prevented in $\Delta Tip41$ cells treated with rapamycin, it is thought that Tip41 removes Tap42 from Sit4 under such conditions. Active Sit4 then dephosphorylates Gln3. In cells depleted of Tip41, Gln3 remains within the cytoplasm, an indication of Sit4 inhibition, thus implicating Tip41 with a role in the activation of Sit4.

In *Saccharomyces pombe*, PP2A activity increased 7 fold in *tip41*⁺ cells (Fenyvuesvolgyi et al., 2005). In this study, induced expression of Tip41 slowed cell

cycle transition following nitrogen starvation, which was overcome by deletion of *PP2A*. Tip41 therefore appears to regulate phosphatase activity in fission yeast in a mechanism analogous to that in budding yeast.

1.3 The mTORC1 complex

The mTORC1 complex is composed of mTOR along with Regulatory Associated Protein of mTOR (Raptor) and mLST8. Additional subunits have recently been identified. Proline-Rich Akt Substrate of 40kDa (PRAS40) and DEPTOR are inhibitory subunits of mTORC1. In addition, the E3 ligase Cullin4-DNA Damage Binding Protein 1 (CUL4-DDB1) and scaffold proteins Telomere Maintenance 2 (Tel2) and Tel2 Interacting Protein 1 (Tti1) are required for mTORC1 activity.

1.3.1 mTOR

As a large 2549 amino acid protein, mTOR was the first mammalian member of the PI3K related kinase (PIKK) family to be cloned. PIKKs share a catalytic domain that contains an ATP binding site, catalytic loop and activation loop. The catalytic domain has two lobes, the N terminal lobe consists of a five-stranded β sheet flanked by three α -helices, along with a larger C terminal lobe. The ATP binding residues are found in the linker region between the two lobes (Walker et al., 2000). Within the C terminal lobe is the activation loop which confers substrate specificity of the kinase (Walker et al., 1999). The catalytic domain is surrounded by the FAT (named after FRAP, ATM and TRAP which all contain the domain) and FATC (an additional FAT domain at the extreme C terminus of the protein) domains. These domains may form a structural scaffold or be involved in protein-protein interactions (Bisotti & Isacchi, 2000). The large number of HEAT repeats at the N terminus of the protein are also involved in protein-protein interactions (Andrade & Bork, 1995, Keith & Schreiber, 1995). As in yeast, mTOR acts in two complexes, namely mTORC1 and mTORC2.

1.3.2 Raptor

Raptor, the mammalian homologue of yeast KOG1 (Table 1.1) (Leowith et al., 2002, Wedaman et al., 2003), interacts with mTOR to form a stoichiometric complex and is required for phosphorylation of mTORC1 substrates. The protein consists of a highly

Yeast	Human orthologue
Pph21/22	PP2Ac
Tpd3	PP2AA subunit
Cdc55	PP2A B subunit
Sit4	PP6c
Tap42	Alpha4
Tip41	Tip41
TOR	mTOR
KOG1	Raptor
LST8	mLST8
AVO1	Rictor

Table 1.1: Yeast proteins and human orthologues relating to TOR/mTOR signalling.

conserved RNC domain that contains three blocks with high sequence similarity within species that is predicted to have a high propensity to form α helices. Overexpression of Raptor S³⁹¹Q to PA mutant within the RNC results in dominant negative inhibition of mTORC1 activity and prevents phosphorylation of eukaryotic Initiation Factor 4E Binding Protein 1 (4EBP1) and ribosomal S6 Kinase 1 (S6K1) (Dunlop et al., 2009). Thus the RNC domain is critical in Raptor mediated mTORC1 substrate recognition. Control of interaction between mTOR and Raptor via the RNC domain is highly sensitive, as point mutations within the domain prevented interaction with mTOR and inhibited mTORC1 kinase activity (Kim et al., 2002).

Following the RNC, Raptor contains HEAT repeats followed by seven WD40 repeats at the C terminus (Kim et al., 2002). Concurrent with mutation of the RNC domain, point mutation within the WD40 domain also prevents interaction with mTOR (Kim et al., 2002). Whereas the HEAT repeats of mTOR are critical for the interaction with Raptor, multiple regions within Raptor are therefore required for interaction with mTOR.

Inhibition of mTORC1 signalling by removal of upstream stimulation inhibits association between mTOR and Raptor to prevent phosphorylation of downstream targets (Kim et al., 2002). Raptor is itself phosphorylated by mTOR at a number of rapamycin sensitive sites including S863. Mutation of S863 to alanine reduced mTORC1 activity towards 4EBP1 and S6K1 showing that Raptor phosphorylation is critical for mTORC1 signalling (Wang et al., 2009, Foster et al., 2010). In addition, mutation of the six identified activating phosphorylation sites inhibited mTORC1 activity *in vitro*, indicating that Raptor phosphorylation acts as a 'biochemical rheostat', regulating mTOR activity in response to upstream signalling (Foster et al., 2010).

1.3.3 mLST8

There is conflicting evidence regarding the requirement of mLST8 for activity of the mTORC1 complex. In one study, mLST8 was shown to bind the kinase domain of mTOR via its WD40 repeats resulting in increased activity of mTORC1. Nutrient sensing by mTORC1 was also shown to require mLST8, as without mLST8, low

amino acid conditions did not change the activity of mTOR-Raptor (Kim et al., 2003). More recently, a murine study concluded that *mLST8* was not essential for mTORC1 activity, as *mLST8*^{-/-} MEFs had no change in S6K1 or 4EBP1 phosphorylation. In addition, the mTOR-Raptor complex still immunoprecipitated from these cells, and phosphorylated S6K1 *in vivo* (Guertin et al., 2006).

1.3.4 DEPTOR

DEPTOR is a newly identified member of the mTORC1 complex. Through interaction with mTOR, DEPTOR inhibits phosphorylation of S6K1. Activity of DEPTOR is controlled by degradation which occurs under mTORC1 activating conditions. Serum stimulation of cells results in post-translational modification of DEPTOR, as shown by gel retardation of the protein, eventually resulting in loss of protein. Reduction of DEPTOR levels is controlled by mTORC1, as the high level of DEPTOR found in *TSC2*^{-/-} MEFs is reduced by inhibition of mTOR. Serum starvation conversely increases levels of the protein (Peterson et al., 2009).

1.3.5 Tel2 and Tti1

Recently stability of the mTORC1 complex has been shown to require Tel2 and Tti1. Tel2 contains HEAT repeats and preferentially binds new proteins (Takai et al., 2010). Tti1 constitutively binds mTOR, and knockdown results in reduced phosphorylation of mTORC1 substrates (Kaizuka et al., 2010). Both are required for the formation of stable mTORC1.

1.3.6 PRAS40

PRAS40 is an inhibitory subunit of mTORC1. Knockdown of PRAS40 increases 4EBP1 binding to mTORC1, implicating PRAS40 as a competitive mTOR inhibitor. In addition, PRAS40 inhibits mTOR autophosphorylation required for activation (Thedieck et al., 2007). Raptor binds PRAS40 via the TOR Signalling (TOS) motif, FVMDE, and interaction is inhibited by stimulation with insulin (Wang et al., 2007). Thus under mTORC1 inactivating conditions, PRAS40 interacts with Raptor to prevent substrate binding and inhibits phosphorylation of downstream mTOR substrates such as S6K1. Activation of mTORC1 by insulin stimulation results in phosphorylation of PRAS40 by mTOR at S183, S212 and S221, and allows activation of mTORC1 (Oshiro et al., 2007, Sancak et al., 2007, Wang et al., 2008).

Following mTORC1 phosphorylation, PRAS40 is released and sequestered by 14-3-3 protein (Fonseca et al., 2007, Wang et al., 2008). PRAS40 may also constitute a method of direct mTORC1 regulation by Akt, as Akt phosphorylates PRAS40 leading to dissociation from mTORC1 (Kovacina et al., 2003).

1.3.7 CUL4-DDB1

Ubiquitination has been shown as essential for mTORC1 signalling. DDB1 serves as an adaptor for the E3 ubiquitin ligase CUL4 (Angers et al., 2006). CUL4-DDB1 has been shown to interact with Raptor, and this interaction is required for phosphorylation of 4EBP1 and S6K1 by mTORC1. CUL4-DDB1 regulates polyubiquitination-mediated proteasomal degradation (Jiang et al., 2011) therefore degradation of an unknown CUL4-DDB1 substrate appears to be involved in mTORC1 signalling (Ghosh et al., 2008).

1.3.7.1 Ubiquitination

Ubiquitination is a post-translational modification resulting in the addition of a large 8kDa ubiquitin molecule to a lysine residue within the target protein. The process of ubiquitination classically involves 3 enzymes in a multi-step reaction. Firstly, a glycine residue within the free ubiquitin molecule is activated by an E1 ubiquitin-activating enzyme. Activated ubiquitin is then transferred to the E2 ubiquitin-conjugating enzyme. Finally, an E3 ubiquitin ligase transfers the ubiquitin molecule from E2 to the target protein (reviewed in Bhat & Greer, 2011).

1.3.7.1.1 Polyubiquitination

Tethering of ubiquitin via K48 within the ubiquitin molecule results in proteasomal degradation of the target protein and typically involves polyubiquitination. Degradation is carried out by the 26 Svedberg (26S) proteasome. This is a 2.3MDa complex consisting of a 20S core with a 19S regulatory particle at one or both ends. The 20S core is a hollow cylindrical structure and is responsible for proteolytic degradation. Once targeted to the proteasome, ubiquitin molecules are removed from the substrate by deubiquitinating enzymes (DUBs) to prevent clogging of the proteolytic chamber. Degradation results in the release of peptides of between 8-12 amino acids in length (reviewed in Bhat & Greer, 2011). Thus ubiquitination provides a means in which to remove proteins that are no longer required by the cell.

Ubiquitination via K63 has diverse consequences within the cell and can either take the form of mono- or polyubiquitination. Protein-protein interaction can be mediated by polyubiquitin which provides a binding platform for complexes thereby resulting in activation of signalling cascades. Interaction with binding proteins takes place via a hydrophobic patch near the C terminus whereas ubiquitin binding domains (UBDs) within signalling proteins mediate binding to ubiquitin (reviewed in Chen & Sun, 2009, Winget & Mayor, 2010).

Polyubiquitin provides a binding platform for proteins involved in signalling to Nuclear Factor Kappa-Light-Chain-Enhancer of Activated B Cells (NF- κ B). Upstream of NF- κ B, Interleukin-1 β (IL-1 β) stimulates activation of the E3 ubiquitin ligase, Tumour Necrosis Factor (TNF) Receptor-Associated Factor 6 (TRAF6), resulting in ubiquitination of Transforming Growth Factor β -Activated Kinase 1 (TAK1) via K63 within ubiquitin (Ninomiya-Tsuji et al., 1999, Deng et al., 2000, Sorrentino et al., 2008, Fan et al., 2010). This provides a binding platform for TAK1 Binding Protein 2 (TAB2) and TAB3 which bind polyubiquitinated proteins (Takesu et al., 2000, Kanayama et al., 2004, Kishida et al., 2005). TAB2 and TAB3 then mediate interaction of TAB1 which stimulates autophosphorylation of TAK1 required for activation (Shibuya et al., 1996, Sakurai et al., 2000).

Tip41 has recently been identified as a ubiquitin binding protein involved in TAK1 activation. In this capacity, Tip41 acts as a TAB and binds polyubiquitinated TAK1 to trigger autophosphorylation. A Phe²⁵⁴-Pro²⁵⁵ motif within Tip41 is essential for ubiquitin binding and is identical to the motif required by TAB2 and TAB3 for interaction with ubiquitinated TAK1. For this reason Tip41 was assigned a new name, TAB4 (Pirckett et al., 2008). Active TAK1 then phosphorylates Inhibitor of NF- κ B (I κ B) Kinase (IKK), leading to phosphorylation and subsequent ubiquitination-mediated proteasomal degradation of I κ B (Wieteck & O'Neill, 2007).

DNA repair also relies on polyubiquitin as a binding platform. K63-linked polyubiquitination by the E3 ubiquitin ligase Ubiquitin Conjugating 13 (Ubc13) is critical in providing a binding platform for proteins involved in double strand break repair following activation of ATM at the damaged site (reviewed in Chen & Sun, 2009).

Polyubiquitination via K63 is also required for Akt activation. Following growth factor stimulation, Akt is polyubiquitinated at K8 and K14 within the Pleckstrin Homology (PH) domain by TRAF6. A *TRAF6*^{-/-} mouse showed reduced Akt ubiquitination and phosphorylation and phosphorylation of Glycogen Synthase Kinase 3 β (GSK3 β). Ubiquitinated Akt may serve as a platform for adaptors of Akt resulting in membrane recruitment therefore allowing activation. The cancer-associated Akt mutant E17K within the PH showed increased ubiquitination and activation, thereby implicating ubiquitination in oncogenic Akt activation (Yang et al., 2009, Yang et al., 2010). Thus polyubiquitination via K63 within ubiquitin has alternative consequences to proteasomal degradation.

1.3.7.1.2 Monoubiquitination

Monoubiquitination has diverse functions including roles in protein trafficking, DNA damage repair and alteration of cellular localisation. The epidermal growth factor receptor (EGFR) is monoubiquitinated provoking internalisation on ligand binding leading to membrane recycling of the receptor rather than lysosomal degradation (reviewed in Acconcia et al., 2009). Ubiquitination also allows protein sorting in the early endosome. Monoubiquitinated proteins within early endosomes are sorted into intraluminal vesicles (ILVs) thereby generating multivesicular bodies (MVBs). Ubiquitinated proteins within the early endosomes are recognised by Endosomal Sorting Complexes Required for Transport (ESCRTs). These include ubiquitin binding proteins which separate ubiquitinated from non-ubiquitinated proteins within the early endosomes ending in scission and formation of an ILV (reviewed in Acconcia et al., 2009).

Within the Fanconi Anaemia pathway involved in DNA repair ubiquitination provides a binding platform recruiting proteins to the damaged site (reviewed in Chen & Sun, 2009).

Ubiquitination can also modulate cellular localisation. Mdm2 is an E3 ligase that regulates both the transcription factor Forkhead Box Protein O4 (FOXO4) and the tumour suppressor p53 (Kubbutat et al., 1997, Lai et al., 2001). Whereas multiple

mono-ubiquitination of p53 results in degradation, single mono-ubiquitination of FOXO4 leads to nuclear translocation. In response to oxidative stress, this results in activation of FOXO4 transcriptional activity (van der Horst et al., 2006, Brenkman et al., 2008).

1.4 Processes regulated by mTORC1

1.4.1 The TOS motif

The mTORC1 complex controls many essential processes in the cell by regulating a number of substrates and effectors (Table 1.2). Two of the best characterised mTORC1 substrates are the 4EBP1 and S6K1, which are involved in translation initiation (Figure 1.3 and 1.4). Both contain a five amino acid sequence known as the TOS motif that is essential for binding to Raptor and subsequent phosphorylation by mTOR following stimulation by upstream signals (Schalm et al., 2003, Wang et al., 2003). Within 4EBP1 this sequence is found at the C terminus encompassing residues F₁₁₄ EMDI. Overexpression of F114A mutant results in decreased cell size and complete abrogation of Raptor binding and mTOR phosphorylation, highlighting the importance of this residue within the motif (Nojima et al., 2003, Schalm et al., 2003, Lee et al., 2008,). Within S6K1, mutation of the N terminal TOS motif F₅DIDL to ADIDL prevented amino acid signalling downstream of mTORC1 (Schalm & Blenis, 2002). Both substrates are essential mediators of cap-dependent translation. Other proteins containing a TOS motif include PRAS40, which is phosphorylated by mTOR (Oshiro et al., 2007, Sancak et al., 2007, Wang et al., 2008), and the transcription factors Hypoxia Inducible Factor 1 (HIF1) and Signal Transducer and Activator of Transcription 3 (STAT3) which are also regulated by mTORC1 (Yokogami et al., 1999, Land & Tee, 2007).

1.4.2 Regulation of translation

1.4.2.1 Translation in eukaryotes

Translation in eukaryotes is a multi-factorial process that requires interaction of transcribed mRNA with ribosomes and a multitude of initiation factors. It occurs in three steps. Firstly, initiation entails the assembly of the ribosome with the initiator-methionyl-transfer-RNA (Met-tRNA^{Met}) in its peptidyl site at the start codon of the mRNA. Polypeptide synthesis follows and constitutes the elongation step. As the

Category	Substrate/ effector	Detail
Translation	4EBP1	Inhibition by mTOR allows eIF4E interaction with eIF4G
	S6K1	Phosphorylates rpS6, eIF4B, SKAR and eEF2K
Transcription	HIF1	Activates hypoxic response including glycolytic genes
	STAT1	mTORC1 inhibition activates STAT1 and IFN γ -responsive genes
	STAT3	Cell-type specific effects
	YY1	Activates mitochondrial genes
	SREBP	Promotes lipogenesis
	GLI3	Activates genes involved in cell cycle regulation
Proliferation	SGK1	Inhibits p27 to allow cell cycle progression
Autophagy	ULK1	Phosphorylated by mTORC1 to inhibit autophagy
	Atg9	Phosphorylated by mTORC1 to inhibit autophagy
	LC3	Phosphorylated by mTORC1 to inhibit autophagy

Table 1.2: mTORC1 effectors and substrates. 'Category' relates to the general cellular process in which the effector/substrate is involved.

Figure 1.3: Insulin signalling to mTORC1. Insulin binds its cognate receptor leading to phosphorylation of IRS-1 and subsequently PI3K. Active PI3K then converts PIP_2 to PIP_3 providing a binding platform for the PH domain-containing proteins PDK1 and Akt at the plasma membrane. PDK1 then activates Akt which phosphorylates TSC2 at a number of Serine residues. TSC2 phosphorylation leads to inactivation of the GAP domain, allowing accumulation of active GTP-Rheb. This allows activation of mTORC1 and phosphorylation downstream substrates including S6K1 and 4EBP1. HIF1 is also activated in the nucleus, as mTORC1 promotes the accumulation of HIF1 α .

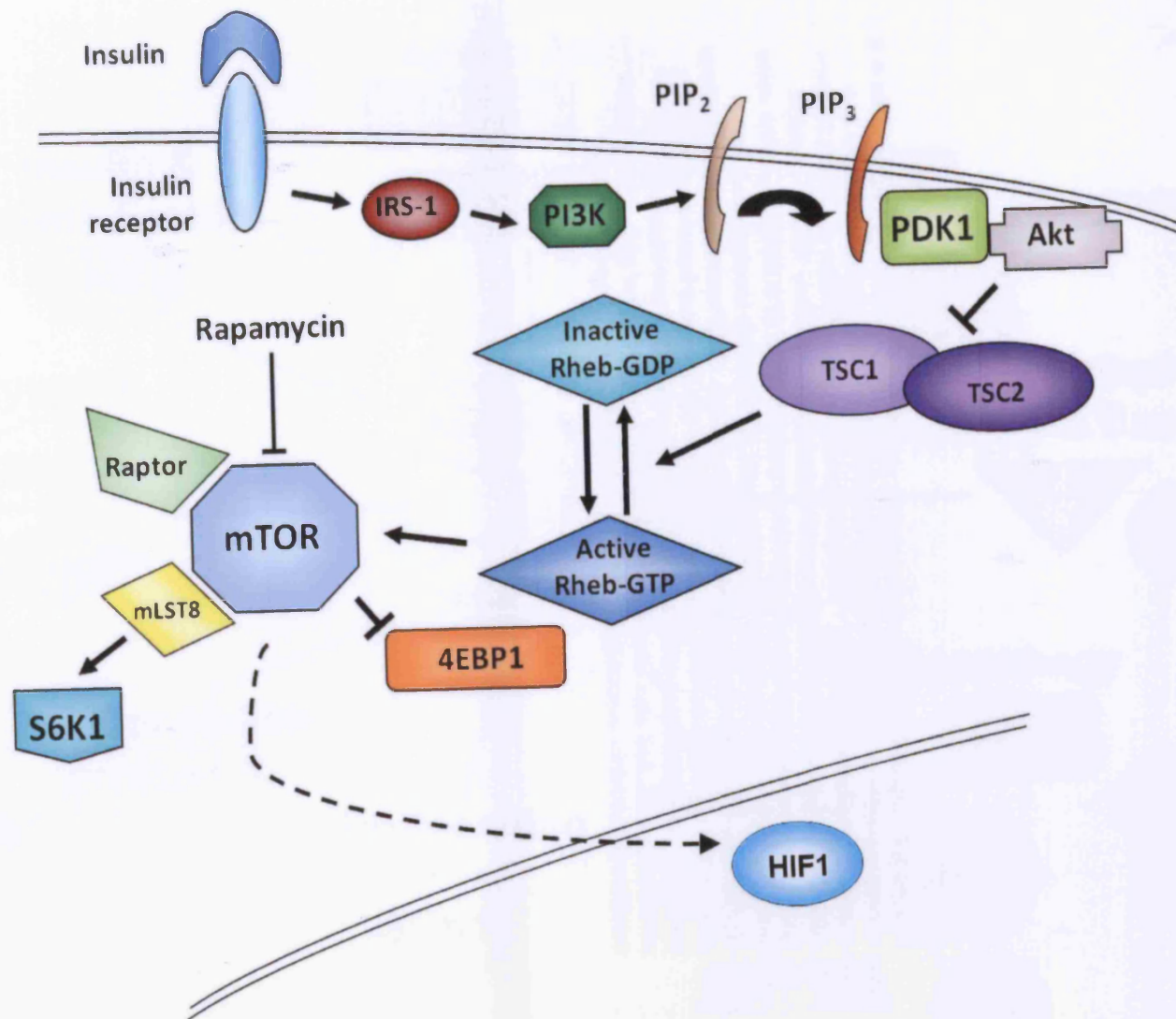
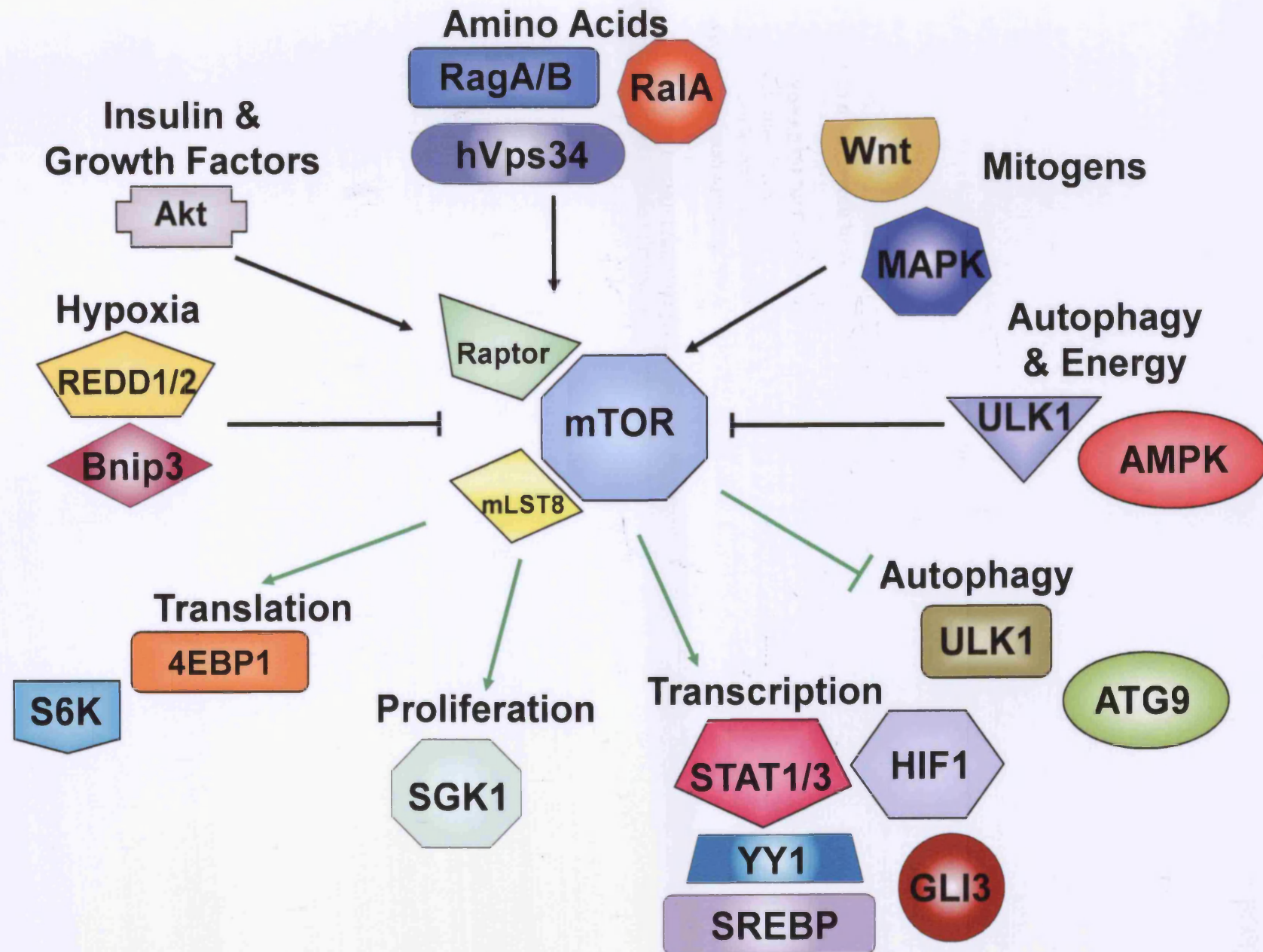


Figure 1.4: Signalling inputs and outputs of mTORC1. The mTORC1 complex is a central regulator of a number of processes and is itself regulated downstream of a number of signalling pathways. Insulin and growth factor activation of mTORC1 takes place via Akt which phosphorylates and inactivates the inhibitory TSC1/2 complex. This then allows accumulation of GTP-Rheb and activation of mTORC1. Amino acids activate mTORC1 via a number of mechanisms and result in localisation of the complex to membrane structures where Rheb resides. AMP accumulation, autophagy and hypoxia all inhibit mTORC1 in order to switch the growth-enhancing effect of the complex. When activated mTORC1 enhances transcription and translation to enhance growth, and also increases proliferation by regulation of p27. The mTORC1 complex also inhibits autophagy which is only required on energy deprivation.



ribosome reaches the stop codon, the completed polypeptide is released leading to termination of translation.

Initiation is itself achieved in four subsequent steps (reviewed in Preiss & Hentze, 2003). The first results in formation of the 43S Pre-Initiation Complex (PIC) consisting of the 40S ribosomal subunit, initiation factors and Met-tRNAi^{Met}. Physiological conditions favour formation of the 80S ribosome, hence the first step in initiation is dissociation into the 40S and 60S subunits by eukaryotic Initiation Factor 3 (eIF3) and eIF1A (reviewed in Dong & Zhang, 2006). Dissociated 40S binds Met-tRNAi^{Met} which is delivered to the subunit by eIF2. As eIF2 is a G protein, activation requires GTP binding mediated by the GEF eIF2B. Activation of eIF2B is regulated by Akt via GSK3. In nutrient deprived cells, GSK3 is active and phosphorylates eIF2B at S535 thereby inhibiting translation (Wang et al., 2001, Wang et al., 2002). Insulin stimulation promotes Akt phosphorylation of GSK3 leading to inactivation of the kinase (Mora et al., 2002, Mora et al., 2005, Mariappan et al., 2008). This leads to the accumulation of hypophosphorylated, and active, eIF2B. The ternary complex (TC) consisting of eIF2, eIF2B and Met-tRNAi^{Met} is then delivered to the 40S subunit aided by eIF1, eIF1A and eIF3. This constitutes the 43S PIC.

The second step in initiation involves the recruitment of 43S to the capped 5' end of the mRNA. This is again mediated by eIF3 which stimulates binding of 43S to mRNA (reviewed in Hinnebusch, 2006). Residing at the 5' end of the mRNA, eIF4E resembles a cupped hand and its concave surface contains a hydrophobic groove for insertion of the mRNA (reviewed in Merrick, 2010). The convex surface binds eIF4G, a subunit of eIF4F, and serves to enhance binding of eIF4F to the 5' end of the mRNA. Also at the 5' end is the RNA helicase, eIF4A unwinds the secondary structure, a process made more efficient by the presence of eIF4F and eIF4B. The 4EBPs act as competitive inhibitors of eIF4G as they block the eIF4G binding region of eIF4E. Phosphorylation by kinases such as mTOR removes them from eIF4E thus allowing binding of the eIF4F complex at the 5' cap of the mRNA.

Scanning of the untranslated region for the AUG start codon constitutes the third phase of initiation, followed by recruitment of the 60S ribosomal subunit to form the complete 80S ribosome. This results in release of the initiation factors and requires

GTP hydrolysis of eIF2. This marks completion of the initiation phase, allowing elongation and finally termination to take place.

1.4.2.2 4EBP1

Phosphorylation of 4EBP1 is sensitive to mTORC1 activation and inhibition (Brunn et al., 1997, Hara et al., 1997). When hypophosphorylated, 4EBP1 binds eIF4E thus inhibiting cap-dependent translation. There are a number of mTORC1 sensitive phosphorylation sites within the protein, which are phosphorylated in sequence. Firstly, T37/46 is phosphorylated, followed by T70 then S65 (Gingras et al., 2001). This results in removal of 4EBP1 from eIF4E thus allowing translation (Brunn et al., 1997, Schalm et al., 2003, Eguchi et al., 2006). A dominant negative 4EBP1 mutant that constitutively binds eIF4E results in decreased cell size, concurrent with inhibition of mTORC1 signalling (Fingar et al., 2002). Additional 4EBP1 phosphorylation sites include S101 and S112. These are constitutively phosphorylated and are required for phosphorylation at S65 and release from eIF4E (Wang et al., 2003).

In relation to mTORC1 signalling, 4EBP1 contains an additional motif at the N terminus termed the RAIP motif after the four residues it encompasses. Mutation I15A within the RAIP motif was shown to reduce Raptor binding and phosphorylation by mTORC1 (Lee et al., 2008). However, alternative studies have failed to show any effect on phosphorylation at T37/46 or T70 following mutation of the same residue (Eguchi et al., 2006). The T37/46 phosphorylation sites of 4EBP1 are insensitive to rapamycin, in contrast to phosphorylation of S65 and T70 which are potently inhibited by the drug (Gingras et al., 2001, Wang et al., 2005).

1.4.2.3 S6K1

S6K1 is activated by mTORC1 following direct phosphorylation at T389. Kinase dead mutants of mTOR result in reduced phosphorylation at this site, whereas rapamycin resistant mTOR mutants results in rapamycin resistance of S6K1 (Brown et al., 1995). An additional R⁴⁰⁹SPRR motif within S6K1 at the C terminus appears to regulate activity of the kinase. Phosphomimetic T389E mutation of the S6K1 TOS mutant F5A only partially rescues catalytic activity of the kinase, whereas complete rescue is achieved by additional mutation of an RSPRR motif (Schalm et al., 2005).

The authors postulated that the RSPRR motif may provide a binding site for an unknown inhibitor of S6K1 activity, PP2A being the prime suspect due to an already identified interaction with the kinase (Westphal et al., 1999). Thus mutation of the RSPRR motif prevents binding of the inhibitor allowing full rapamycin resistant catalytic activity on combination of mutated F5A and phosphomimetic T389E. Following mTORC1 activation, S6K1 enhances translation through a number of mechanisms.

1.4.2.3.1 Ribosomal protein S6

The first identified substrate of S6K1 was the ribosomal protein S6, after which it was named (reviewed in Fenton & Gout, 2011). Activation of S6 requires phosphorylation of several residues at the C terminus including S235, S236, S240, S244 and S247 (Kreig et al., 1988, Ferrari et al., 1991, Bandi et al., 1993). S6K1 can phosphorylate all of these sites leading to full activation of S6. Numerous mouse studies have concluded that the primary role of S6 lies in control of cell growth. Mutation of S6 phosphorylation sites to Alanine within a mouse model results in smaller rodents due to stunted growth (Ruvinsky et al., 2009). Cultured MEFs from this model are also smaller and the size is not decreased further by treatment with rapamycin, indicating that S6 is the principle method of control of cell growth by mTOR (Ruvinsky et al., 2005). S6K1 deficient mice are also smaller and have reduced levels of phosphorylated S6 (Pende et al., 2000, Pende et al., 2004).

1.4.2.3.2 eIF3-PIC

S6K1 activates translation through interaction with the eIF3-PIC. eIF3 is composed of 13 subunits and tethers the PIC (including the 40S ribosomal subunit) to the 5' cap of mRNA via interaction with eIF4F. The complex blocks premature binding of the 60S ribosomal subunit and enhances binding of the ternary complex GTP-eIF2-Met-tRNA (reviewed in Peterson & Sabatini, 2005). Binding of eIF3-PIC with S6K1 is regulated by amino acids and rapamycin. S6K1 mutants F5A and T389A are both constitutively bound to eIF3-PIC, indicating that following phosphorylation by mTORC1, S6K1 dissociates from eIF3-PIC. Following dissociation, S6K1 phosphorylates S6 and eIF4B (Shahbazian et al., 2006). Phosphorylated eIF4B can then bind the eIF3-PIC. Phosphorylation of eIF3 is essential in regulating association with the eIF3-PIC as phosphomimetic S422D (the eIF4B phosphorylation

site) is constitutively bound to eIF3-PIC whereas S422A is unable to associate (Holz et al., 2005). Activation of mTORC1 also results in increased association of eIF4G with eIF3 (Harris et al., 2006).

1.4.2.3.3 SKAR

Another mechanism by which S6K1 regulates translation is through the novel S6K1 interacting protein S6K1 Aly/REF-like target (SKAR). This was discovered following a two hybrid screen using S6K1 as bait, and was subsequently found to be an S6K1 substrate. The exon-junction complex (EJC) is a multi-subunit protein recruited to mRNA following intron splicing, which functions to eliminate mRNAs with premature termination codons (PTCs). Following the first round of translation, the EJC is removed from the mRNA (Ishigaki et al., 2001). On discovery of a PTC however, the EJC is not removed triggering nonsense mediated decay of the mRNA (reviewed in Lejeune & Maquat, 2005). SKAR binds to mRNA via the EJC. Following interaction with the RNA recognition motif (RRM), S6K1 phosphorylates SKAR at S383 and S385 at the C terminus of the protein. Phosphorylation of these sites is sensitive to insulin and rapamycin and to knockdown of S6K1, implicating SKAR as a target of S6K1 downstream of mTORC1 (Richardson et al., 2004). Following phosphorylation, S6K1 remains bound to SKAR and increases the translation efficiency of spliced mRNAs (Ma et al., 2008). Knockdown of SKAR reduced the amount of S6K1 bound to mRNA and reduced phosphorylation of mRNA binding proteins (Ma et al., 2008). Therefore S6K1 binds SKAR in order to localise phosphorylation of mRNA associated proteins.

1.4.2.3.4 eEF2K

S6K1 also regulates the elongation phase of translation by phosphorylating eukaryotic Elongation Factor 2 Kinase (eEF2K). Elongation constitutes addition of amino acids (in the form of aminoacyl-tRNA) to the nascent polypeptide, mediated by the ribosome. Correct codon-anticodon interactions between mRNA and tRNA respectively result in conformational changes within the ribosome leading to peptide bond formation and extension of the peptide (Doudna & Rath, 2002, Ramakrishnan 2002). Elongation factors mediate this stage of translation. Whereas eEF1 delivers aminoacyl-tRNA to the ribosome, eEF2 aids translocation of the nascent polypeptide within the ribosome to allow accommodation of subsequent aminoacyl-tRNAs (Li et

al., 2005, Taylor et al., 2006). Phosphorylation of eEF2 by eEF2K prevents binding to the ribosome and inhibits elongation (Lie et al., 2005). Activated S6K1 phosphorylates and inhibits eEF2K at S366 allowing the accumulation of dephosphorylated eEF2 and activation of elongation (Wang et al., 2001). Thus mTORC1 is a powerful regulator of eukaryotic translation at both the initiation and elongation phases.

1.4.3 Regulation of transcription

1.4.3.1 HIF1

Transcription factor activation also comprises the mTORC1 mediated response to hypoxia, which involves the transcription factor HIF1. HIF1 activates the expression of Vascular Endothelial Growth Factor (VEGF) in order to promote angiogenesis (Kim et al., 2001). In addition, HIF1 activates genes to promote glucose uptake and glycolysis (Semenza et al., 1994, Chen et al., 2001). This allows the hypoxic cell to upregulate anaerobic metabolism in the absence of a plentiful supply of oxygen.

HIF1 is a heterodimeric complex consisting of HIF1 α and HIF1 β , which are encoded by separate genes (Iyer et al., 1998). HIF1 α contains a number of domains that regulate the protein. The PER-ARNT-SIM (PAS) motif constitutes the HIF1 β binding site at the N terminus. The two transactivation domains (TADs) bind cofactors and increase transcriptional activity. Finally, the oxygen dependent degradation (ODD) domain mediates inhibition under normoxic conditions (reviewed in Semenza, 2001). HIF1 α also contains a TOS motif at the N terminus, implicating the protein as a bona fide substrate of mTORC1 (Land & Tee, 2007).

Whereas HIF1 β is constitutively expressed, HIF1 α expression is induced in hypoxic conditions. Under normoxic conditions, HIF1 α is hydroxylated at a number of proline residues within the ODD domain and TAD that are recognised by von-Hippel Lindau protein (VHL) (Bruick & McKnight, 2001, Epstein et al., 2001, Lando et al., 2002). Binding of VHL mediates interaction of HIF1 α with an E3 ligase, resulting in ubiquitination within the ODD domain and TAD and proteolytic degradation (Mason et al., 2001). Under hypoxic conditions, hydroxylation is inhibited leading to accumulation of HIF1 α and subsequently HIF1 complex.

HIF1 α expression is activated by mTORC1 (Laughner et al., 2001). Under hypoxic conditions, rapamycin decreased HIF1 α expression in PC3 cells, which was reversed on expression of a rapamycin resistant mutant mTOR (Hudson et al., 2002). TSC2^{-/-} MEFs have increased HIF1 activity, which is reduced by rapamycin (Burgarolas et al., 2003, Land & Tee, 2007). In addition, Raptor interacts with HIF1 α WT but not a TOS mutant form of the protein (Land & Tee, 2007). Recently, HIF1 α activation was shown to require S6K1 downstream of mTORC1. Knockdown of S6K1 in Phosphatase and Tensin Homologue (*PTEN*) null cells reduced the high levels of HIF1 α expression seen in these cells, as a result of decreased accumulation of the protein (Tandon et al., 2011). Previous studies have shown mTORC1 mediates the effect on HIF1 α via the ODD domain (Hudson et al., 2002). Still, the exact mechanism of mTORC1 mediated activation of HIF1 remains unclear.

1.4.3.2 STAT1

Interferon- γ (IFN- γ) stimulates STAT1 dimerisation, nuclear translocation and activation of target genes. Recently, this was found to be mediated by mTORC1. Inactivation of mTORC1 following treatment with rapamycin increased mTORC1-STAT1 interaction and nuclear translocation. Therefore inactivation of mTORC1 mediates STAT1 activation and expression of IFN- γ responsive genes (Fielhaber et al., 2009).

1.4.3.3 STAT3

STAT3 is activated in response to cytokines and growth factors and promotes cell-type specific activation of transcription. Both serine and tyrosine phosphorylation are required for full activation of transcription factor function (Wen et al., 1995). Phosphorylation of Y705 by the MAPK c-Jun N terminal Kinase (JNK) is required for nuclear translocation and DNA binding, whereas S727 phosphorylation increases transcriptional activation by possibly increasing interaction with cofactors. Activation of STAT3 has diverse consequences, including proliferation of B lymphocytes and growth arrest in monocytes. Transformation resulting from expression of constitutively active STAT3 has lead to identification of the transcription factor as an oncogene (reviewed in Levy & Lee, 2002).

Phosphorylation of S727 was found to be mediated by mTORC1, as a peptide containing the S727 site was phosphorylated by mTORC1 (Yokogami et al., 1999). Interestingly, mTORC1 was also found to promote Y705 phosphorylation of STAT3 in neuronal progenitor cells as phosphorylation of both S727 and Y705 were sensitive to rapamycin. Differentiation of the progenitor cells was reversed by rapamycin, implicating mTORC1 in neuronal differentiation. A direct interaction between mTORC1 and STAT3 was also discovered, indicating STAT3 may be a substrate for mTORC1 (Wang et al., 2008). STAT3 activation mediated by mTORC1 also increased tumorigenicity of breast cancer cells, providing further evidence for the role of STAT3 as an oncogene (Zhou et al., 2007).

1.4.3.4 YY1

The transcription factor Yin Yang 1 (YY1) is so called due to its dual action as a transcriptional activator and repressor. For example, YY1 may activate or repress the adenoviral-associated virus P5 promoter depending on the presence of adenoviral E1A protein (Shi et al., 1991). YY1 may also repress or activate IFN β as dictated by cofactor binding. Repression of transcription can occur by YY1 competing with activating cofactors or by interaction with repressors. On the other hand YY1 can directly activate transcription, or indirectly activate by removal of inhibitory function via cofactor recruitment thus unmasking the activating N terminus of the protein.

Activation of transcription by YY1 can also occur by recruitment of co-activators, such as peroxisome-proliferator-activated receptor co-activator 1 α (PGC1 α). PGC1 α controls mitochondrial function through interaction with transcription factors. The potential role of mTORC1 in mitochondrial function was investigated following data showing rapamycin reduces mitochondrial membrane potential, oxygen consumption and ATP generation. In addition, mTORC1 was found to localise to mitochondria (Schieke et al., 2006). Thus it appears that mTORC1 may have a positive regulatory role within mitochondrial function. Later studies found that rapamycin reduces expression of mitochondrial genes encoded by PGC1 α . YY1 binding motifs were enriched in mitochondrial genes regulated by PGC1 α , and rather than modulating PGC1 α directly, mTORC1 was found to modulate activity of YY1.

PGC1 α was therefore identified as a co-activator of YY1 to activate transcription of mitochondrial genes. YY1 binds both Raptor and PGC1 α , and whilst mTORC1-YY1 interaction remains unchanged by rapamycin treatment, PGC1 α binding is prevented. Thus mTORC1 controls YY1 activation of mitochondrial genes by mediating binding with the co-activator PGC1 α .(Cunningham et al., 2007). This may allow the cell to activate mitochondrial function to provide energy following a plentiful supply of nutrients.

1.4.3.5 SREBP

Although mTORC1 promotes cell growth through activation of translation resulting in protein biosynthesis, this does not complete the story. Recently, mTORC1 has been implicated in lipid biosynthesis, thus providing a second point of cell growth augmentation. The transcription factor sterol regulatory element binding protein (SREBP) controls fatty acid and sterol synthesis, which are the major lipids found in eukaryotic membranes. SREBP is synthesised as an inactive precursor and is anchored in this state at the endoplasmic reticulum bound to sterol cleavage activating protein (SCAP). Low cholesterol levels result in translocation of SREBP/SCAP to the golgi resulting in proteolytic cleavage and release of the DNA binding domain. This then shuttles into the nucleus to activate genes with sterol regulatory elements (SREs) in the promoter region, which are involved in cholesterol and fatty acid synthesis (reviewed in Powers, 2008). SREBP has been identified as a target of mTORC1 downstream of Akt. Activation of Akt results in increased cell size, which is prevented by knockdown of SREBP. The downstream effector of SREBP was identified as mTORC1 as rapamycin inhibits the Akt induced nuclear accumulation of SREBP (Porstmann et al., 2008). Thus Akt mediated activation of mTORC1 can promote nuclear accumulation of active SREBP, providing a link between mTORC1 and lipogenesis.

1.4.3.6 GLI3

In the absence of activating signals, the transcription factor Glioma-Associated Oncogene Family Zinc Finger 3 (GLI3) is subject to proteolytic cleavage, followed by nuclear translocation of the N terminal peptide resulting in transcriptional repression. Activation of GLI3 genes follows activation of upstream signalling pathways, leading

to the nuclear translocation of the full length protein and activation of cyclin D1 and other responsive genes (Krauß et al., 2008). Inhibition of mTORC1 with rapamycin was found to promote the nuclear translocation of active GLI3 and transcription of the cyclin D1 gene (Krauß et al., 2008). This implies that mTORC1 negatively regulates GLI3 and therefore the transcription of *cyclin D1*.

1.4.4 Regulation of proliferation

A link between mTORC1 signalling and proliferation has long been recognised since the discovery that rapamycin inhibits cell cycle progression by binding mTOR kinase (Brown et al., 1994, Sabatini et al., 1994). Cell cycle progression is controlled by Cyclin-Dependent Kinases (Cdks) that are activated by cyclin binding and inhibited by Cdk inhibitors, including p27 (reviewed in Sherr & Roberts, 1999). By regulating G₀ to S phase transitions, p27 binds Cdks and acts as a tumour suppressor. Following contact inhibition, loss of focal adhesion, activation by the growth inhibitory signal TGFβ or UV radiation, p27 inhibits cyclin D-Cdk complexes that mediate progression from G₀ to G₁. During G₁, p27 can inhibit Cdk2 complexes that control the expression of proteins required for G₁ to S transition (reviewed in Chu et al., 2008).

Control of p27 is achieved by cellular localisation and accumulation. Phosphorylation of p27 at T187 results in ubiquitination and proteolytic degradation of the protein (reviewed in Woods, 2010). In order to inhibit Cdks, p27 must first localise to the nucleus. Phosphorylation at T157 occurs downstream of Akt and results in cytosolic shuttling of p27. Overexpression of a constitutively active Akt mutant resulted in complete cytosolic translation of p27, whereas a T157A mutant of p27 was constitutively nuclear and caused a reduction in cell proliferation (Liang et al., 2002, Shin et al., 2002, Viglietto et al., 2002).

Whilst rapamycin inhibits p27 accumulation and TSC2^{-/-} MEFs contain exclusively cytoplasmic p27, a link between mTORC1 and p27 in order to execute cell cycle progression was investigated (Nourse et al., 1994, Soucek et al., 1998). Recently the link between mTORC1 and p27 has been identified as serum- and glucocorticoid-inducible kinase 1 (SGK1), a member of the AGC kinase family named after three members therein; protein kinase A, G and C (reviewed in

Peterson & Schreiber, 1999). SGK1 was found to be an inhibitor of p27, phosphorylating the protein at T157 resulting in cytosolic accumulation following activation by mTORC1. Overexpression of mTOR resulted in phosphorylation at T157 and cytoplasmic shuttling of p27 which was prevented by knockdown of SGK1. Following immunoprecipitation studies, SGK1 was identified as a Raptor interacting protein.

Although a putative TOS motif is found within SGK1, mutational analysis showed that this is not required for the interaction. The mTORC1 phosphorylation site within SGK1 was identified as S422, which is hyperphosphorylated in TSC2^{-/-} MEFs and sensitive to rapamycin (Hong et al., 2008). In summary, mTORC1 controls cell cycle progression by phosphorylating SGK1, which in turn phosphorylates p27 resulting in cytoplasmic shuttling of p27. This in turn allows activation of Cdks, by removing the inhibitory effect of p27, and cell cycle progression.

1.4.5 Regulation of autophagy

Macroautophagy (hereafter autophagy) is a non-selective process whereby the contents of a portion of the cytosol are degraded to provide macromolecules during periods of nutritional stress (reviewed in Xie & Klionsky, 2007, Pattingre et al., 2008, Longatti & Tooze, 2009, Mizushima, 2010). The process begins with the formation of an isolation membrane or phagophore which expands to form a double-membrane bound vesicle, the autophagosome. These then fuse with lysosomes resulting in degradation of the contents and release of the resulting macromolecules into the cytosol for recycling. A number of proteins localise to the isolation membrane and thus the autophagosome. These include, among others, the Unc51-like kinase 1 (ULK1) complex, the transmembrane protein Autophagy Related 9 (Atg9) and microtubule-associated protein 1 light chain 3 (LC3). Evidence suggests that mTORC1 regulates each of these components.

1.4.5.1 ULK1

Initially cloned in murine cells (Yan et al., 1998), ULK1 has subsequently been shown to localise to autophagosomes and promote their formation. ULK1 acts as a kinase within an intricately regulated complex with Atg13 (Autophagy-related 13), Focal Adhesion Kinase Family Interacting protein of 200kDa (FIP200) and Atg101

where Atg13 mediates the interaction between ULK1 and FIP200/Atg101 (Hara et al., 2008, Jung et al., 2009). Atg13 and FIP200 are both substrates of ULK1 (Jung et al., 2009) and their depletion by shRNA reduces ULK1 kinase activity and localisation to autophagosomes (Ganley et al., 2009). Thus ULK1 requires Atg13 and FIP200 for full activation. In addition, ULK1 appears to undergo autophosphorylation following activation (Jung et al., 2009). Atg101 was identified as an Atg13 binding protein essential for Atg13 and ULK1 stability and autophagosome formation (Mercer et al., 2009, Hosokawa et al., 2009). Therefore, Atg101 is another essential component of the ULK1 complex in the induction of autophagy.

1.4.5.2 Atg9

The mTORC1 complex phosphorylates ULK1 and Atg13 *in vitro* (Hosokawa et al., 2009, Jung et al., 2009) and *in vivo* studies show that rapamycin leads to dephosphorylation of the proteins (Ganley et al., 2009). Following activation of the pathway, ULK1 and Atg13 are phosphorylated by mTORC1 resulting in inhibition of autophagy. Following mTORC1 inhibition, by cell starvation or rapamycin treatment, ULK1 kinase is activated resulting in autophosphorylation, in addition to phosphorylation of Atg13 and FIP200. This allows migration of the ULK1 complex to autophagosomes and promotes autophagy. Thus it appears that mTORC1 negatively regulates ULK1 and Atg13 in order to inhibit autophagy.

The exact mechanism of ULK1 mediated promotion of autophagy is not clear. Currently the only known function of ULK1 is in the redistribution of the transmembrane protein Atg9 from the trans-golgi network to peripheral endosomes that are positive for GFP-tagged LC3 (Young et al., 2006). Knockdown of ULK1 prevents Atg9 shuttling under nutrient starvation. Thus the ULK1 complex, and by extension mTORC1, may regulate autophagy via the shuttling of Atg9 from the trans-golgi network to autophagosomes.

1.4.5.3 LC3

The mTORC1 complex also appears to regulate autophagy via phosphorylation of LC3. Cytosolic LC3-I is converted to LC3-II following conjugation to phosphatidylethanolamine (PE) which localises to autophagosomal membranes. Rapamycin reduces phosphorylation of LC3 which allows recruitment of the protein to autophagosomes and thus induces autophagy (Cherra et al., 2010).

Phosphorylation of LC3 therefore appears to act as an additional regulatory mechanism for the protein.

In summary, regulation of autophagy by mTORC1 appears to comprise of three principle mechanisms: (1) phosphorylation and localisation of the ULK1 complex to the phagophore, (2) localisation of Atg9 via the ULK1 complex, and (3) phosphorylation of LC3. As mTORC1 is controlled downstream of a number of signals relating to the nutritional status of the cell, including amino acid availability, the AMP/ATP ratio and growth factor signalling, it is well placed as a sensor for the requirement of autophagy.

1.4.6 Hierarchical phosphorylation of substrates

In contrast to the expected outcome, cap dependent translation is only marginally inhibited following treatment of cells with rapamycin (Choo et al., 2008). After 24h rapamycin treatment, following initial dephosphorylation, 4EBP1 becomes hyperphosphorylated and resistant to treatment with an additional dose of rapamycin. Interestingly this revived activity of 4EBP1 requires mTORC1 and is not due to initiation of the negative feedback loop as wortmannin inhibition had no effect. On the other hand, inhibition of protein synthesis using cyclohexamide did prevent hyperphosphorylation of 4EBP1 (Choo et al., 2008). The reason for the revival in 4EBP1 phosphorylation may be due to hierarchical binding of mTORC1 substrates, which places 4EBP1 as the most favoured substrate. Binding of 4EBP1 to mTORC1 is strong and withstands minor structural rearrangement of the kinase, such as occurs on FKBP12/rapamycin binding. Conversely, S6K1 binds relatively weakly, and is difficult to purify with mTORC1 from cell lysates. The hyperphosphorylation of 4EBP1 that follows long term treatment with rapamycin may be as a result of incomplete inhibition of mTORC1 mediated 4EBP1 phosphorylation by rapamycin. Rapamycin inhibition may completely abrogate phosphorylation of weaker mTORC1 substrates leaving 4EBP1 as the sole substrate for the kinase (Choo & Blenis, 2009).

1.5 Signalling inputs to mTORC1

1.5.1 The TSC1/2 complex

The 140kDa Tuberous Sclerosis Complex 1 (TSC1) and 200kDa TSC2 proteins interact via coiled coil domains to form a large heterodimeric complex at the plasma

membrane (Plank et al., 1998, van Slegterhorst et al., 1998). The stability of TSC2 relies on TSC1. Overexpression of TSC1 increases levels of endogenous TSC2 *in vivo*. It is known that free TSC2 is highly ubiquitinated and thus unstable while TSC1 bound TSC2 is not (Benvenuto et al., 2000). The reliance of TSC2 stability on TSC1 is explained by later findings that TSC1 prevents interaction of the ubiquitin ligase Homologous to E6-AP Carboxy Terminus and Regulator of Chromosome Condensation 1 (HERC1) with TSC2 (Chong-Kopera et al., 2006). In support of this, disease causing mutations in TSC2 that cause destabilisation of the protein do so by allowing HERC1 binding in the presence of TSC1 (Benvenuto et al., 2000, Chong-Kopera et al., 2006).

1.5.2 Rheb

The principal role of the TSC1/2 heterodimer relies on the presence of a GTPase activating protein (GAP) domain within TSC2 which increases the intrinsic GTPase activity within the GTP binding protein Ras homologue enriched in brain (Rheb) (Figure 1.4). TSC2, therefore, serves as a negative regulator of the Rheb protein (Tee et al., 2003(a), Inoki et al., 2002). The fact that patient derived TSC2 GAP mutants failed to inactivate Rheb *in vivo* demonstrates the clinical importance of this GAP domain (Inoki et al., 2003, Garami et al., 2003, Tee et al., 2003(a), Zhang et al., 2003). In unstimulated cells, TSC1/2 is found at endoplasmic membranes, with TSC2 in an active and hypophosphorylated state. Following activation of upstream regulatory signalling pathways, TSC2 is phosphorylated at a number of sites causing its release from TSC1 into the cytosol, thus relieving Rheb of its GAP mediated inhibition. Cytosolic phosphorylated TSC2 is sequestered by 14-3-3 protein to provide protection from proteasomal degradation (Li et al., 2002, Shumway et al., 2003, Cai et al., 2006). The use of patient derived mutations of TSC2 also identified the mTORC1 signalling pathway as an effector of TSC1/2 signalling, as such mutations resulted in aberrant phosphorylation of mTORC1 substrates (Tee et al., 2003(1)).

Rheb binds mTORC1 via mTOR and mLST8 independently of guanine nucleotide which only serves to increase mTORC1 activity when in the triphosphate rather than diphosphate state (Long et al., 2005, Smith et al., 2005, Sato et al., 2009). Overexpression of Rheb increases phosphorylation of S6K1 and 4EBP1, and

expression of the constitutively GTP bound Rheb mutant Q64L further promotes phosphorylation of these mTORC1 substrates (Tee et al., 2003(b), Sato et al., 2009).

Rheb acts upstream of mTORC1 to activate downstream signalling events. Activation of mTORC1 substrates caused by overexpression of Rheb is sensitive to rapamycin and is blocked by expression of a dominant negative mutant of mTOR (Inoki et al., 2003). The cellular localisation of Rheb is also important in activating mTORC1 signalling as a farnesyl-binding mutant stimulated S6K1 phosphorylation less effectively than wild type Rheb (Tee et al., 2003(b)).

1.5.3 Rheb-mediated mTORC1 activation

1.5.3.1 FKBP38

It is not clear exactly how Rheb activates mTORC1, although it may be attributable to an increase in substrate binding. When GTP-Rheb binds mTORC1, a subsequent increase in substrate binding is observed (Avruch et al., 2009, Sato et al., 2009). Recently, FKBP38 was shown to bind both Rheb and mTOR, and the possibility that FKBP38 provided the link between Rheb and mTORC1 was investigated. FKBP38 overexpression inhibited phosphorylation of S6K1 and the non-hydrolysable GTP γ S Rheb showed increased binding to FKBP38 compared to GDP-Rheb. Thus a model was put forward where active GTP-Rheb displaces inhibitory FKBP38 from mTOR and allows signalling of downstream substrates (Bai et al., 2007, Ma et al., 2008). In support of this, studies *in vitro* showed inhibition of purified mTORC1 by FKBP38 (Dunlop et al., 2009). Conflicting evidence from further studies failed to show interaction between Rheb and FKBP38, nor did FKBP38 potentially inhibit mTORC1 (Wang et al., 2008, Uhlenbrock et al., 2009).

1.5.3.2 PLD1

Phospholipase D1 (PLD1) was also proposed to link Rheb with mTORC1. Rheb binds PLD1 specifically when GTP bound. Overexpression of Rheb activates PLD1 whereas TSC2 overexpression had the reverse effect. Finally, knockdown of PLD1 inhibited phosphorylation of S6K1 (Sun et al., 2008). Therefore, PLD1 appears to bind active Rheb in order to activate downstream mTORC1 phosphorylation events.

1.5.3.3 TCTP as a Rheb GEF

Although TSC2 has been identified as the GAP for Rheb, the counteracting guanine exchange factor (GEF) that promotes the accumulation of active GTP-Rheb has not been conclusively found. A *Drosophila* study identified translationally controlled tumour protein (TCTP) as a potential Rheb GEF. A dTCTP knockout had reduced phosphorylation of dS6K1, and dRheb co-immunoprecipitated dTCTP from cell lysates. Studies *in vitro* showed enhanced GDP/GTP exchange of dRheb on addition of dTCTP, which was mirrored in human cells where knockdown of TCTP reduced the levels of GTP-Rheb (Hsu et al., 2007).

1.5.3.4 An mTORC1-independent function of Rheb

In addition to activity towards PLD1, Rheb also inhibits B-Raf and C-Raf within the ERK pathway, possibly mediating a negative feedback loop to prevent aberrant activation of MAPK. Overexpression of Rheb reduces B-Raf activity by reducing phosphorylation and heterodimerisation with C-Raf. This is rapamycin resistant so appears to be an mTORC1-independent function of Rheb (Karbowiczec et al., 2004, Karbowiczec et al., 2006).

1.5.4 Amino acids

Activity of mTORC1 is sensitive to a number of upstream signals. Activation in response to amino acids activation provides a foundation for further activation by other stimuli. Amino acid withdrawal results in loss of 4EBP1 and S6K1 phosphorylation which is unresponsive to insulin. As this does not alter insulin stimulation of RTKs, PI3K, Akt or MAPK, this appears to act directly on the mTORC1 complex (Hara et al., 1998). S6K1 phosphorylation at T389 is sensitive to amino acid withdrawal in TSC2^{-/-} MEFs, indicating that amino acid activation of mTORC1 is independent of insulin stimulation (Smith et al., 2005).

Amino acids activate mTORC1 by a number of pathways, which result in localisation of the complex to endomembranes. Therefore localisation of mTORC1, rather than kinase activity *per se*, appears central to activation in response to amino acids.

Rheb still binds mTORC1 when not guanosine nucleotide-bound (Smith et al., 2005). As Rheb similarly localises to membranes, via the farnesylated residue, it is thought that mTORC1 localisation permits Rheb-mediated activation of the complex.

Therefore localisation to the membrane with Rheb initiates the first stage of mTORC1 activation, followed by inhibition of Rheb GAP activity by removal of TSC1/2.

Localisation of yeast TOR1 to endosomes and golgi requires LST8, raising the possibility that mLST8 plays a similar role in mTORC1 (Chen & Kaiser, 2003). This may explain why purified mTOR-raptor complexes, without mLST8, actively phosphorylate mTORC1 substrates (Guertin et al., 2006). Mammalian LST8 may therefore be required for membrane localisation of mTORC1 in response to amino acids.

1.5.4.1 hVps34

The class III PI3K human vacuolar protein sorting 34 (hVps34) forms part of a complex with its associated kinase hVps15 and is involved in endosome to golgi retrograde transport (Burda et al., 2002). Activity of hVps34 is sensitive to amino acid availability. In response to amino acid stimulation PIP₃, the product of active hVps34, increases staining as punctuate spots on addition of amino acids to growing cells. This is reduced by both the PI3K inhibitor wortmannin and amino acid withdrawal (Nobukuni et al., 2005). Insulin does not alter the activity of hVps34, although the protein is sensitive to glucose stimulation and energy status of the cell via 5' Adenosine Monophosphate-Activated Protein Kinase (AMPK) (Byfield et al., 2005).

The link between hVps34 and mTORC1 activity was established following evidence that overexpression of the hVps34 associated kinase hVps15 increased S6K1 phosphorylation at T389 (Byfield et al., 2005). Following this, knockdown of hVps34 was found to reduce phosphorylation of 4EBP1 and S6K1 in response to amino acid stimulation (Byfield et al., 2005, Nobukuni et al., 2005). A direct link between mTORC1 and hVps34 was found in discovery that the two proteins interact (Gulati et al., 2008). Amino acid withdrawal blocks S6K1 phosphorylation independent of TSC1/2 activity (Byfield et al., 2005, Nobukuni et al., 2005).

The hVps34 mediated mTORC1 response to amino acids appears to be sensitive to intracellular Ca²⁺ levels. On amino acid stimulation intracellular Ca²⁺ levels increase,

leading to activation of calcium modulated protein (CaM), which binds and activates hVps34. This process is essential for mTORC1 activation by hVps34, as expression of hVps34 mutants that don't bind CaM results in S6K1 immunity to activation by amino acids (Gulati et al., 2008).

1.5.4.2 Rag GTPases

The Ras-related Small GTP-Binding Protein (Rag) GTPases, of which there are four, act as heterodimers consisting of RagA/B along with RagC/D (Schurmann et al., 1995, Hirose et al., 1998, Sekiguchi et al., 2001). In response to amino acid stimulation, GTP bound Rag GTPase dimers accumulate and bind to Raptor, and are essential for amino acid stimulated activation of mTORC1 (Sancak et al., 2008). Expression of constitutively GTP-bound RagB (^{GTP}Rag) activates mTORC1 and is insensitive to amino acid deprivation, whereas dominant negative constitutively GDP bound RagB (^{GDP}RagB) prevents amino acid stimulated S6K1 phosphorylation at T389 (Kim et al., 2008, Sancak et al., 2008).

After binding the mTORC1 complex, active Rag dimers aid translocation of mTORC1 from the cytosol to vesicles and perinuclear regions. In the absence of amino acids, mTORC1 accumulates in the cytosol. Under the same conditions, GTP-bound RagB expression promotes mTORC1 accumulation at perinuclear regions and vesicles. As kinase activity of mTORC1 is not stimulated by ^{GTP}RagB *in vitro*, localisation appears to be key in this process (Sancak et al., 2008).

1.5.4.3 RalA

A second G protein involved in amino acid signalling to mTORC1 is RalA, a member of the Ras superfamily. RalA is involved in exocytosis, cell adhesion, membrane dynamics and cell migration and is critical in Ras-induced tumorigenesis of human cells (Ohta et al., 1999, Moskalenko et al., 2001, Lim et al., 2005). Activation of RalA requires Ral Guanine nucleotide dissociation stimulator (RalGDS) which binds the Ras binding domain to promote GTP-binding of RalA (Gonzalez-Garcia et al., 2005, reviewed in Ferro & Trabalzini, 2010). On amino acid stimulation, GTP-RalA accumulates allowing mTORC1 mediated phosphorylation of S6K1. Knockdown of RalA and RalGDS abolish mTORC1 directed phosphorylation of S6K1 in response to amino acids. RalA-induced mTORC1 activity acts in parallel or downstream of Rheb,

as an overactive Rheb mutant fails to stimulate S6K1 activity on knockdown of RalA (Maehama et al., 2008). RalA localises to internal membranes (perinuclear and vesicular) which is critical for its effects within the cell (Shipitsin & Feig, 2004, Vitale et al., 2005). As RalA does not directly bind to or activate mTORC1, it may act in a similar way to hVps34 and the Rag GTPases by localising mTORC1 from the cytosol to internal membranes.

1.5.5 Growth factor and insulin signalling

Growth factor and insulin-mediated activation of mTORC1 is mediated by a PI3K/Akt cascade stimulated by RTK activation (Figure 1.4). PI3K activation by RTKs may occur through direct binding or via adaptors such as insulin receptor substrate 1 (IRS-1) in the case of insulin signalling. IRS-1 is phosphorylated at a number of tyrosine residues at the C terminus following insulin binding to the insulin receptor (IR) and is required for signalling events downstream of insulin (Mendez et al., 1996, Stenkula et al., 2007, reviewed in Boura-Halfon & Zick, 2009). This provides a docking platform for PI3K, which converts PIP₂ within the plasma membrane to the second messenger PIP₃ (Chung et al., 1994, Mendez et al., 1996, Maehama & Dixon, 1998, Aoki et al., 2001). PI3K is a heterodimer consisting of a p85 regulatory subunit and p110 catalytic subunit. Proteins containing PH domains bind PIP₃, recruiting them to the membrane where they are activated (Engelman et al., 2006).

Akt and PIP₃-Dependent Protein Kinase 1 (PDK1) both contain PH domains. Recruitment to the membrane allows PDK1 mediated phosphorylation of Akt at T308 (Alessi et al., 1997). Full activation of Akt then requires phosphorylation at S473 by mTORC2. PDK1 association with Akt alone is sufficient to promote phosphorylation of downstream targets, indicating that PIP₃ merely serves as a docking messenger to co-localise Akt and PDK1 (Ding et al., 2010).

Recent studies indicate PDK1 bypasses Akt in response to amino acid stimulation and directly phosphorylates PRAS40 and mTOR leading to activation of S6K1. Amino acid stimulation results in activation of mTORC1 without activation of Akt and *PDK1* knockout mice abrogated the activation of S6K1 by amino acid sufficiency (Ding et al., 2010). This provides another mechanism for activation of mTORC1 in response to amino acid sufficiency.

Activated Akt phosphorylates a number of downstream substrates including mTOR, PRAS40 and TSC2 (reviewed in Manning & Cantley, 2007, Canedo et al., 2010). Phosphorylation of mTOR at S2448 may result in increased activity of the kinase (Scott et al., 1998, Nave et al., 1999, Reynolds et al., 2002), although studies have shown this is redundant for phosphorylation of 4EBP1 and S6K1 (Sekulic et al., 2000).

Phosphorylation of TSC2 at a number of serine residues, including S939, S981 and S1462, affects both binding and therefore stability of TSC1/2 and cellular localisation of the complex. Mutation of Akt phosphorylation sites within TSC2 increases stability of the TSC1/2 complex, and inhibits signalling to S6K1 and 4EBP1, indicating that Akt phosphorylation promotes TSC1/2 instability (Dan et al., 2002, Inoki et al., 2003, Potter et al., 2003).

Phosphorylation also results in translocation of TSC1/2 from membranes to the cytosol. Phosphorylation of TSC2 at S939 and S981 doesn't alter GAP activity of TSC2 but results in translocation from the membrane to the cytosol where it is sequestered by 14-3-3. As Rheb is membrane-associated, this prevents the inhibitory activity of TSC1/2 (Cai et al., 2006). Evidence therefore suggests that Akt phosphorylation of TSC2 does not alter GAP activity but instead prevents interaction with Rheb.

Finally, phosphorylation of PRAS40 results in dissociation of the inhibitor from the mTORC1 complex allowing activation of mTORC1 (Kovacina et al., 2003). Active Akt therefore promotes mTORC1 activity by relieving upstream inhibition and possibly activating the mTOR kinase itself.

1.5.6 Mitogenic stimuli

1.5.6.1 PA

The lipid second messenger phosphatidic acid (PA) is created by PLD1 and provides an additional path to mTORC1 activation induced by mitogenic stimuli. Cdc42 is involved in regulation of the cell cycle. Induced activation of Cdc42 stimulates PLD1 activity and increases phosphorylation of S6K1. Expression of a Cdc42 mutant,

which is deficient in PLD1 binding, reduces S6K1 phosphorylation, which is overcome by addition of extracellular PA (Fang et al., 2003). Accumulated PA binds the FKBP12-Rapamycin Binding (FRB) domain of mTOR and competes with FKBP12 to activate mTORC1. As the catalytic activity of mTORC1 is unchanged by PA *in vitro*, this competition may provide the principle mechanism of PA induced S6K1 and 4EBP1 activity (Fang et al., 2002). Alternatively, PA binding to mTOR may localise mTORC1 to the membrane thus promoting binding to Rheb (Sun et al., 2008). PLD1 synthesises PA and, as discussed in Section 1.5.3.2, interacts with Rheb in order to activate mTORC1. Rheb activation may therefore stimulate PLD1 to produce PA which, due to its localisation to membranes, then binds and activates mTORC1.

1.5.6.2 MAPK

MAPK activity has also been shown to stimulate mTORC1. Five families of MAPKs exist, one of which is the ERK1&2 family. G-protein coupled receptors, RTKs and phorbol esters such as phorbol 12-myristate 13-acetate (PMA) transmit signals to ERK via Raf followed by MAPK/ERK kinase (MEK). Activated ERK then phosphorylates a number of substrates including p90 ribosomal S6 kinases (RSKs) and MAPK interacting kinase (MNKs) (reviewed in Roux & Blenis, 2004). Activation of the classical Raf/MEK/ERK cascade by PMA promotes phosphorylation of S6K1 and 4EBP1. Further investigation identified TSC2 and Raptor as MAPK targets. Overexpression of TSC2 reverses PMA-induced S6K1 phosphorylation. The inhibitory effect of TSC2 is removed by RSK1 by phosphorylation at S1798, and S1798A mutation prevents PMA-induced S6K1 phosphorylation (Roux et al., 2004).

RSK activation takes place via MEK/ERK as inhibition of either results in reduction of S6K1 and 4EBP1 phosphorylation induced by PMA (Roux et al., 2004, Rolfe et al., 2005). The finding that Akt activity was unaffected by PMA indicated that activation of mTORC1 by MAPK is independent of insulin signalling (Roux et al., 2004, Rolfe et al., 2005).

MAPK also induces phosphorylation of Raptor and S719, S721 and S722 by RSK. Knockdown of RSK1 or 2 results in reduced Raptor phosphorylation. Although Raptor phosphorylation had no effect on mTOR-Raptor-substrate binding, mTOR

kinase activity was reduced by mutation of the three phosphorylation sites to alanine. Thus, mTORC1 activity is also activated by MAPK via Raptor phosphorylation (Carriere et al., 2008), providing a dual mechanism for activation of translation by ERK.

MAPK also activates mTORC1 effectors independently of the complex. Both rpS6 and eIF4E are phosphorylated in response to ERK activation, reiterating the importance of translational control by MAPK (Rolfe et al., 2005, Roux et al., 2007).

1.5.6.3 AMPK

On depletion of ATP, mTORC1 must be inhibited in order to preserve energy within the cell. Accumulation of AMP results in activation of the AMPK by Liver Kinase B1 (LKB1) mediated phosphorylation of T172 (Shaw et al., 2004). AMPK inhibits mTORC1 signalling to S6K1 and 4EBP1 to inhibit the high energy input process of translation (Bolster et al., 2002, Krause et al., 2002, Kimura et al., 2003). This is achieved through two known mechanisms.

Firstly, AMPK phosphorylates TSC2 at T1227 and S1345 leading to activation of TSC1/2 and inhibition of mTORC1 by GDP-Rheb accumulation. Knockdown of TSC2 prevents the reduction in S6K1 phosphorylation resulting from accumulation of AMP. Inhibition of S6K1 is restored by expression of WT TSC2 but not an AMPK phosphorylation site mutant of the protein, indicating that AMPK mediated phosphorylation at T1227 and S1345 is critical in AMPK mediated mTORC1 inhibition (Inoki et al., 2003).

Another mechanism of AMPK-mediated inhibition of mTORC1 occurs by direct phosphorylation of Raptor. As TSC2 deficient cells retain energy sensitivity, a screen for AMPK substrates discovered Raptor phosphorylation sites at S792 and S722 were sensitive to AMPK. Mutation of these sites prevented AMPK mediated inhibition of mTORC1. Inhibition appears to result from binding of Raptor within the complex to 14-3-3 in the cytosol (Gwinn et al., 2008).

A further link between mTORC1 activation and AMPK occurs via Akt. In addition to activating mTORC1 by phosphorylation and inhibition of TSC1/2, Akt also inhibits

AMPK by maintaining cellular ATP levels by increasing glycolysis and oxidative phosphorylation. Knockdown of Akt results in depletion of ATP followed by activation of AMPK and inhibition of mTORC1 (Hahn-Windgassen et al., 2005).

1.5.6.4 Wnt

Canonical Wnt signalling leads to activation of T cell factor transcription factors. In the absence of upstream signals GSK3 β , in complex with adenomatous polyposis coli, axin and PP2A, phosphorylates β catenin leading to its ubiquitination and degradation. Binding of the Wnt ligand to its receptor leads to inhibition of GSK3 β by Dishevelled leading to accumulation of β catenin, which interacts with T cell factor transcription factors to activate transcription from promoters with TCF sites (reviewed in Smalley & Dale, 1999). Wnt signalling positively regulates mTORC1. In the absence of the Wnt ligand, GSK3 β was found to phosphorylate TSC2 at the activating residues S1337 and S1341, leading to inhibition of mTORC1. Inactivation of GSK3 β following activation of canonical Wnt signalling lead to activation of mTORC1 due to the absence of these TSC2 phosphorylation events (Inoki et al., 2006).

1.5.7 Hypoxic input

1.5.7.1 REDD1

Although expression of hypoxia-induced genes is controlled by mTORC1 via HIF1, chronic hypoxia inhibits mTORC1 signalling. Hypoxia results in inhibition of S6K1 and 4EBP1 phosphorylation independent to the activation status of Akt, AMPK and HIF1 (Arsham et al., 2002). Two methods of mTORC1 inhibition during hypoxia have been uncovered. Regulated in development and DNA damage responses 1 (REDD1) expression is induced by hypoxia and inhibits mTORC1 by activating TSC1/2. Cells lacking REDD1 fail to reduce S6K1 phosphorylation during hypoxia, including head and neck squamous cell carcinoma which frequently lack expression of the protein (Brugarolas et al., 2004, Schneider et al., 2008). In addition, knockout of TSC2 prevents hypoxia-induced inhibition of mTORC1, as does disruption of the TSC1/2 complex (Brugarolas et al., 2004).

Inhibition of TSC2 occurs by sequestering the protein in the cytosol by 14-3-3 as active TSC1/2 is located at membranes (Cai et al., 2006). REDD1 binds 14-3-3 to remove TSC2 and allowing localisation of TSC2 to membranes. Mutation of the 14-3-3 binding domain of REDD1 prevents hypoxia-induced inhibition of mTORC1 by maintaining interaction between 14-3-3 and TSC2 in the cytosol (DeYoung et al., 2008). During recovery from hypoxia, mTORC1 inhibition is removed by proteasomal mediated degradation of REDD1, induced by ubiquitination (Katiyar et al., 2009).

1.5.7.2 Bnip3

By inhibiting accumulation of GTP-Rheb, Bcl-2/adenovirus E1B 19kDa interacting protein 3 (Bnip3) also inhibits mTORC1 in response to hypoxia. Rheb is tethered to membrane structures via farnesylation (Tee et al., 2003(b)). Bnip3 targets Rheb by localising to the same membranes via the transmembrane domain. Deletion mutants of Bnip3 without the transmembrane domain fail to interact with and inhibit Rheb, and do not inhibit S6K1 or 4EBP1 phosphorylation in response to hypoxia. Bnip3 inhibits Rheb by activating GTPase activity, as wild-type but not transmembrane deletion mutant Bnip3 reduced GTP-Rheb accumulation (Li et al., 2007). Thus in response to hypoxia, Bnip3 localises with Rheb at endomembranes and activates GTPase activity resulting in inhibition of mTORC1.

1.5.8 Autophagic input

1.5.8.1 ROS

A number of upstream pathways have been shown to inhibit mTORC1 in order to activate autophagy. Reactive oxygen species (ROS) have been shown to activate Ataxia-Telangiectasia Mutated (ATM), which in turn activates AMPK. As an inhibitory pathway upstream of mTORC1, AMPK activation subsequently activates autophagy. Thus ROS positively regulate autophagy via mTORC1 inhibition (Alexander et al., 2010). The purpose of this mechanism is not well understood, but may be a method of releasing macromolecules to replace those damaged by high levels of ROS within the cell.

1.5.8.2 ULK1 and AMPK

AMPK has also been shown to activate autophagy by inhibition of mTORC1. AMPK is known to interact and phosphorylate ULK1 at S317 and S777, which further

activates ULK1 and enhances autophagy (Egan et al., 2011, Kim et al., 2011). Activated ULK1 then phosphorylates Raptor, leading to inhibition of mTOR substrate binding (Dunlop et al., 2011). Therefore activation of ULK1 in response to nutrient limiting conditions promotes a negative feedback loop towards mTORC1 thus inhibiting protein synthesis.

SV40 Small T Antigen (ST) induces tumorigenicity in cells by inhibition of PP2A. Cells infected with the antigen have increased activity of AMPK, which is further increased by glucose depletion, and rely on autophagy induction by AMPK mediated mTORC1 inhibition as a nutrient source on cell starvation (Kumar & Rangarajan, 2009). Inhibition of AMPK in these cells results in rapid cell death. Thus ST appears to induce autophagy as an alternative energy source for cancer cells on nutrient deprivation.

1.5.9 Inhibitors of mTORC1

1.5.9.1 PI3K inhibitors

Wortmannin and LY294002 are two specific Phosphatidylinositol-3-Kinase (PI3K) inhibitors with an IC_{50} of 4.2nM and 1.4 μ M respectively. Both bind the ATP binding site and act as a competitive inhibitor for the enzyme. Wortmannin irreversibly inhibits PI3K as it results in conformational rearrangement of the active site due to covalent attack on the ATP binding site. There is a high degree of complementarity between PI3K and wortmannin, and the inhibitor binds more deeply in the ATP binding pocket than ATP itself. Inhibition by LY294002 on the other hand is not irreversible, as interaction takes place mainly via hydrogen bonds (Walker et al., 2000).

Although useful in research, neither is a suitable drug to treat diseases caused by aberrant PI3K activity. Wortmannin is unstable and LY294002 is a relatively weak inhibitor. In addition, both display a number of off-target effects. For these reasons, wortmannin and LY294002 have instead provided a useful starting point for the development of the PI3K inhibitor GDC-0941 that is now in use in clinical trials towards breast and lung carcinoma (O'Brien et al., 2010, Soria et al., 2010).

Both wortmannin and LY294002 inhibit 4EBP1 phosphorylation and mTOR autophosphorylation in vivo, indicating inhibition of mTOR (Brunn et al., 1996). Staurosporine is a general kinase inhibitor and has a variety of affinities ranging from nanomolar to micromolar.

The IC₅₀ for PI3K by staurosporine is 9 μ M (Walker et al., 2000). Implications in vivo include broad spectrum inhibition of kinases resulting in a number of off-target effects. Previous studies indicate that staurosporine does not inhibit mTOR directly (Tee & Proud, 2001).

1.5.9.2 Rapamycin

The drug rapamycin inhibits mTORC1 and mTORC2 by binding FKBP12, which interacts with mTOR at the FRB domain (Chen et al., 1995, reviewed in Foster & Toschi, 2009). PA is generated by PLD1 in response to mitogenic stimuli and activates mTORC1 and mTORC2 by binding the FRB domain of mTOR (Fang et al., 2001, Hornberger et al., 2006). This results in increased association of mTOR with Raptor and Raptor-Independent Companion of mTORC2 (Rictor) and increased phosphorylation of the respective substrates S6K1 and Akt (Toschi et al., 2009). It is thought that rapamycin inhibits the complexes by competing with PA-mTOR interaction. Competition with PA provides a reason for the higher concentration of rapamycin required to inhibit mTORC2 as opposed to mTORC1. Higher concentrations of rapamycin are required to compete with mTORC2-PA binding than PA binding to mTORC1 (Toschi et al., 2009). Although a potent inhibitor of mTOR, it does not inhibit all mTORC1 phosphorylation events and resultant processes, including some phosphorylation sites within 4EBP1 such as the priming sites, T36 and T45 (Choo et al., 2008).

1.5.9.3 TorinI

The effectiveness of mTORC1 inhibition could be improved with the use of catalytic inhibitors rather than rapamycin based therapy (reviewed in Efeyan & Sabatini, 2010). Thoreen *et al.* developed the mTOR ATP-competitive inhibitor, TorinI. Whereas rapamycin only resulted in a modest induction of macroautophagy, TorinI resulted in intense formation of autophagosomes which was mirrored by a depletion

of Raptor protein using siRNA (Thoreen et al. 2009). Torin1 also reduced phosphorylation of all 4EBP1 phosphorylation sites.

1.5.10 Feedback inhibition

1.5.10.1 Feedback to IRS-1

A negative feedback loop attenuates PI3K signalling in response to insulin induced mTORC1 activation via IRS-1 downregulation (reviewed in Manning, 2004, Carracedo & Pandolfi, 2008, Grant, 2008). This occurs by direct phosphorylation by mTORC1 and S6K1 at a number of serine residues. Active mTORC1 and S6K1 phosphorylate IRS-1 at S636/639 and S1101, respectively (Veileux et al., 2010). Knockdown of mTOR, Raptor or S6K1 or treatment with rapamycin reduces the insulin induced phosphorylation of IRS-1 at these residues, providing further evidence for the existence of a feedback loop.

Serine mediated phosphorylation of IRS-1 results in attenuation of signalling to PI3K and therefore Akt. Studies *in vitro* showed direct binding between IRS-1 and Raptor, and phosphorylation of IRS-1 by purified mTORC1 (Tzatsos & Kandror, 2006). The residues surrounding S636/639 are composed of a common mTORC1 phosphorylation motif and fall within the binding site of the p85 regulatory subunit of PI3K. Thus serine phosphorylation of IRS-1 may prevent interaction with PI3K causing a reduction in downstream signalling.

Direct phosphorylation of IRS-1 by mTORC1 appears to be controlled by PP2A (Hartley & Cooper, 2002). The rapamycin induced decrease in IRS-1 phosphorylation was reversed by okadaic acid treatment, but inhibition of S6K1 phosphorylation was still observed. Thus a PP2A complex may inhibit mTORC1 phosphorylation of IRS-1 independently of S6K1.

Further evidence for the role of mTORC1 in an insulin induced negative feedback of PI3K signalling comes from studies in adipocytes and neuronal cells. Rapamycin prevents the insulin induced phosphorylation of IRS-1 in 3T3-L1 adipocytes leading to rescue of PI3K activity (Haruta et al., 2000, Veileux et al., 2010). On studying the effect of hyperinsulinaemia on neuronal cells, Mayer & Belsham similarly discovered

that rapamycin reversed the S6K1 mediated phosphorylation of IRS-1 in an immortalised hypothalamic cell line (Mayer & Belsham, 2010).

Amino acid activation of mTORC1 also results in negative feedback via IRS-1 phosphorylation. Amino acid stimulation increases S6K1 mediated phosphorylation of IRS-1 at S1101 and mutation of this residue maintains Akt activation in response to stimuli (Tremblay et al., 2007). Activation of the feedback loop again results in reduced binding of the PI3K p85 regulatory subunit thus reducing activity of PI3K by 70% (Tremblay & Marette, 2001). This feedback mechanism was prevented by treatment with rapamycin.

IRS-1 phosphorylation downstream of mTORC1 results in depletion of IRS-1 which similarly reduces PI3K signalling. Phosphorylation of IRS-1 at S312 results in ubiquitination mediated proteasomal degradation of IRS-1 (Zhende et al., 2002, Greene et al., 2003). Proteasome inhibitors prevent IRS-1 degradation and maintain signalling to Akt (Haruta et al., 2000).

Loss of the inhibitory TSC1/2 complex also results in negative feedback via IRS-1. IRS-1 levels are depleted in *TSC1*^{-/-} and *TSC2*^{-/-} MEFs (Harrington et al., 2004, Shah et al., 2004). As a result cells are unresponsive to IGF-1. Over-expression of a catalytically active PI3K p110 subunit in these cells increases Akt activation indicating that the same feedback loop is active. In addition, IRS-1 mRNA is reduced in *TSC1*^{-/-} and *TSC2*^{-/-} MEFs (Harrington et al., 2004, Shah et al., 2004). Thus chronic hyperactivation of mTORC1 reduces both IRS-1 stability and transcription. This reduces signalling through PI3K to Akt and provides a mechanism by which tumours lacking *TSC2* are predominantly benign. *TSC2*^{+/-} mice develop slow growing tumours due to inactivation of Akt signalling. *PTEN* haploinsufficiency restores Akt signalling a greatly enhances their severity (Manning et al., 2005).

1.5.10.2 PDGFR

An alternative negative feedback loop following mTORC1 hyperactivation results in downregulation of Platelet-Derived Growth Factor Receptor (PDGFR). *TSC1*^{-/-} and *TSC2*^{-/-} MEFs are defective in PI3K signalling in response to serum, PDGF, EGF and

insulin due to reduced levels of PDGFR β (Zhang et al., 2003). This occurs via reduced transcription or mRNA half-life and is rescued by treatment with rapamycin (Zhang et al., 2007). Deregulation of mTORC1 therefore reduces PI3K signalling by attenuating IRS-1 and PDGFR β .

1.5.10.3 S6K1 and GSK3

Two additional feedback mechanisms involving S6K1 and eIF4E exist independent of those discussed above. On hyperactivation of mTORC1, the resulting decrease in Akt activity is overcome by the ability of S6K1 to phosphorylate GSK3 (Zhang et al., 2006). In cells and tumours lacking TSC1 or TSC2, GSK3 phosphorylation is sensitive to rapamycin. Following knockdown of S6K1, GSK3 becomes hypophosphorylated. Thus S6K1 is the direct kinase of GSK3 following Akt inactivation induced by the negative feedback loop following hyperactivation of mTORC1.

1.5.10.4 eIF4E

The translational initiation factor eIF4E also exerts a negative feedback loop to control translation, again following mTORC1 activation. Following induced expression of eIF4E, 4EBP1 and S6K1 phosphorylation was reduced, whereas Akt phosphorylation remained unchanged (Khaleghpour et al., 1999). Thus active eIF4E appears to induce a negative feedback loop downstream of Akt to downregulate translation.

1.5.10.5 PP2A β and PDK1

Silencing of the *PPP2R2B* gene, which encodes the B β subunit of PP2A, is present in over 90% of colon cancers (Tan et al., 2010). PP2A β was subsequently found to regulate a novel mTORC1 feedback loop resulting in Myc activation in these cancers. Loss of PPP2R2B is sufficient to promote cellular transformation, and re-expression in colorectal cancer cells reduces cell proliferation. PP2A β was shown to dephosphorylate S6K1 downstream of mTORC1, and appears to act in response to rapamycin inhibition as re-expression of B β in colorectal cancer cells sensitised the cells to the drug. Loss of PP2A β by silencing of the gene resulted in induction of Myc phosphorylation following rapamycin treatment, which was abolished by re-

expression of B β . As knockdown of PDK1 prevented rapamycin-induced Myc phosphorylation, the effect of B β silencing was found to be mediated by PDK1. Indeed B β was shown to interact with PDK1 and prevent membrane localisation following treatment with rapamycin. This provides a novel feedback mechanism where, in the absence of PP2A_{B β} regulation, rapamycin results in membrane recruitment of PDK1 which promotes phosphorylation of Myc (Tan et al., 2010).

1.5.10.6 Feedback following chronic inhibition

Chronic mTORC1 inhibition also results in upregulation of Akt via an IRS-1-PI3K independent mechanism. Rapamycin increases phosphorylation of Akt, as does Raptor knockdown (Wang et al., 2008, Chen et al., 2010). This is not abrogated by IRS-1 or TSC1 depletion, indicating a mechanism independent of the relief IRS-1 mediated feedback, although inhibition of IGF-IR reversed the effect (O-Reilly et al., 2006, Wan et al., 2007). Thus chronic mTORC1 inhibition appears to induce additional signalling cascades to increase signalling to Akt.

1.6 Phosphatase regulation of mTORC1

1.6.1 PP2A

The role of PP2A as the phosphatase within the mTORC1 signalling pathway is well documented. Studies *in vitro* have shown decreased PP2A activity on insulin or Insulin-Like Growth Factor 1 (IGF-1) stimulation which is increased following treatment with rapamycin (Liu et al., 2010, Peterson et al., 1999, Begum & Ragola, 1996). Overexpression of a rapamycin resistant mTOR mutant prevented the rapamycin induced increase in PP2A activity (Liu et al., 2010). In addition, PP2A inhibition using calyculin A increased phosphorylation of S6K1 under amino acid starved conditions or following treatment with rapamycin (Peterson et al., 1999, Bielinski & Mumby 2007). Therefore a PP2A complex appears to be directly controlled by mTORC1. A direct interaction between PP2Ac and S6K1 has been observed and the S6K1 binding region within PP2Ac mapped to between the 88th amino acid and the C terminus of the protein (Yamashita et al., 2005, Peterson et al., 1999). This indicates that PP2A acts directly on mTORC1 substrates.

The exact method of PP2Ac regulation employed by mTORC1 is unclear. PP2Ac is phosphorylated by mTORC1 *in vitro* (Peterson et al., 1999). Activity of the catalytically inactive S6K1 TOS mutant (F5A) is rescued by additional phosphomimetic mutation of the mTORC1 phosphorylation site T389 and the putative PP2A binding motif RSPRR. This S6K1 mutant is also resistant to inhibition with rapamycin (Schalm et al., 2005). This points to a model where active mTORC1 binds to and phosphorylates S6K1 thus displacing PP2A from the RSPRR motif. PP2Ac activity relies on regulatory subunit binding, therefore the key to uncovering PP2Ac activity in mTORC1 signalling lies in discovering the relevant regulatory subunit involved.

1.6.2 Alpha4

1.6.2.1 General introduction

Alpha4, the human homologue of yeast Tap42 (Table 1.1), is a regulatory subunit of PP2Ac that plays a critical role in controlling many signalling events in the cell. In fact, deletion of the Alpha4 gene leads to cell death by induction of apoptosis (Kong et al., 2004). Alpha4 is intrinsically unstructured as a monomer (Smetana et al., 2006). However, Alpha4 becomes structured upon substrate binding allowing high specificity on interaction with other proteins (Smetana et al., 2006). The function of Alpha4 is executed by interaction with PP2Ac via amino acid residues 198 to 202, where Alpha4 both stabilises and inhibits the phosphatase (Nanahoshi et al., 1998, Chen et al., 1998, Nanahoshi et al., 1999). Inhibition of PP2Ac is allosteric, as Alpha4 does not obstruct the active site of PP2Ac (Prickett & Brautigan, 2004, Prickett & Brautigan, 2006). Furthermore, Alpha4 binds independently of the A and B subunits of PP2A thereby acting as an independent regulatory subunit (Prickett & Brautigan, 2004, Chung et al., 1999).

1.6.2.2 Functions

1.6.2.2.1 Regulation of mTORC1

Much conflicting data exists as to the role of Alpha4 in complex with PP2Ac. Whilst Alpha4 acts as an activating PP2Ac subunit to direct phosphatase activity towards the mTORC1 substrates 4EBP1 and S6K1 (Nien et al., 2007), over-expression of Alpha4 also leads to a decrease in 4EBP1 and S6K1 phosphorylation (Nanahoshi et al., 1998, Grech et al., 2008). Therefore whether Alpha4 is an activating or inhibitory

PP2Ac subunit is not clear. Studies *in vitro* indicate that Alpha4 inhibits PP2Ac activity (Chung et al., 1999, Prickett & Brautigan, 2004, Prickett & Brautigan, 2006). There is also conflicting data as to the regulation of PP2A_{Alpha4} in response to mTORC1, with some studies observing reduced interaction on inhibition of mTORC1 (Murata et al., 1997, Yamashita et al., 2005), and others seeing no change (Nanahoshi et al., 1998, Nanahoshi et al., 1999, Yoo et al., 2008). It is therefore clear that although a great deal of investigation into the role of Alpha4 towards PP2Ac has been done, there is much left to ascertain.

PP2A_{Alpha4} positively regulates STAT1 in complex with mTOR. Inactivation of mTOR amplifies the induction of IFN γ stimulated genes. In fact, rapamycin increases mTOR binding to STAT1, mediated by PP2Ac-Alpha4, leading to nuclear accumulation of the complex (Fielhaber et al., 2009). In support of this, dominant negative PP2Ac mutation L119A prevented the rapamycin induced nuclear translocation of STAT1. This is analogous to the regulation of the stress response proteins MSN2 and MSN4 in yeast, which are subject to TORC1 mediated nuclear translocation without phosphorylation (Crespo et al., 2002). Interestingly, Alpha4 translocates to the nucleus following post-translational modification with O-linked β -N-acetylglucosamine, mediated by O- β -N-acetylglucosaminyltransferase (OGT) (Dauphinee et al., 2005). The reason or function of this post-translational modification is currently unknown but likely is necessary for the nuclear translocation of Alpha4.

1.6.2.2.2 Independent regulation of STAT1

PP2A_{Alpha4} also negatively regulates signal transducer and activator of transcription 1 (STAT1) activity without altering phosphorylation of the protein. Cytokines such as IFN γ secreted by activated T lymphocytes and natural killer cells activate STAT1 by causing dimerisation of their cognate receptors. This leads to phosphorylation of the receptor associated Janus kinases and tyrosine kinases and finally tyrosine phosphorylation at the C terminus of STAT1. Phosphorylated STAT1 then translocates to the nucleus to bind promoters of IFN γ responsive genes (reviewed in Kramer & Heinzl, 2010). Alpha4 over-expression decreases STAT1 activity by

promoting binding to its inhibitor Protein Inhibitor of Activated STAT1 (PIAS1) (Nien et al., 2007).

1.6.2.2.3 Apoptosis

The protective role of Alpha4 towards PP2Ac is key in preventing apoptosis in response to stress stimuli (Prickett & Brautigan, 2006). PP2A complexes are sensitive to such stimuli, where stress signals can lead to their disintegration. Alpha4 then sequesters inactive PP2Ac, thus protecting it from proteasomal degradation and ensuring its availability to dephosphorylate stress-induced phosphorylation events following removal of upstream signals (Kong et al., 2009). In support of this theory, studies show that heat shock increases PP2A_{Alpha4} complex formation and over-expression of Alpha4 leads to an increased rate of reversal of stress induced phosphorylation events (Kong et al., 2009).

A ubiquitin interacting motif (UIM) encompassing amino acids 46 to 60 within Alpha4 is essential for the protective role of Alpha4 towards PP2Ac (LeNoue-Newton et al., 2011). The UIM was shown to be necessary for monoubiquitination of Alpha4 and also to block the interaction of the E3 ubiquitin ligase Mid1 with PP2Ac (McConnell et al., 2010). In these studies, over-expression of wild-type, but not a UIM mutated Alpha4, reduces PP2A polyubiquitination and degradation of the phosphatase (McConnell et al., 2010). This indicates that Alpha4 sequesters PP2A in an inhibitory state and prevents proteasomal degradation. Following removal of stress signals, Alpha4 then acts as a regulatory subunit directing PP2Ac phosphatase activity towards stress induced phosphoproteins. For example, Alpha4 binds MEK3 and directs dephosphorylation by PP2Ac thus reducing MAPK p38 activation following Tumour Necrosis Factor α (TNF α) stimulation (Prickett & Brautigan, 2007). Similarly, Alpha4 directs dephosphorylation of the stress-activated transcription factors c-Jun and p53 (Kong et al., 2004, Saleh et al., 2005,).

1.6.2.2.4 Memory and learning

Alpha4 is also implicated in memory and learning, with a specific form of the protein expressed exclusively in the brain and testis (Maeda et al., 1999). Knockout of murine Alpha4 specifically in the central nervous system reduced learning ability and

impaired memory (Yamashita et al., 2006). In this instance, PP2A_{Alpha4} mediated dephosphorylation of calcium-calmodulin-dependent protein kinase II (CaMKII) was implicated as playing a key role in such processes.

1.6.3 Tip41

1.6.3.1 General introduction

The role of Tip41 within the PP2A_{Alpha4} complex is less well understood. While Tip41 has been identified in complex with Alpha4 and PP2Ac, the exact nature of the interactions taking place has not been conclusively shown, and the question as to whether Tip41 binds Alpha4, PP2Ac or both remains (Smetana & Zanchin, 2007). Tip41 has also been identified as a negative PP2Ac regulator *in vitro* and interaction between Tip41 and PP2Ac is insensitive to treatment with rapamycin (McConnell et al., 2007). Further investigation is therefore required into the role of Tip41 within the PP2A_{Alpha4} complex. Tip41 contains a putative TOS motif and shares considerable sequence identity with the yeast counterpart, although the protein is smaller with a molecular weight of 32kDa, making the protein an ideal target for phosphatase regulation in relation to mTORC1 signalling (Figure 1.5)

1.6.3.2 Functions

Although studies into the role of Tip41 within the PP2A_{Alpha4} complex are inconclusive, other roles for Tip41 have been identified within the ATM/ATR and NF- κ B signalling pathways.

1.6.3.2.1 ATM/ATR

Ataxia-telangiectasia (A-T) is an autosomal recessive disorder with phenotypic characteristics ranging from lymphoid tumours to radiosensitivity, cell cycle checkpoint defects and insulin-resistant diabetes. The disease is caused by a defective *ATM* gene. ATM is a member of the PIKK family of S/T kinases and is involved in DNA damage response. DNA double strand breaks (DSBs) result in ATM association with the MRE11-RAD50-NBS1 (MRN) complex at the damaged site

Figure 1.5: Sequence alignment of mouse, human and yeast Tip41. Protein sequence alignment shows 33% sequence identity maintained between yeast and mammalian (both human and murine) Tip41, and 95% sequence identity between the sequences of human and mouse Tip41. Critically, the 'FEDEL' TOS motif is conserved and is highlighted in green.

(Carney et al., 1998, Varon et al., 1998, Stewart et al., 1999, reviewed in Lavin, 2008).

Full activation of ATM requires post-translational modification via phosphorylation and acetylation. Inactive ATM forms homodimeric complexes where the kinase domain is bound to an internal domain of another ATM molecule (Bakkenist & Kastan, 2003). Following ionising radiation and thus formation of double strand breaks (DSBs), one ATM molecule within the complex phosphorylates the other at S1981 leading to dissociation and activation of the protein. Autophosphorylation appears to be negatively regulated by PP2A (Goodarzi et al., 2004). Okadaic acid leads to an increase in ATM S1981 phosphorylation and in non-irradiated cells ATM associates with the A subunit of PP2A. Thus, PP2A appears to maintain ATM in an unphosphorylated state and DSBs result in dissociation of PP2A allowing autophosphorylation of ATM. Acetylation at Y3016 is also required for dissociation of ATM homodimers (Sun et al., 2007). Mutation of this residue prevents ATM upregulation, and reduces phosphorylation of ATM substrates. The activation of ATM results in phosphorylation of up to 700 proteins involved in DNA replication, DNA repair and cell cycle regulation (Matsuoka et al., 2007), but the principle substrate appears to be p53 (Canman et al., 1998, Moyal et al 1998).

ATM and RAD3-related (ATR) is activated in response to single strand DNA breaks (reviewed in Cimprich & Cortez, 2008) and shares many biochemical and functional similarities to ATM. Replication protein A (RPA) coats most ssDNA within the cell to prevent hairpin formation and protects ssDNA from nucleases (reviewed in Fanning, Klimovich & Nager, 2006), and is recognised by ATR-interacting protein (ATRIP) (Ball et al., 2007). ATR and ATRIP are functionally co-dependent, and loss of expression of either protein leads to complete abrogation of expression and function of the other (Cortez et al., 2001). Full activation of ATR requires the activator topoisomerase-binding protein 1 (TOPBP1), which is recruited by the Rad9-Hus1-Rad1 (9-1-1) complex (Parrilla-Castellar et al., 2004, Kumagai et al., 2006, Delacroix et al., 2007, Lee et al., 2007, Mordes et al., 2008). Activated ATR phosphorylates Checkpoint Homologue 1 (Chk1) which is released from chromatin leading to transmission of DNA damage signals and cell cycle arrest (Smits et al., 2006). ATM appears to partially regulate ATR (Yoo et al., 2007). In response to DSBs in

Xenopus, ATM phosphorylates TOPBP1 at S1131 and strongly enhances interaction of TOPBP1 with ATR. Mutation of S1131 prevents phosphorylation of Chk1 in response to DSBs showing that ATM-mediated phosphorylation of TOPBP1 is required for full activation of ATR. The activation of ATR is therefore closely related to that of ATM.

1.6.3.2.2 *mTORC1 and ATM/ATR*

There are a number of potential links between ATM/ATR signalling and the mTORC1 pathway. Analysis of ATM responsive phosphorylation events revealed an enrichment for substrates within the IGF-1 pathway, including IRS2, TSC1 and 4EBP1 (Matsuoka et al., 2007). Additional evidence comes from studies using *ATM*^{-/-} mice, which provide a mammalian model for A-T (Barlow et al., 1996). Thymocytes derived from *ATM*^{-/-} mice showed increased phosphorylation of 4EBP1 as a consequence of reduced PTEN activity. (Kuang et al., 2009). Of interest, c-Myc was upregulated in these thymocytes as a consequence of increased mTORC1 signalling. Inhibition of mTORC1 with rapamycin attenuates c-Myc expression and reduces the development of lymphomas. This finding infers a model where ATM deregulation results in increased mTORC1 mediated c-Myc expression resulting in malignant thymic lymphomas. Similarly, activation of ATM in breast cancer cell lines decreases 4EBP1 phosphorylation and reduces cap-dependent translation (Braunstein et al., 2009). Regulation of mTORC1, therefore, appears to play a crucial role in signalling downstream of ATM.

Although a direct link between mTORC1 and ATR signalling has not been identified, inhibition of Akt has been shown to activate DNA damage repair via Chk1. Xu *et al.* found reduced association of ATR and RPA to chromatin, and reduced Chk1 phosphorylation, in late G2 arrested cells following radiation-induced DNA damage. Depletion of Akt restored radiation induced Chk1 phosphorylation, whilst a *PTEN*^{-/-} cell line showed attenuation of Chk1 phosphorylation under the same conditions. These data show that Akt activation suppresses ATR mediated Chk1 activation, and may indicate a role for mTORC1 upstream of ATR.

1.6.3.2.3 *Tip41 and ATM/ATR*

The precise role of Tip41 within ATM/ATR signalling is unknown, but an unidentified 33kDa substrate of ATM/ATR showed reduced phosphorylation on knockdown of Tip41 (McConnell et al., 2007). As okadaic acid treatment increased phosphorylation of this substrate, Tip41 appears to sequester PP2A to regulate dephosphorylation towards the unknown protein component within this pathway. Further investigation is clearly necessary to identify the protein and elucidate the role of Tip41 within this pathway.

1.6.3.2.4 *NF-κB*

The NF-κB family of transcriptional regulators is involved in a number of processes including immunity and inflammation (reviewed in Wietek & O'Neill, 2007). There are five members within the family that homo- and hetero-dimerise to form a variety of complexes that recognise specific motifs within various genes. One such dimer, namely p50-p65, is activated in response to IL-1β. Without activating signals, p50-p65 is sequestered in the cytosol in an inactive state by IκB. On binding of IL-1β to the IL-1 Receptor (IL-1R), IKK phosphorylates IκB at specific sites resulting in its ubiquitination and degradation. Following release from the inhibitor, p50-p65 activates target genes (Wietek & O'Neill, 2007).

Activation of IKK involves a complex formation based on polyubiquitin binding. Following stimulation by IL-1β, TRAF6 polyubiquitinates TAK1 leading to the subsequent activation of IKK (Ninomiya-Tsuji et al., 1999, Deng et al., 2000, Sorrentino et al., 2008, Fan et al., 2010). TAB1 binds to and triggers autophosphorylation of TAK1 required for activation (Shibuya et al., 1996, Sakurai et al., 2000). Other members of the complex include TAB2 and TAB3, which mediate binding between TAK1 and TRAF6 by binding polyubiquitin chains (Takesu et al., 2000, Kanayama et al., 2004, Kishida et al., 2005). PP6c is required for inactivation of the complex, which dephosphorylates TAK1 (Kajino et al., 2006). Interestingly, recent findings indicate that whilst TAB2 and TAB3 are redundant in the activation of TAK1, TAB2 plays an additional role in deactivation of TAK1 by recruiting PP6 to the complex (Broglie et al., 2010).

1.6.3.2.5 *mTORC1 and NF-κB*

A link between mTORC1 signalling and the NF- κ B pathway is implicated in the development of insulin resistance due to obesity. Two modes have been identified. Firstly, IKK directly phosphorylates TSC1 at the inactivating sites S487 and S511 resulting in activation of mTORC1 by removal of the inhibitory TSC1/2 complex (Lee et al., 2007). This results in angiogenesis and is associated with increased tumour progression in breast cancer patients. The same mechanism results in insulin resistance due to induction of the negative regulatory feedback downstream of mTORC1. Here, IKK mediated activation of mTORC1 results in phosphorylation of IRS-1 at S307 and S636/639, preventing interaction with PI3-K thus rendering downstream proteins resistant to stimulation by the insulin receptor, which lies upstream (Hartley & Cooper, 2002, Lee et al., 2008). The second link between NF- κ B and insulin resistance stems from the ability of IKK to directly phosphorylate IRS-1 at S312 (Gao et al., 2002). This results in proteasomal degradation of IRS-1 following ubiquitination (Zhande et al., 2002, Greene et al., 2003).

IKK also binds mTOR directly and appears to act both up- and downstream of the mTORC1 complex. In PTEN null prostate cancer cells, IKK associates with mTOR and knockdown of IKK results in a decrease of mTOR activity (Dan et al., 2007). Thus IKK appears to activate mTOR downstream of Akt. In the same cell line, the interaction between mTOR and IKK results in IKK and subsequent NF- κ B activation (Dan et al., 2008). In addition, rapamycin inhibits NF- κ B activation, providing further evidence for a role of IKK downstream of mTOR. Thus IKK binds mTOR leading to activation of mTORC1 activity, whilst also resulting in activation of the protein itself.

1.6.3.2.6 Tip41 and NF- κ B

Tip41 has recently been renamed TAB4, due to its identification as a novel member of the TAK1 activation complex (Prickett et al., 2008). On expression of HA-Tip41, autophosphorylation of FLAG-TAK1 (but not endogenous) is induced resulting in increased activation. Mutation analysis showed that Tip41 binds ubiquitinated TAK1 via conserved Phe²⁵⁴Pro²⁵⁵ motif, also found in TAB2 and TAB3. Interestingly, although Tip41 directly binds TAK1, an overlay assay showed that binding was dependent on the phosphorylation state of TAK1. A 'Hit and Run' model was hypothesised, where Tip41 binds inactive (dephosphorylated) TAK1 to cause autophosphorylation of TAK1 and subsequent release of Tip41 from the complex

(Prickett et al., 2008). It is important to note that in this study, overexpressed HA-Tip41 failed to influence NF- κ B activation in the absence of co-expressed FLAG-TAK1. Thus Tip41 overexpression does not affect endogenous NF- κ B signalling when expressed alone.

1.6.4 B α

1.6.4.1 Inhibition by E4ORF4

DNA tumour viruses have historically been useful in uncovering mechanisms of phosphatase regulation in the cell. As viruses must overcome checkpoints to facilitate replication, this often means inhibiting regulatory phosphatase activity. For example, the SV40 small T antigen binds and forms a stable complex with PP2Ac leading to release of the regulatory B subunit and enhanced cell survival (reviewed in Branton & Roopchand, 2001). The adenovirus is able to maintain protein synthesis within the cell even in PBS (O'Shea et al., 2005(b)). Two proteins encoded by the virus achieve this, namely Early Region 4 Open Reading Frame 1 (E4ORF1) and E4ORF4. As a master regulator of translation, mTORC1 is targeted by both these viral proteins, as expression of E4ORF1 and E4ORF4 result in increased phosphorylation of S6K1 and 4EBP1, and rapamycin inhibits viral replication (O'Shea et al., 2005(a), Li et al., 2009). The method of E4ORF1 action is relatively simple, as it binds and activates PI3K to increase GTP-loading of Rheb (O'Shea et al., 2005(a)). E4ORF4 on the other hand interacts with both the B α regulatory subunit of PP2Ac and cellular Sarcoma (c-Src).

1.6.4.2 Functions

1.6.4.2.1 Regulation of mTORC1

As E4ORF4 binds B α resulting in enhanced phosphorylation of mTORC1 substrates, it was proposed that PP2A_{B α} may be involved in attenuation of mTORC1 signalling on removal of stimulatory signals. E4ORF4 is a small 114 residue polypeptide and deletion of even small portions reduces stability indicative of considerable tertiary structure (reviewed in Branton & Roopchand, 2001). As E4ORF4-bound PP2A_{B α} still retains catalytic activity towards the peptide substrate, modulation of PP2Ac activity may take place by inhibition of substrate interaction (Li et al., 2009).

Binding of E4ORF4 to B α does not alter Rheb GTP-loading and mutants that don't bind B α (Δ 359 and L51/54A) don't induce phosphorylation of S6K1 and don't associate with PP2A α . E4ORF4-mediated activation of S6K1 is sensitive to rapamycin. Therefore, E4ORF4 appears to act in a parallel pathway to PI3K, upstream of mTORC1 (O'Shea et al., 2005(a)). This notion is backed up as both E4ORF1 and E4ORF4 are required for full activation of S6K1 (O'Shea et al., 2005(a)). Whereas E4ORF1 is required for Rheb-induced activation of mTORC1, PP2A α may act within the amino acid induced activation of mTORC1 which does not alter Rheb loading but localises mTORC1 to membranes where it may be activated by Rheb (O'Shea et al., 2005(a)). Studies using E4ORF4 have therefore convincingly implicated PP2A α in inhibition of signalling to mTORC1.

A little understood function of E4ORF4 inhibition of PP2A α also results in downregulation of cellular Myelocytomatosis (c-Myc) protein and transcription (Ben-Israel et al., 2008). The transcription factor c-Myc is activated by growth factors via the Ras/MEK/ERK pathway and activates genes involved in proliferation, survival and angiogenesis (reviewed in Wolfer & Ramaswamy, 2011). As mTORC1 is known to activate translation of c-Myc, inhibition by E4ORF4 appears contradictory and therefore requires further investigation (West et al., 1998).

1.6.4.2.2 *Cdk1*

An additional role of B α in cell cycle regulation was also uncovered using E4ORF4. The cell cycle is controlled by cyclin-Cdk complexes, and different cyclins are associated with different phases of the cell cycle. For example, the G1 phase is controlled by cyclin D whereas DNA replication in the S phase is controlled by cyclin A and cyclin B. Mitosis is controlled by cyclins B and A. Mitotic exit requires ubiquitination of cyclin B which is achieved by the E3 ligase anaphase promoting complex (APC). This is activated on the onset of anaphase. APC recognises substrates via the subunits Cdc20 and Cadherin 1 (Cdh1) which are activated by Cdk1. When all chromosomes are positioned on spindle microtubules Cdc20 is activated leading to degradation of the separase inhibitor securin. This allows separase-mediated cleavage of sister chromatids and exit from mitosis. Following mitosis, APC interacts with Hypertrichosis 1 (Htc1) which is inhibited by Cdk1. This

ensures that on onset of the following cell cycle, APC is inhibited until its activity is required again (reviewed in Murray, 2004).

On over-expression of E4ORF4, Cdk1 activity is increased and knockdown of B α resulted in cell cycle arrest at G2 to M transition (Li et al., 2009). E4ORF4 therefore activates Cdk1 by inhibiting PP2A_{B α} in order to control the cell cycle. During mitosis, B α is phosphorylated at S167 resulting in reduced binding to the A and PP2Ac subunits. This reduces PP2A_{B α} activity towards Cdk1 phosphorylated substrates (Schmitz et al., 2010). On exit of mitosis, PP2A_{B α} dephosphorylates Cdk1 substrates downstream of Cdc20, which is required for mitotic exit (Manchado et al., 2010).

In yeast, E4ORF4 affects the spindle assembly checkpoint by binding APC and recruiting the Pph21/22-Cdc55 complex (Kornitzer et al., 2001). PP2A_{B α} may therefore control mitotic exit by dephosphorylating the activating Cdc20 and Cdh1 subunits of APC thus inhibiting Cdk1 activated mitotic exit. This role of PP2A_{B α} is concurrent with a role of nuclear PP2A_{B α} in a cellular checkpoint induced by ionising radiation. Ionising radiation decreases PP2A_{B α} trimer in the nucleus, which is prevented in ATM deficient cells (Guo et al., 2002). Loss of PP2A_{B α} may therefore trigger a cell cycle/DNA damage checkpoint.

1.6.4.2.3 Wnt signalling

PP2A_{B α} is also implicated in inhibition of canonical Wnt signalling. Without upstream stimuli, GSK3 β in complex with adenomatous polyposis coli, axin and PP2A phosphorylate β catenin leading to its ubiquitination and degradation. Binding of the Wnt ligand to the cognate receptor leads to inhibition of GSK3 β by Dishevelled leading to accumulation of β catenin, which interacts with T cell factor transcription factors to activate transcription from promoters with TCF sites (reviewed in Smalley & Dale, 1999). Knockdown of PP2Ac or B α increases β catenin phosphorylation at S552 and S675, highlighting an activating role of PP2A_{B α} in canonical Wnt signalling (Zhang et al., 2009). Thus PP2A_{B α} may be involved in β catenin dephosphorylation allowing accumulation of the protein and transcription of TCF genes.

1.6.5 B'

More recently, another PP2Ac regulatory subunit has been implicated in regulation of S6K1 activity downstream of mTORC1. Flies with mutated B' regulatory PP2Ac subunit developed a phenotype indicative of defective insulin signalling (Hahn et al., 2010). Tissue staining showed elevated S6K1 phosphorylation with no alteration to Akt or 4EBP1 phosphorylation. Investigations using HeLa cells mirrored these results, as cells with mutated *PPP2R5D* (human B') had higher levels of S6K1 phosphorylation with no change in the phosphorylation status of 4EBP1. Direct interaction between S6K1 and B' was also demonstrated. It is probable that B' acts as the regulatory PP2Ac subunit directing phosphatase activity towards S6K1 downstream of mTORC1.

1.6.6 B β

PP2AB β has also been implicated in regulation of substrate phosphorylation downstream of mTORC1, as discussed in Section 1.5.10.5.

1.6.7 PP6c

The potential role of PP6c in mTORC1 signalling has not been investigated extensively. Calyculin A is a general PP2A and PP1 phosphatase inhibitor. Treatment with the drug has been shown to increase S6K1 phosphorylation, therefore a role for the other PP2A family members, such as PP6, cannot be discounted (Bielinski & Mumby, 2007, Peterson et al., 1999).

1.6.7.1 Structure

PP6c was discovered as the functional homologue of yeast Sit4 (Table 1.1), with a role in cell cycle regulation (Bastians & Ponstingl, 1996, Stefansson & Brautigan, 2007). Later, a number of regulatory and scaffold subunits were identified to comprise the PP6 holoenzyme. The scaffold subunits of PP6 include PP6 regulatory subunits 1 (PP6R1), 2 and 3. These are orthologues of the Sit4 associated proteins (SAPs) in yeast which constitutes the regulatory subunits of the phosphatase. PP6R2 and PP6R3 expression in yeast can rescue the phenotype observed in knockout of all SAP proteins (Morales-Johansson et al., 2009). Preliminary data indicate the role of PP6R proteins to be as a scaffold, connecting PP6c to the

regulatory subunits. Analysis of PP6R sequences predicts that a high degree of alpha helix formation allows presentation of residues required for protein-protein interaction within the PP6 holoenzyme (Guerignon et al., 2009).

The regulatory subunits of PP6c include three ankyrin repeat proteins, Ankrd28, Ankrd44 and Ankrd52. The three proteins share extensive sequence identity although they derive from separate branches of the phylogenetic tree. Ankrd28 was found to mediate the function and specificity of PP6c, and interacts with all three PP6R proteins in addition to PP6c. As the C terminus of PP6R1 bound Ankrd28 but not PP6, this provided evidence for the role of PP6R proteins as scaffold subunits (Stefansson et al., 2008).

1.6.7.2 Functions

1.6.7.2.1 NF- κ B

PP6 is involved in attenuation of signalling to NF- κ B. Activation of NF- κ B requires phosphorylation and subsequent degradation of its inhibitor I κ B by IKK. Activation of IKK involves the TAK1 complex. PP6 associates with TAK1 and knockdown of PP6 reduced IL-1 induced activation of TAK1 measured by phosphorylation at T187 (Kajino et al., 2006). PP6 is therefore involved in reducing signalling to NF- κ B on removal of cytokine signals. Knockdown of PP6R1 and Ankrd28 prevented the TNF α induced degradation of I κ B, indicating that both subunits form part of the PP6 complex involved in TAK1 dephosphorylation, supporting the evidence of these subunits in a PP6 holozyne (Stefansson et al., 2008).

1.6.7.2.2 DNA repair

The identification of PP6 as a binding protein of DNA protein kinase (DNA-PK) following ionising radiation implicated PP6 in repair of DSBs. Repair of DSBs can take place via non-homologous end joining (NHEJ) or homologous repair. In the case of NHEJ, DSBs are detected by the Ku70/80 heterodimer which leads to the recruitment of DNA-PK. Like mTOR, DNA-PK is a PIKK. DNA-PK binds to broken DNA ends where kinase activity is activated. One DNA-PK binds at each free end of DNA at the break. Interaction between the DNA-PKs via HEAT repeats is required to align the two strands for repair.

Autophosphorylation of DNA-PK leads to conformational change and dissociation of DNA-PK allowing other DNA repair proteins to access the damaged site. H2AX is also a substrate for DNA-PK. Phosphorylated H2AX acts as a recruitment platform for other proteins involved in the DNA damage response (reviewed in Dobbs et al., 2010).

Ionising radiation induces DSBs which promotes translocation of DNA-PK to the nucleus. PP6c, PP6R1, PP6R2 and PP6R3 bind to DNA-PK, and translocate to the nucleus with DNA-PK following ionising radiation. In cells lacking DNA-PK, the level of PP6 in the nucleus is reduced. PP6 appears to be required for the activation of DNA-PK as knockdown of PP6R1 or PP6 reduces activation of DNA-PK following ionising radiation (Mi et al., 2009). Phosphorylation of H2AX is sustained on knockdown of PP6 or PP6R1 following ionising radiation (Douglas et al., 2010). PP6 therefore appears to translocate to the nucleus with DNA-PK on DSB formation and locate to the site of DSB, where it may be involved in dephosphorylation of DNA-PK substrates following repair of the DSB.

1.7 mTORC2

1.7.1 The mTORC2 complex

The mTORC2 complex consists of mTOR along with Raptor Independent Companion of mTORC2 (Rictor), mLST8 and mammalian Stress-Activated Protein Kinase Interacting Protein 1 (mSin1). Protein Observed with Rictor (Protector) and Heat Shock Protein 70 (Hsp70) have also been observed in complex with mTORC2. Similarly to mTORC1, mTORC2 is activated by PI3K via an unknown mechanism, although in contrast, activity is unaffected by amino acids (Pearce et al., 2007). The principle substrate of mTORC2 is Akt, which is phosphorylated at S473 in response to mTORC2 activation (Sarbasov et al., 2005). Full activation of Akt requires additional phosphorylation at T308 by PDK1. Phosphorylation of the hydrophobic motif of Akt by mTORC2 provides a docking site for PDK1 to phosphorylate the activation loop. Thus mTORC2 phosphorylation of Akt primes the kinase for full activation by PDK1 (Bondi, 2004). Concurrent with this, pre-incubation of Akt with mTORC2 in vitro boosts PDK1 mediated activation (Sarbasov et al., 2005).

Dephosphorylation of Akt may be mediated by protein phosphatase 1 (PP1) (Gupta et al., 2009).

1.7.1.1 *Rictor and mSin1*

Rictor and mSin1 are exclusive members of the mTORC2 complex. Substrate binding may be mediated by mSin1, which binds Akt directly and is required for phosphorylation at S473 (Jacinto et al., 2006). Five mSin1 isoforms exist due to alternative splicing, although only isoforms 1,2 and 5 bind mTORC2. Knockdown of mSin1 results in reduced phosphorylation of Akt, highlighting the importance of mSin1 in mTORC2 kinetics.

The three mTORC2-binding isoforms of mSin1 form individual complexes. Isoforms 1 and 2 form complexes that are responsive to insulin and PI3K signalling, whereas mTORC2 complexes containing isoform 5 are not. Activation of mTORC2 in response to upstream signals may therefore be controlled by response from mSin1. The activation of mTORC2 containing mSin1 isoform 5 is unknown, but may involve localisation of mTORC2 mediated by PIP3 in order to phosphorylate Akt (Frias et al., 2006).

1.7.1.2 *mLST8*

Although mLST8 is also considered a member of the mTORC1 complex, studies using mouse models implicate a more central role in mTORC2 activity. The mLST8 knockout mouse has a similar phenotype to the Rictor but not Raptor knockout model. In addition, knockdown of mLST8 prevents interaction between mTOR-Rictor and results in a mobility shift of Rictor (Guertin et al., 2006). An explanation for this may therefore be that knockdown of mLST8 may result in accumulation of free Rictor resulting in targeting for proteasomal degradation by ubiquitination.

1.7.1.3 *Protor*

Protor is a novel component of mTORC2 with a currently unknown function (Pearce et al., 2007).

1.7.1.4 Hsp70

Hsp70 has been implicated in the mTORC2 signalling pathway. Heat shock proteins are induced in response to a wide variety of stressors including temperature, oxidative stress and UV radiation. They are grouped according to molecular weight, with the best characterised group being Hsp70. Both soluble and membrane bound forms of the protein exist, and many functions have been ascribed including assisting in protein folding, preventing aggregation and membrane transport (Shin et al., 2003, Arispe et al., 2004, Horvath et al., 2008).

Immunoprecipitation studies identified a temperature sensitive interaction between Rictor and Hsp70. Further studies found that knockdown of Hsp70 reduced Akt phosphorylation at S473, the mTORC2 phosphorylation site (Martin et al., 2008), and that mTORC2 mediated Akt phosphorylation is also sensitive to heat shock (Oehler-Janne et al., 2008). Hsp70 therefore appears to positively regulate mTORC2 phosphorylation of Akt, in a temperature sensitive manner.

1.7.2 Functions of mTORC2

1.7.2.1 Actin remodelling

Disruption of mTORC2 signalling interferes with actin remodelling via PKC along with the GTPases Rho and Rac. Knockdown of Rictor results in morphological defects due to F-actin defects. Normal filamentous (F) actin is distributed throughout the cytosol and at the cell corex. Following Rictor knockdown, thick actin fibres are present in the cytosol with few at the cell cortex. In addition paxillin phosphorylation is reduced and is present at the ends of the thick F-actin fibres rather than at focal adhesions. Knockdown of PKC results in a similar phenotype to Rictor knockdown indicating that the defect in F-actin seen on knockdown of Rictor may be caused by defective PKC activation (Sarbasov et al., 2004). Although similar, the phenotype is not identical indicating that mTORC2 has other substrates involved in actin remodelling.

The Rho and Rac GTPases regulate actin assembly and disassembly. Knockdown of Rictor results in decreased GTP-bound Rho and Rac whereas constitutively active Rho and Rac relieve the defect in F-actin seen on knockdown of Rictor (Jacinto et al., 2004). This suggests that mTORC2 leads to the accumulation of GTP-bound Rho

and Rac resulting in F-actin remodelling. By controlling remodelling of the cytoskeleton, mTORC2 promotes cell migration.

1.7.2.2 Angiogenesis

Prostaglandin E2 (PGE2) promotes angiogenesis in endothelial cells, partly by inducing cell migration, and also induces activity of Rac via mTORC2. Knockdown of Rictor prevented endothelial cell migration induced by PGE2, and reversed PGE2 induced Rac activity (Dada et al., 2008). Angiogenesis is therefore another process positively regulated by mTORC2.

1.7.2.3 Akt

Other processes downstream of mTORC2 are mediated by activation of Akt, which has numerous substrates in the cell. Although the best characterised substrate is GSK3, phosphorylation is not activated by mTORC2 (Guertin et al., 2006). Substrates of Akt that are phosphorylated in response to mTORC2 activation include the FOXO transcription factors. Akt phosphorylates FOXO1, FOXO3a and FOXO4 in the nucleus leading to 14-3-3 binding and translocation to the cytosol. This prevents the transcription of pro-apoptotic genes (reviewed in Manning & Cantley, 2007). As an activator of Akt, the mTORC2 complex is well-placed to encourage tumorigenesis. Indeed glioma have a high incidence of Akt activation. In glioma cell lines and primary tumour cells, Rictor mRNA and protein levels are elevated, concurrent with increased mTORC2 activity and increased anchorage dependent growth (Masri et al., 2007).

1.7.3 Regulation of mTORC2

1.7.3.1 Feedback from mTORC1

A negative feedback loop between mTORC1 and mTORC2 places Rictor downstream of S6K1. Phosphorylation of Rictor at T1135 reduces phosphorylation of Akt at S473 and promotes Rictor binding to 14-3-3. Expression of Rictor mutant T1135A prevents inhibition, indicating that the phosphorylation event is inhibitory to mTORC2 (Dibble et al., 2009). This therefore provides a method of downregulating Akt in response to mTORC1 activation to prevent aberrant activation of Akt signalling.

1.7.3.2 TSC1/2

Although the inhibition of mTORC1 by TSC1/2 is well documented, recent evidence shows that the complex activates mTORC2. *TSC2*^{-/-} MEFs have reduced activity of mTORC2, without any defect in mTOR-Rictor binding, which is unresponsive to insulin. Raptor knockdown did not restore mTORC2 activity therefore inhibition of mTORC2 activity appeared to be a direct consequence of TSC2 loss, not due to a negative feedback loop involving mTORC1. Overexpression of a TSC2 GAP mutant had no effect on mTORC2, indicating the effect of TSC1/2 is independent of the GTPase activating function of TSC2. In addition, mTORC2 was found to bind directly to TSC2. Thus the TSC1/2 complex is required for activation of mTORC2, mediated by direct binding, providing another mechanism for activation of Akt (Huang et al., 2008). These findings may partly explain why *TSC1* and *TSC2* are not associated with malignancy in human cancer.

1.8 PP2A and disease

The extensive nature of PP2A regulation indicates the importance of the protein within the cell, and indeed dysregulation is implicated within many pathologies. For instance, alteration of PP2A function has been found within many cancer types, usually resulting in a removal of PP2A function leading to hyperphosphorylation of oncoproteins.

1.8.1 Genetic disease

1.8.1.1 Opitz Syndrome

OS is an X-linked syndrome caused by mutations in the *MID1* gene. The disease is characterised by diverse symptoms including cleft palate, heart defects and mental retardation. By mediating interaction between Midline1 and PP2A, Alpha4 plays a crucial role in some cases of OS. Mutations of Midline1 that prevent interaction with PP2A_{Alpha4} result in the microtubule destabilisation that underlies the pathogenic phenotype of the disease (Troddenbacher et al., 2001). In support of this, Alpha4 expression in the developing mouse embryo coincides with tissues affected by OS, such as the heart and brain (Everett & Brautigan, 2002). In addition, Midline1 protein that is deficient in Alpha4 binding could not move along MTs and permanent phosphorylation of S96 within Midline1 had the same effect (Aranda-Orgilles et al., 2008). OS patient mutations within the Alpha4 binding site may therefore be pathogenic due to reduced migration of Midline1 along MTs.

1.8.2 Neurological disease

1.8.2.1 Alzheimer's Disease

The implication of PP2A in the pathogenesis of AD is also well documented. Within the AD brain reside a number of extracellular senile plaques made up of amyloid β -peptide ($A\beta$ - a fragment of amyloid precursor protein) along with hyperphosphorylated Tau leading to the formation of neurofibrillary tangles within neurons. In healthy brain tissue, Tau stabilises MTs and is regulated by phosphorylation, which decreases with age. In the AD brain Tau is abnormally hyperphosphorylated. Tau phosphorylation is controlled by GSK3 β and PP2A, where GSK3 β phosphorylates Tau within its MT binding domain and prevents interaction with MTs leading to their decreased stability (Martin et al., 2009). An unidentified PP2A complex dephosphorylates GSK3 β in order to maintain kinase activity (Qian et al., 2010).

Tau phosphorylation is controlled by PP2A_{B α} and dysfunction of the protein is implicated in AD progression. PP2A_{B α} dephosphorylates Tau *in vitro* and the PP2A binding site within Tau has been uncovered (Xu et al., 2008). The expression of PP2Ac L309A defective in methylation and therefore B α binding results in MT destabilisation, as does knockdown of B α , indicating a crucial role of PP2A_{B α} in AD pathogenesis (Evans & Hemmings, 2000, Nunbhakdi-Craig et al., 2007). Within the pR5 mouse model of AD, expression of methylation deficient PP2Ac L309A exacerbated the formation of neurofibrillary tangles (Deters et al., 2009). In addition, within rat brain slices PP2A was found to reduce Tau phosphorylation directly at several phosphorylation sites (Qian et al., 2010).

The identification of AD as a state of insulin resistance within the brain has led to the term 'Type III diabetes' being used to describe the disease (Castri et al., 2003, Gupta et al., 2011,). The two presenting pathologies of AD are linked via the insulin signalling pathway which, when active, results in inactivation of GSK3 β . $A\beta$ prevents GSK3 β inactivation via Akt by acting as an insulin antagonist where the peptide binds the receptor and interferes with its autophosphorylation (Townsend et al., 2007, Lee et al., 2009, Hernandez et al., 2010,). Further evidence for the role of insulin

signalling in AD comes from cultured mouse neurons where inhibition of PI3K/mTORC1 signalling with LY294002 increased PP2A and GSK3 β activity towards Tau (Meske et al., 2008). Insulin stimulation had the reverse effect. In addition the AMPK-activating drug metformin reverses the neuropathological change seen in an AD model and induces Tau dephosphorylation by PP2A via inhibition of mTORC1 signalling (Gupta et al., 2011, Kickstein et al., 2010). Thus hyperactive insulin signalling is critical in the development of AD.

Activation of insulin signalling also increases translation of Tau via mTORC1. Analysis of AD brains showed higher phosphorylation of eEF2 and 4EBP1 than in control samples, indicating that mTORC1 is hyperactive in AD. As one theory for the formation of neurofibrillary tangles is that they develop due to increased translation of Tau, this provides evidence that increased activity of mTORC1 may cause this phenomenon (Li et al., 2005). Therefore the development of neurofibrillary tangles may be caused by increased translation of Tau, caused by hyperactivation of mTORC1.

PP2A is also central in acting between A β interference at the cell surface and the hyperphosphorylation of Tau within neurons. The presence of A β reduced PP2A_C methylation and therefore activity of the PP2A_{B α} holoenzyme was observed within human and murine brain samples (Zhou et al., 2008). In addition to studies with E4ORF4 (O'Shea et al., 2005(a)) this provides evidence that PP2A_{B α} acts within the insulin signalling pathway, as PP2A_{B α} is also affected by A β and contributes to Tau hyperphosphorylation.

1.8.3 Cancer

Mutations within the A subunit of PP2A appear to be particularly common in cancerous cells, being implicated in melanoma along with breast, lung and colon cancers. Cancerous mutations to the A subunit result in an inability to bind the B' regulatory subunit (Calin et al., 2000, Reudiger et al., 2001(a), Esplin et al., 2006) whilst those found in lung and colon cancer prevent binding of the B'' or C subunits (Wang et al., 1998, Calin et al., 2000, Takegi et al., 2000, Ruediger et al., 2001(b)).

All of these result in removal of PP2A activity and thus hyperphosphorylation of target proteins.

A pathogenic mutation within the A subunit of PP2A of particular importance to mTORC1 signalling is the mutation resulting in defective binding to RalA. Growth factor activation results in the accumulation of active GTP-RalA which promotes receptor mediated endocytosis, secretion and the formation of filopodia (Feig, 2003). RalA activates mTORC1 signalling to S6K1 (Maehama et al., 2008) and its activity is controlled via dephosphorylation by PP2A. Cancer-associated mutations in the A subunit of PP2A reduce binding to RalA, resulting in constitutive activation of RalA and downstream signalling, including mTORC1, and cellular transformation (Sablina et al., 2007).

Alteration to B regulatory subunits within cancerous cells is thought to occur due to changes in miRNA levels. Within lung and hepatic cell carcinoma levels of miRNA31 and miRNA222, both of which degrade B α , are increased leading to lower availability of the regulatory subunit and finally hyperphosphorylation of PP2A_{B α} targets (Liu et al., 2010, Wong et al., 2010). The increased availability of both these miRNAs was associated with increased tumorigenicity and reduced life expectancy, highlighting the importance of PP2A regulation within the cell.

Mutations to PP2Ac are less frequently encountered within cancer, although a short nucleotide polymorphism has been found that increases the risk of breast cancer (Dupont et al., 2010). This is probably due to the essential nature of PP2Ac within the cell, and loss of function mutation would almost certainly result in cell death.

1.9 Diseases and mTORC1

1.9.1 Genetic disease

1.9.1.1 Tuberous Sclerosis Complex

Mutations of *TSC1* and *TSC2* that affect interaction between the proteins are found in patients with the disease Tuberous Sclerosis Complex (TSC) (Hodges et al., 2007). TSC is characterised by the development of hamartomas in the brain, heart, skin and kidneys. Mental retardation, autism and epilepsy are common

manifestations of the disease, in addition to the diagnostic markers including facial angiofibromas and calcified retinal hamartomas.

Familial TSC is caused by mutation of *TSC1* or *TSC2* in equal measures, as opposed to sporadic TSC in which *TSC2* mutation is five times more common. Although no link between the point of mutation and severity of the disease has been documented, increased severity of TSC is associated with mutations within *TSC2*. Increased risk is possibly due to a higher rate of secondary hits in the *TSC2* gene as it is larger. TSC patients carry a mutation in a single copy of either the *TSC1* or *TSC2* gene. Tumour development is thought to arise following a second hit event resulting in loss of heterozygosity.

1.9.1.2 Lymphangi leiomyomatosis

LAM is a TSC-associated disease and develops in approximately 30% of women with TSC. It is characterised by proliferation of abnormal smooth muscle cells and cyst formation in the lungs (reviewed in Inoki et al., 2005, Tee & Blenis, 2005, Rosner et al., 2008).

1.9.1.3 von Hippel-Lindau disease

The VHL protein mediates the degradation of HIF1. Mutation of *VHL* occurs in the namesake von Hippel-Lindau disease, resulting in aberrant accumulation of HIF1 and deregulation of HIF1 responsive genes. The disease is characterised by tumour formation in the brain, retina, kidneys and pancreas (reviewed in Inoki et al., 2005, Tee & Blenis, 2005, Rosner et al., 2008).

1.9.1.4 PTEN syndromes

Activity of PI3K is counteracted by PTEN which converts PIP_3 to PIP_2 (Maehama & Dixon, 1998). PTEN-hamartoma syndromes include Cowden syndrome, Bannayan-Riley-Ruvalcaba syndrome, Proteus syndrome and Lhermite-Duclos disease. All are autosomal dominant and are caused by loss of tumour suppressor function of PTEN. The site of tumour formation varies widely, but hyperactivation of Akt provides a common feature (reviewed in Inoki et al., 2005, Tee & Blenis, 2005, Rosner et al., 2008).

1.9.1.5 Peutz- Jeghers syndrome

Peutz- Jeghers syndrome (PJS) is caused by mutation in the *LKB1* gene and results in formation of hamartomas in the gastrointestinal tract, along with clinical features overlapping with those of TSC. This is unsurprising as loss of inhibitory MAPK via *LKB1* loss of function removes mTOR repression in response to AMP accumulation (reviewed in Inoki et al., 2005, Tee & Blenis, 2005, Rosner et al., 2008). Both TSC and PJS tumours therefore comprise deregulated mTORC1.

1.9.2 Neurological disease

Dysregulation of proteasomal degradation and autophagy are becoming increasingly recognised in the development of neurological diseases. This is often due to accumulation of aggregated proteins, leading to neuronal cell death. As an inhibitor of autophagy, inhibition of mTORC1 may be of therapeutic benefit for the treatment of such diseases by upregulating degradation of accumulated proteins.

1.9.2.1 Parkinson's disease

Parkinson's disease arises due to mutation of the α -synuclein gene, associated with an increase in the intracellular concentration of both mutant and wild type α -synuclein. This is caused by the inability of the cell to degrade mutant α -synuclein, as the protein cannot translocate into lysosomes. Instead mutant α -synuclein binds to lysosomal membrane receptors, thus blocking the receptors and preventing interaction with other proteins, including wild type α -synuclein (reviewed in Pan et al., 2008). As rapamycin has been shown to result in degradation of α -synuclein in cell models (Webb et al., 2003), inhibition of mTORC1 could provide a potential therapy for Parkinson's disease.

1.9.2.2 Huntington Disease

An autosomal dominant disorder, Huntington disease (HD) is caused by polyglutamine expansion at the N terminus of huntingtin protein. The N terminal repeats are cleaved from huntingtin and aggregate with a number of proteins within the cell, including transcription factors, leading to dysregulation of transcription (reviewed in Rubinsztein, 2002). Rapamycin may provide a potential therapy for HD as a derivative of the drug was found to enhance clearance of aggregated polyglutamine within the cell by upregulation of autophagy (Ravikumar et al., 2004).

1.9.3 Intractable epilepsy

Cortical dysplasia (CD) in children is associated with development of intractable epilepsy, which is unresponsive to antiepileptic medication and instead requires surgery to remove abnormal cells. Although a variety of abnormal cell types have been observed in CD, increased cell size provides a common link between them. As mTORC1 controls cell size via S6K1 (Ruvinsky et al., 2005), CD cells were analysed for activation of mTORC1 substrates and indeed S6 was found to be hyperphosphorylated (Ljungberg et al., 2006). Inhibition of mTORC1 could therefore be used as a therapy to treat intractable epilepsy in children.

1.9.4 Type II diabetes

Activation of the negative feedback loop is at the root of insulin resistant Type II diabetes. Initial insulin resistance is met with an increase in insulin production by pancreatic β -cells resulting in hyperinsulinaemia. Type II diabetes occurs when this fails to rescue the uptake of glucose in adipose and muscle tissue. Skeletal muscle biopsies from patients with Type II diabetes showed reduced association between IRS-1 and PI3K concurrent with increased phosphorylation of IRS-1 at S636 (Bouzakri et al., 2003). Further evidence stems from studies using *S6K1*^{-/-} mice, which remain sensitive to insulin due to reduced serine phosphorylation of IRS-1 (Um et al., 2004). These mice are also resistant to IRS-1 phosphorylation at S1101 induced by high fat diet (Tremblay et al., 2007).

In human L6 muscle cells, chronic activation of mTORC1 using amino acids reduced IRS-1 protein and reduced glucose uptake in response to insulin (Tremblay & Marette, 2001 & Priola et al., 2003). Berg *et al.* also found that in 3T3-L1 adipocytes, rapamycin rescues the insulin induced increase in Akt phosphorylation, resulting in improved glucose uptake. Thus hyperactivation of mTORC1 results in IRS-1 phosphorylation and degradation leading to the reduced glucose uptake underpinning Type II diabetes.

In addition, chronic activation of mTORC1 leads to reduced membrane translocation of the Glucose Transporter 4 (GLUT4) (Taha et al., 1999, Gaster et al., 2001, Garcia-Souza et al., 2008). This is associated with glucose-mediated tissue damage

observed in patients with Type II diabetes. Therefore dysregulation of insulin signalling underpins the pathological phenotype of Type II diabetes due to the inability of the cell to respond to insulin.

1.9.4.1 Hsp70 and diabetes

Hsp70 has been shown to interact with mTORC2. Expression of Hsp70 is altered in patients with Type II diabetes, although there is deliberation as to whether levels are higher or lower (Kurucz et al., 2002, Bruce et al., 2003, Chung et al., 2008, Nakhjavani et al., 2010). Recent murine and primate studies have found that increased serum Hsp70 is associated with a reduction in insulin resistance (Chung et al., 2008, Kavanagh et al., 2011). In addition, SNPs within Hsp70 (Bouassida et al., 2004) and its regulatory region (Marucci et al., 2009) are associated with increased risk of developing Type II diabetes, with the latter reducing mRNA stability.

Two explanations exist as to why Hsp70 may be involved in the development of Type II diabetes. The first involves the membrane protein ectonucleotide pyrophosphatase phosphodiesterase 1 or plasma cell membrane glycoprotein-1 (ENPP1 or PC-1), an inhibitor of insulin signalling. Knockdown of ENPP1 increases insulin sensitivity and glucose tolerance in a mouse model, whilst mutating the pyrophosphatase/phosphodiesterase domain prevents inhibition of insulin signalling in HEK293 cells (Chin et al., 2009, Zhou et al., 2009). Therapeutic intervention of ENPP1 is therefore being explored as a method of controlling Type II diabetes. Hsp70 binds the 3'UTR of ENPP1 mRNA thus stabilising the transcript and increasing levels of ENPP1 protein. This in turn reduces IR and IR-1 phosphorylation (Marucci et al., 2009). Hsp70 therefore appears to negatively regulate insulin signalling by increasing levels of ENPP1.

The second explanation for the association between Hsp70 and Type II diabetes involves the more typical role of Hsp70 in protein folding. Misfolding of the islet β -cell peptide human amylin into oligomers is linked with the pathogenesis of Type II diabetes. This is prevented by Hsp70, which can seemingly detect misfolded human amylin (Chien et al., 2010). Thus in conflict with the first role, Hsp70 in this instance appears to prevent pathogenesis of Type II diabetes.

1.9.5 Cancer

Upregulation of mTORC1 is observed in a number of human cancers. Upregulated S6K1 is a marker for mTORC1 activation. Therefore in order to uncover whether mTORC1 is upregulated in cancer cells, S6K1 activity is assayed. Indeed, analysis of breast cancer biopsies showed upregulation of S6K1. This was associated with poor prognosis, indicating that upregulation of mTORC1 in breast carcinoma results in aggressive phenotype of the disease (Barlund et al., 2000). Therefore mTORC1 may play an important role in the progression of breast cancer. Indeed phosphorylation of mTOR itself is associated with poor prognosis in colon cancer (Slattery et al., 2010).

1.9.5.1 TSC1/2

Mutations in *TSC1* and *TSC2* have been correlated with a number of human cancers. In a sample of pancreatic cancers, 57% were negative for *TSC2* expression, which was associated with a more aggressive phenotype and poor survival rate (Kataoka et al., 2005). Loss of *TSC2* was seen in 13% of endometrial carcinomas in one study, and in the remaining samples phosphorylation of *TSC2* at S939 was observed. This indicates that activation of mTORC1 is a key step in the development of endometrial carcinoma (Lu et al., 2008). Mutation of *TSC2* was also associated with colon cancer specifically (Slattery et al., 2010). The level of *TSC2* mRNA was lower in breast cancer biopsies as opposed to normal tissue. This was associated with poor survival and recurrence of the disease, as was reduced expression of *TSC1*. Hypermethylation of the *TSC1* promoter was associated with reduced levels of *TSC1* mRNA in these biopsies, whereas hypermethylation of the *TSC2* promoter was seen less frequently (Jiang et al., 2005). *TSC1* haploinsufficiency is also correlated with bladder cancer (Knowles et al., 2003).

1.9.5.2 Rheb

The farnesyl transferase inhibitor SCH66336 (Lonafarnib) was designed to treat such tumours containing active Ras. Although the drug inhibited growth of tumours in preclinical studies, it was found not to abrogate prenylation (and therefore activation) of Ras or Raf as predicted. Instead, Lonafarnib was found to inhibit tumour growth by preventing prenylation of Rheb, indicating the importance of Rheb activation in

cancer cells. Further study noted increased Rheb mRNA transcript levels in cancer cell lines, and expression of a prenylation-deficient Rheb mutant prevented Lonafernib induced cell death (Basso et al., 2005). This implies that Rheb activation is a critical inducer of carcinogenesis in human cancer. Indeed Rheb mRNA and protein levels are positively correlated with aggressive phenotype of prostate cancers (Kobayashi et al., 2010).

1.9.5.3 SGK1

Hyperactivation of mTORC1 can promote carcinogenesis by inactivation of p27. By inhibiting cyclin D-Cdk complexes, p27 inhibits cell proliferation (reviewed in Sherr & Roberts, 1999). Therefore progression of the cell cycle requires inactivation of p27. This is achieved by SGK1, which phosphorylates p27 at T157 resulting in cytosolic accumulation. SGK1 is activated downstream of mTORC1, and may be a direct substrate of the complex (Hong et al., 2008). Therefore mTORC1 promotes cell cycle progression by activating SGK1, which in turn inhibits p27 allowing activation of cyclin D-Cdk complexes. Inactivation of p27 is associated with a number of human cancers (Rosner et al., 2006). A low level of nuclear p27 was associated with poor survival rate in breast cancer sufferers, particularly in patients less than 45 years old (Alkarain et al., 2004). A direct link between mTORC1 and carcinogenesis promoted by inactivation of p27 was found in a sample of human bladder tumours. In this study, mutations in *TSC1* were associated with suppression of p27 activity (Adachi et al., 2003).

1.9.5.4 Ras

The oncogene *RAS* activates mTORC1 signalling via the Raf/MEK/ERK cascade (reviewed in Roux & Blenis, 2004). Activating mutations in *RAF* are found in over 60% of malignant melanomas and in many tumours of the colon, thyroid and lung (reviewed in Shaw & Cantley, 2006). In addition, mutation of the Ras GAP, Neurofibromatosis 1 (NF1), results in accumulation of active Ras which is at the root of the disease NF (reviewed in Inoki et al., 2005). Ras promotes cell proliferation by inhibition of apoptosis through inactivation of TSC2 by phosphorylation at S1798 (Roux et al., 2004, Freilinger et al., 2008). To inhibit apoptosis, TSC1/2 triggers phosphorylation of Bcl-2 Associated Agonist of Cell Death (BAD) and upregulation of the proapoptotic heterodimerisation of BAD/BCL-2 and BAD/BCL-XL (Freilinger et al.,

2006). Therefore tumours containing active Ras or Raf are resistant to apoptosis by preventing the inhibitory activity of TSC1/2.

1.9.5.5 *PLD1*

Increased expression of PLD1 has been associated with breast cancer. In the MDA-MB-321 breast cancer cell line, PLD1 expression was 10 fold higher than in another breast cancer cell line, MCF-7. Rapamycin inhibits mTORC1 and mTORC2 by competing with PA binding which is required for mTOR-Raptor and mTOR-Rictor interaction (reviewed in Foster & Toschi, 2009). Breast cancer cells displaying high levels of *PLD1* expression, and therefore PA accumulation, were highly resistant to rapamycin, which was reversed by inhibition of PLD1. In contrast, elevating PLD1 activity in MCF-7 cells leads to rapamycin resistance (Chen et al., 2003). This reveals that PLD1 expression may be an indicator of rapamycin resistance in breast cancer.

1.9.5.6 *Feedback loops – and therapeutics*

The mTORC1 negative feedback loop has implications for mTORC1 inhibition in cancer therapeutics. Inhibition of mTOR with the rapamycin analogues temsirolimus and everolimus has resulted in modest success in clinical trials. In fact, temsirolimus increased the rate of disease progression in patients with advanced pancreatic cancer (Javle et al., 2010). It is likely that this effect is due to inadvertent activation of PI3K signalling by abrogation of the negative feedback loop.

Biopsies post-treatment showed that everolimus increased IRS-1 protein and Akt activation in human liver, colon and breast carcinoma (O'Reilly et al., 2006). In tumour cell lines this was reversed by IGF-IR inhibition. Everolimus also increased signalling within the MAPK pathway, as indicated by enhanced ERK phosphorylation in post-treatment breast and colon carcinoma biopsies (Carracedo et al., 2008). In tumour cell lines, ERK and Akt phosphorylation induced by everolimus or rapamycin was inhibited by LY294002 and wortmannin, indicating activation via IRS-1 and PI3K (Sun et al., 2005).

Combination treatment with everolimus and the MEK1/2 inhibitor PD0325901 reduced proliferation and increased apoptosis of tumour cell lines to a greater

degree than treatment with either drug alone. This provides evidence that combinatory treatment may be required to inhibit mTORC1 whilst preventing PI3K signalling via the negative feedback loop. In human neuroendocrine tumour cell lines, everolimus in combination with the PI3K inhibitor NVP-BEZ235 prevented feedback activation of Akt induced by everolimus alone (Zitzmann et al., 2010).

Combination therapy may also be of use in the treatment of Acute Myeloid Leukaemia (AML), a disease in which the PI3K/Akt and mTORC1 pathways are frequently activated. Blast cells from bone marrow samples taken from AML patients showed increased Akt activation following incubation with everolimus (Tamburini et al., 2008). Akt phosphorylation decreased when a combination of everolimus and the PI3K inhibitor IC87114 was used. Therefore, although inhibition of mTORC1 increases PI3K/Akt signalling via prevention of the negative feedback loop, this can be overcome by combination therapy with PI3K inhibitors.

1.10 Project aims

Although the kinase events leading to activation of mTORC1 are well understood, the phosphatase regulation that is required to prevent aberrant signalling has been granted little attention. Although the role of PP2A in mTORC1 signalling has been documented (Peterson et al., 1999, Schalm et al., 2005, Yamashita et al., 2005, Bielinski & Mumby, 2007), the key to uncovering phosphatase regulation lies in the identification of target regulatory subunit(s). This project aims to analyse a number of regulatory subunits that potentially control PP2A activity within the mTORC1 signalling pathway.

In budding yeast, phosphatases are essential in regulating TORC1 substrate phosphorylation in the absence of stimulatory signals. The transcription factor Gln3 activates expression of nitrogen-regulated genes (Beck & Hall, 1999, Cardenas et al., 1999, Duvel et al., 2003). In response to good nitrogen supply, TORC1 phosphorylates Gln3 to promote its binding to the repressor Ure2 in the cytosol. Conversely, TORC1 inhibition by limited nitrogen supply leads to dephosphorylation of Gln3 by Sit4 and Pph21/22, resulting in nuclear translocation and activation (Cardenas et al., 1999, Bartram et al., 2000, Komeili et al., 2000, Crespo et al.,

2002). Inhibition of Pph21/22 and Sit4 is required to prevent activation of nitrogen-responsive genes in the presence of sufficient nitrogen supply. This is achieved by the inhibitory subunit Tap42, which prevents Pph21/22 and Sit4 binding to regulatory subunits (Wang et al., 2003). During repression of TORC1 signalling, Tap42 is sequestered by Tip41, allowing Pph21/22 and Sit4 activation. Yeast TORC1 promotes Tap42-mediated repression of phosphatase activity by phosphorylating Tip41 (Jacinto et al., 2001). Phosphorylation of Tip41 causes dissociation of Tap42 from Tip41, which allows free Tap42 to then sequester and inhibit Sit4 and Pph21/22.

Mammalian Tip41, with a molecular weight of 32kDa, shares much sequence identity with the yeast counterpart and, crucially, the TOS motif is conserved. As a TOS motif is required for optimal phosphorylation by mTOR, I hypothesise that Tip41 might be regulated by mTORC1 in a similar manner to that in yeast. In addition, Tip41 was shown to purify with the mammalian Tap42 and Pph21/22 orthologues Alpha4 and PP2Ac respectively (McConnell et al., 2007). Studies of Alpha4 indicate conflicting roles in relation to mTORC1, where it has been placed as both an activating and inhibitory PP2A subunit towards mTORC1 substrates (Nanahoshi et al., 1998, Nien et al., 2007, Grech et al., 2008). Nevertheless, PP2A_{Alpha4} has been shown to regulate phosphorylation of mTORC1 substrates. These pieces of evidence poised human Tip41 as a potential functional homologue of the yeast counterpart. Human Tip41 inhibits PP2Ac *in vitro* but a role in the regulation of mTORC1 substrates has not been investigated (McConnell et al., 2007). This project set out to explore the role and regulation of Tip41 in mTORC1 signalling.

As the functional homologue of yeast Sit4, PP6c is perfectly placed to play a role in mTORC1 signalling in human cells (Bastians & Ponstingl, 1996). In addition, Tip41 binds PP6c although the functional relevance of this remains unknown. As the role of PP6c in mTORC1 has not been investigated, this project also contains a brief study into the role of this PP6c phosphatase in relation to mTORC1 substrates. The decision to concentrate on the catalytic rather than regulatory subunits of PP6c was taken. The rationale for this was that, in contrast to PP2Ac, no data has been published linking PP6c with mTORC1 signalling. Therefore, this project conducted some preliminary experiments to determine whether PP6c indeed impacted on mTORC1 activity.

Classically, PP2Ac requires regulatory and scaffold subunits for dephosphorylation of substrates. The regulatory, or B, subunit can be one of at least 20 proteins that can be further sub-grouped into four families based on sequence similarity. These regulatory subunits control substrate specificity of PP2Ac. The A scaffold subunit functions to bridge the interaction between the B regulatory subunit and PP2Ac. DNA viruses often inhibit PP2A activity in order to promote replication in the absence of stimulating signals. The adenoviral protein E4ORF4 is no exception, and was found to activate mTORC1 by inhibiting a PP2A complex specifically containing the B α regulatory subunit. Rather than inhibiting catalytic activity, E4ORF4 acts as a non-competitive inhibitor preventing substrate recognition by B α (Li et al., 2009). The exact substrate of PP2A_{B α} was not found, although E4ORF4 did not alter Rheb GTP-loading and its resulting activation of S6K1 was sensitive to rapamycin (O'Shea et al., 2005(a)). Therefore PP2A_{B α} was proposed to inhibit mTORC1 signalling in a parallel pathway to that of insulin/growth factor signalling. The role of PP2A_{B α} was explored in relation to mTORC1 indirectly using the E4ORF4 protein in this project. This project also sets out to study the regulation and role of PP2A_{B α} directly in relation to mTORC1 signalling.

CHAPTER 2 - MATERIALS AND METHODS

2.1 Suppliers

Consumables and equipment used in this study were purchased from the following companies.

Abcam, Cambridge, UK

AbGene Surrey, UK

Applied Biosystems, Cheshire, UK

ATCC, Middlesex, UK

Bibby Sterilin, Staffordshire, UK

Binder, Venrey, Netherlands

BioRad Laboratories Ltd., Hertfordshire, UK

Cambridge Bioscience, Cambridge, UK

Calbiochem, Nottingham, UK.

Cell Signalling Technologies, Danvers, USA

Corning Costar, Amsterdam, Netherlands

Enzo Life Sciences, Exeter, UK

Eurofins MWG Operon. Ebersberg, Germany

FujiFilm UK Ltd, Bedfordshire, UK

GE Healthcare, Buckinghamshire, UK

Helena Biosciences Europe. Gateshead, UK

Hoefer, Holliston, USA

Invitrogen Life Sciences Ltd. Paisley, UK

Konica Minolta, Basildon, UK

Lonza Vervieres, Cambridge, UK

Millipore, Edinburgh, UK

National Diagnostics, Atlanta, USA

New England Biolabs Ltd., Hertfordshire, UK

Perkin Elmer, Massachusetts, USA

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
Sartorius, Epsom, UK
Sigma-Aldrich Company Ltd. Dorset, UK
Starlabs, Milton Keynes, UK
Ted Pella Inc., California, USA
Thermo Fisher Scientific, Surrey, UK
VWR International. Leicestershire, UK

2.2 Materials

2.2.1 Chemicals

Analytical grade Ethanol and Methanol were obtained from Thermo Fisher Scientific. All other chemicals were obtained from Sigma Aldrich Company Ltd unless otherwise indicated.

2.2.2 Plasmid details

The pcDNA₃-(HA)₃-Tip41 and pcDNA₃-(HA)₃-Tip41(F156A) were produced by Dr A. Tee. Site directed mutagenesis was used to create (HA)₃-Tip41 mutants D71L, Y79H and M196V (Smetana & Zanchin, 2007). The pcDNA₃-FLAG-Alpha4, pKH3-(HA)₃-PP2Ac and pKH3-(HA)₃-PP2Ac(E42A) were kind gifts from Prof. D. L. Brautigan at the University of Virginia (Prickett & Brautigan, 2004). Prof. J. Blenis at Harvard University kindly supplied pcDNA₃-GST-4EBP1, pGEX-GST-4EBP1 and pcDNA₃-Myc-4EBP1, which were used to create pcDNA₃-GST-4EBP1(F114A) and pcDNA₃-Myc-4EBP1(F114A) by Dr. E. Dunlop using site directed mutagenesis. Dr. S. Schalm of Harvard University donated pRK7-HA-S6K1 (Schalm et al., 2005). The pRK5-Myc-mTOR and pRK5-HA-Raptor vectors were provided by Dr. D. Sabatini at the Whitehead Institute, and Raptor mutants as detailed here (Kim et al., 2002) were created by Dr. E. Dunlop using site directed mutagenesis. Mutants are detailed below in Table 2.1.

Raptor Mutant	Mutation	Domain
1	¹⁹⁴ YDC ₁₉₆ to AAA	RNC
2	²⁶¹ DLF ₂₆₃ to AAA	RNC
3	³¹³ NWIF ₃₁₆ to AAAA	RNC
4	³⁹¹ SQ ₃₉₂ to PA	RNC
7	⁷³⁸ SLQN ₇₄₁ to PAAA	Uncharacterised
9	¹¹⁹¹ RVYDRR ₁₁₉₆ to DAAAADD	WD40

Table 2.1: Raptor mutant details

Prof. C. Walker at the MD Anderson Centre kindly supplied pcDNA_{3.1}-FLAG-TSC2 which was used to create pDEST27-GST-TSC2 using the Gateway Cloning system by Dr E. Dunlop. The pRK5-Myc-Rictor vector was obtained from Addgene (plasmid 11367). The pcDNA₃-HA-E4ORF4, -HA-E4ORF4(L51/54A) and -HA-E4ORF4(Δ359) plasmids were kindly provided by Dr. D. Stokoe (University of California). The pRK7-GST-Rheb and pRK7-GST-Rheb(Q64L) were kindly provided by Dr. E. Dunlop. The HIF1α luciferase reporter was purchased from Promega UK Ltd. (Cat. No. LR0128). As detailed in Materials and Methods, unless specified otherwise above, genes were subcloned into the pDEST27-GST and pcDNA_{3.1}-nV5DEST vectors using the Gateway Cloning system (Invitrogen) as described in manufacturer's protocol. All shRNA clones were obtained from Sigma Aldrich Company Ltd., and are cloned within the pLKO.1-puro vector. Non-coding shRNA, also purchased from Sigma Aldrich Company Ltd., was used as a negative control in all shRNA experiments. Clone sequences are detailed in Table 2.2.

2.2.3 Primers

Primers used for QPCR of VEGF expression were of the sequences 5'-ACTCCAGGGCTTCATCGTTA-3' (reverse) and 5'-GGAGAGCAGAAGTCCCATGA-3' (forward) were used (Garcia et al., 2009). Other primers were obtained from Sigma with catalogue numbers as follows: β-actin QT01680476, Tip41 QT00035497, Bα QT00024500 and PP6c QT00015666.

For site directed mutagenesis of Tip41, the following primers were used. The forward primer used to generate V5-Tip41(D71L) was 5'-GA ATT GAG TTC AAT GCT ACA **CTT** GCG TTA AGA TGT GTA AAC-3' and the reverse 5'-GTT TAC ACA TCT TAA CGC **AAG** TGT AGC ATT GAA CTC AAT TC-3'. The forward primer used to generate V5-Tip41(M196V) was 5'-GAT GGG GTG CTT ATC AGA **GTG** AAT GAC ACG AGA CTT TAC-3' and the reverse 5'-gta AAG TCT CGT GTC ATT CAC TCT GAT AAG CAC CCC ATC-3'. To generate V5-Tip41(Y79H) the forward primer used was 5'-G TTA AGA TGT GTA AAC AAC CAC CAA GGA ATG CTT AAA G-3' and the reverse primer 5'-C TTT AAG CAT TCC TTG **GTG** GTT GTT TAC ACA TCT TAA C-3'. Nucleotides outlined in bold indicate mutations in comparison to wild type sequence.

2.2.4 Antibodies

Tip41 antibody was obtained from Cambridge Bioscience. Santa Cruz Biotechnology supplied Alpha4 antibody. Ubiquitin antibody was obtained from Enzo Life Sciences. Antibody directed to HA was obtained from Roche. Anti PP6c was obtained from Millipore. Anti B α and IKK β were obtained from Abcam. Secondary antibodies were purchased from Sigma Aldrich Company Ltd. All other antibodies were purchased from Cell Signalling.

2.2.5 Molecular biology and cloning

Phusion DNA Polymerase was purchased from Thermo Fisher Scientific. Dpn1 was purchased from New England Biolabs. All Gateway cloning products were purchased from Invitrogen along with One Shot OmniMAX 2-T1 and One Shot TOP10 Chemically Competent *E coli*. QIAprep Spin Miniprep Kit and HiSpeed Plasmid Maxi Kit were both purchased from Qiagen.

2.2.6 Cell culture

Human Embryonic Kidney 293 (HEK293) were purchased through ATCC. TSC2-/- (p53-/-) MEF cells were kindly donated by Dr D. Kwaitowski at Harvard University. Dulbecco's Modified Eagle Medium (DMEM) was obtained from Lonza Vervieres whilst Fetal Bovine Serum (FBS), penicillin/streptomycin and Trypsin 0.25% with EDTA 4Na were obtained from Invitrogen

	shRNA Clone	Sequence
Tip41	1	CCGGGCCTTGTTTATGTACAGATTTCTCGAGAAATCTGTACATA ACAAGGCTTTTTG
	2	CCGGCCTAATGAAATATCCCAGTATCTCGAGATACTGGGATAT TCATTAGGTTTTTG
	3	CCGGCGAGAATATACGTACGAGAACTCGAGTTCTCGTGACG TATATTCTCGTTTTG
	4	CCGGACCTAATGAAATATCCCAGTACTCGAGTACTGGGATATT TCATTAGGTTTTTG
	5	CCGGGTGGAGAAATTAGCCGATGAACTCGAGTTCATCGGCTA ATTTCTCCACTTTTTG
Bα	1	CCGGAGAAACACAAAGCGAGACATACTCGAGTATGTCTCGCTT TGTGTTTCTTTTT
	2	CCGGGATCCCAGTAACAGGTCATTTCTCGAGAAATGACCTGTT ACTGGGATCTTTTT
	3	CCGGTCCTGCTTAGTTGAGATAGTTCTCGAGAACTATCTCAAC TAAGCAGGATTTTT
	4	CCGGGTAGATGATGATGTAGCAGAACTCGAGTTCTGCTACATC ATCATCTACTTTTT
	5	CCGGGCAAGTGGCAAGCGAAAGAACTCGAGTTTCTTTGCTT GCCACTTGCTTTTT
PP6c	1	CCGGGAGTCAAATGTTCAGCCAGTACTCGAGTACTGGCTGAA CATTTGACTCTTTTT
	2	CCGGCCAGAACGACAACGCCATATTCTCGAGAATATGGCGTT GTCGTTCTGGTTTTT
	3	CCGGCCAAAGTTATTCCGGGCAGTTCTCGAGAACTGCCCGGA ATAACTTTGGTTTTT
	4	CCGGGCTTCGATCATGGTCTTCAAACCTCGAGTTTGAAGACCAT GATCGAAGCTTTTT
	5	CCGGGCTTATTTACTGGTCTGGGAACTCGAGTTCCAGACCAG TAAATAAGCTTTTT

Table 2.2: Sequences of shRNA clones. Each shRNA is cloned in the pLKO.1-puro vector as supplied by Sigma Aldrich.

2.2.7 Transfection and cell lysis

Lipofectamine 2000 was obtained from Invitrogen.

2.2.8 Protein purification, cell fractionation and associated techniques

Protein G Sepharose 4 Fast Flow beads and GST SpinTrap columns were purchased from GE Healthcare. All radioactive chemicals were obtained from Perkin Elmer. The Catch and Release Kit v2.0 was purchased from Millipore, as was Inactive Akt. Qiagen supplied the Qproteome Cell Compartment Kit.

2.2.9 SDS PAGE

NuPAGE Novex 4-12% Bis-Tris, 3-8% Tris-Acetate and 4-12% Bis-Tris Zoom Gels (1.0mm) were purchased from Invitrogen along with NuPAGE LDS Sample Buffer (4X). ProtoGel reagents (30% ProtoGel) were purchased from Fisher Scientific, including Protein Loading Buffer Blue (2X).

2.2.10 Isoelectric Focussing, electrotransfer and western blotting

NuPAGE Sample Reducing Agent (10X) was purchased from Invitrogen. Immobilon-P PVDF Transfer Membrane was purchased from Millipore. GE Healthcare supplied the Amersham ECL Western Blotting Detection Reagents along with the 2D Clean-Up Kit and 7cm Immobiline Drystrip pH3-10 NL used for IEF. FujiFilm Super RX supplied by FujiFilm UK Ltd. was used to visualise results.

2.2.11 Gel staining and Mass Spectrometry

Colloidal Blue Staining Kit was purchased from Invitrogen. Trypsin was purchased from Promega UK Ltd.

2.2.12 mRNA extraction and quantitative PCR

Extraction of mRNA and Q-PCR reagents were purchased from Qiagen, including the RNeasy Mini Kit, QIAshredders, QuantiTect Reverse Transcription Kit and QuantiTect SYBR Green PCR Kit.

2.3 Equipment

2.3.1 Plastics and glassware

Sterile Gilson pipette tips were supplied by StarLab. Sterile 5ml, 10ml and 25ml stripettes were from Corning CoStar. Microcentrifuge tubes were supplied by Sigma Aldrich Company Ltd. Plastic strip tubes (0.2ml), 96 well Thermo-fast skirted detection plates and adhesive PCR sealing sheets were from ABGene. Sterile universal tubes were obtained from Sterilin. Glassware was purchased from VWR International Ltd. and Thermo Fisher Scientific. Optilux 96 well luminometer plates were purchased from VWR international. Tissue culture flasks and plates were purchased from Helena Biosciences. Nunc CryoTube vials used for cell storage in liquid nitrogen were supplied by Thermo Fisher Scientific.

2.3.2 Molecular biology and cloning

A Horizon 11.14 gel tank (Invitrogen) was used for DNA electrophoresis. Visualisation of gels was attained using a BioRad GelDoc XR transilluminator. Power packs were supplied by BioRad. The GeneAmp PCR System 9700 was supplied by Applied Biosystems.

2.3.3 Cell culture

The CB Series CO₂ incubator was supplied by Binder. The Motic AE30 microscope was supplied by Ted Pella Inc.

2.3.4 SDS PAGE and electrotransfer

The XCell Surelock Minicell was supplied by Invitrogen, and the Consort EV261 Electrophoresis Power Supply was obtained from Sigma. The Hoefer miniVE Electrotransfer Unit, used for electrotransfer of NuPAGE gels, was supplied by Thermo Fisher Scientific. The omniPAGE Maxi Vertical Unit, used for SDS PAGE of 4EBP1 and subsequent electrotransfer in order to visualise mobility shift, was obtained from VWR International Ltd. The Konica Minolta SRX-101A Film Processor was obtained from Konica Minolta. The Model 583 Gel Drier was purchased from BioRad.

2.3.5 Mass Spectrometry

A 4800 MALDI TOF/TOF Analyser was purchased from Applied Biosystems.

2.3.6 Luciferase assays

The TR717 Microplate Luminometer was obtained from Applied Biosystems.

2.3.7 Q-PCR

The Applied Biosystems GeneAmp 9700 was used for real-time Q-PCR.

2.3.8 General equipment

The Hereaus Pico 17 Centrifuge, the Jenway 3510 pH Meter, the Eppendorf Thermomixer Compact and the Stuart Mini Orbital Shaker SSM1 were supplied by Thermo Fisher Scientific. The NanoDrop 8000 used to quantify DNA, RNA and protein was obtained from Thermo Fisher Scientific. Sartorius supplied the B1200 balance.

2.3.9 Software

ImageJ v1.44 was used for densitometry analysis. The MASCOT Database search engine v2.1 used for MS/MS queries was obtained from Matrix Science Ltd and the Global Proteome Server Explorer software v3.6 was obtained from Applied Biosystems. Tropix WinGlow software was used for collecting luminescence data. Statistics and graphing was carried out using Microsoft Excel and Minitab 15.

2.4 Methods

2.4.1 General reagents

Solutions were made in MilliQ water and autoclaved at 15lb/sq.in. at 121°C for 40 minutes where necessary.

2.4.2 Buffers and solutions

3x mTOR Kinase Buffer: 75mM HEPES pH 7.4, 60mM KCl, 30mM MgCl₂.

Blenis Lysis Buffer: 10mM KPO₄, 5mM EGTA pH 7.2, 10mM MgCl₂, 50mM β-Glycerophosphate.

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.

Cross-Linking Buffer A: 40 mM Hepes pH 7.5, 120 mM NaCl, 1 mM EDTA, 50 mM NaF, 50 mM β -Glycerophosphate, 0.3%(w/v) CHAPS.

GTP Loading Buffer: 25 mM HEPES pH 7.4, 5 mM EDTA , 0.5 mg/ml BSA.

HEPES/KCL Wash Buffer: 25mM HEPES pH 7.4, 20mM KCl.

High Salt Wash Buffer: 40mM HEPES pH7.4, 2mM EDTA, 10mM β -Glycerophosphate, 400nM NaCl.

Low Salt Wash Buffer: 40mM HEPES pH 7.4, 2mM EDTA, 10mM β -Glycerophosphate, 150mM NaCl, 0.3% CHAPS.

Luciferase Reagent: 50mM Tricine pH 7.8, 15mM MgSO₄, 15mM KH₂PO₄, 4mM EGTA, 2mM ATP, 1mM LuciferinCell Culture.

Luria Agar (1L): 10g Tryptone, 5g Yeast Extract, 10g NaCl, 1g Glucose, 1g Anhydrous MgCl₂. Adjusted to pH 7.0 before adding 15g Agar, 2ml of 1M NaOH and autoclaving.

Luria Broth (1L): 10g Tryptone, 5g Yeast Extract, 10g NaCl, 1g Glucose, 1g Anhydrous MgCl₂. Adjusted to pH 7.0 and autoclaved.

MgCl₂ Loading Buffer: 25 mM HEPES pH. 7.4, 5 mM MgCl₂.

mTOR/Raptor Lysis Buffer: 40mM HEPES pH 7.4, 2mM EDTA, 10mM β -Glycerophosphate, 0.3% CHAPS.

NP-40 Lysis buffer: 20mM Tris pH 7.4, 150mM NaCl, 1mM MgCl₂, 1% Nonidet P-40, 10% Glycerol, 1mM DTT, 50mM β -glycerophosphate, 50mM NaF.

Radiolabelling Buffer A: 50 mM HEPES pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 1 mg/ml BSA, 1 mM DTT, 1% Triton.

Radiolabelling Buffer B: 50 mM HEPES pH 7.4, 100 mM NaCl, 10 mM MgCl₂, 0.1% Triton.

Raptor Lysis Buffer: 50 mM β -Glycerol Phosphate, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100 .

Rehydration Buffer: 7M Urea, 2M Thiourea, 2%(w/v) CHAPS.

Rheb Lysis Buffer: 40mM HEPES pH 7.4, 10mM β -Glycerophosphate, 5mM MgCl₂, 0.3% CHAPS.

Rheb Storage Buffer: 20mM HEPES pH 8.0, 200mM NaCl, 5mM MgCl₂.

Rictor Lysis Buffer: 40mM HEPES pH7.5, 120mM NaCl, 1mM EDTA, 10mM pyrophosphate, 10mM β -glycerophosphate, 50mM NaF, 0.3%(w/v) CHAPS.

Running Buffer 10X (1L): 144.07g Glycine, 30.285g Tris-Base, 10g SDS.

ST Buffer - S6K1 Kinase Assay: 50mM Tris-HCl pH 7.2, 5mM Tris, 150mM NaCl.

Start Buffer: 25mM HEPES pH 7.4, 10mM MgCl₂.

TAE Buffer: 40 mM Tris acetate, 1 mM EDTA.

TBS-T (1L): 2.42g Tris-base, 8g NaCl adjusted pH to 7.6 followed by addition of 0.1%(v/v) Tween-20.

Western Transfer Buffer 10X (1L): 144.07g Glycine, 30.285g Tris-Base, 2g SDS.

2.4.3 Molecular biology

2.4.3.1 PCR

DNA primers were synthesised by Eurofins MWG Operon. Genes required for cloning were amplified by PCR for 23 cycles under the following conditions:

Denaturation 98°C, 30s

Annealing 52°C, 30s

Polymerisation 72°C, 3mins

PCR product was treated with 1µl Dpn1 for 1h at 37°C, and purified as per Gateway Technology with Clonase II protocol.

2.4.3.2 Agarose gel electrophoresis

Electrophoresis was carried out on an agarose gel (1%(w/v) agarose in TAE, 5ng/ml ethidium bromide) at 100V.

2.4.3.3 Cloning

Gateway cloning was used for GST- and V5-tagging using the Gateway pDEST27 and pcDNA3.1/V5-DEST vectors respectively. The pENTR221 vector was used with the BP Clonase Enzyme Mix to create an entry vector using the purified PCR product in the initial cloning stage. The LR Clonase II Enzyme Mix was then used in the second recombination step.

2.4.3.4 Transformation and selection of competent cells

Following Gateway cloning One Shot® OmniMAX™ 2-T1 *E. coli* were transformed as detailed in manufacturer's protocol. To replenish vector stocks, One Shot TOP10 Chemically Competent *E. coli* were used as detailed in manufacturer's protocol. Successful transformants were selected by growing the cells overnight on

LB/1.5%(w/v) agar plates with the appropriate antibiotic at 37°C. Isolated colonies were then grown overnight in a shaking incubator in LB containing the appropriate antibiotic.

2.4.3.5 Plasmid DNA preparation

For small scale purification, the QIAprep Spin Miniprep Kit was used as per manufacturer's protocol. Following large scale plasmid amplification, the HiSpeed Plasmid Maxi Kit was used according to manufacturer's protocol. If verification that cloning had been successful was required, DNA was sent for sequencing at Eurofins MWG Operon.

2.4.3.6 Site-directed mutagenesis

Site-directed mutagenesis was used to create mutations within clones. The PCR reaction was set up as detailed in Section 2.4.3.1. The following PCR cycle was used to amplify product.

Initial denaturation	98°C for 5min
Then 18 cycles of:	
Denaturation	98°C for 1min
Annealing	52°C for 1min
Polymerisation	72°C for 15min
Then a final polymerisation	72°C for 18min

PCR products were then treated as in Section 2.4.3.1 and used to transform One Shot® TOP10 Chemically Competent *E. coli* as in Section 2.4.3.3. Successful mutagenesis was determined by sequencing carried out by Eurofins MWG Operon.

2.4.4 Cell culture

HEK293 and TSC2^{-/-} MEF cells (as indicated) were cultured in 75cm² flasks using DMEM supplemented with 10%(v/v) FBS and 1%(v/v) penicillin/streptomycin. Flasks were incubated at 37°C, 5%(v/v) CO₂.

Cells were regularly passaged to avoid problems associated with over-confluency.

Firstly, cells were washed twice in Trypsin 0.25% with EDTA 4Na which was

aspirated before incubating the flask for 5 minutes at 37°C. Cells were then diluted in DMEM and transferred to a new flask or plate as required.

Cells required for storage were resuspended in FBS supplemented with 8%(v/v) DMSO and frozen in liquid nitrogen.

2.4.5 Transfection

2.4.5.1 CaCl_2 precipitation transfection protocol

Transfection of cells in 100mm or 150mm plates was achieved using CaCl_2 precipitation. Cells were plated and transfected 4 hours later after ensuring cells were adhered. The transfection mixture was prepared with the volume of DNA diluted in the required volume of water as detailed in Table 2.3. CaCl_2 was then included followed by dropwise addition of 2xBES whilst aerating the mixture using a drawn-out glass Pasteur pipette.

	100mm plate	150mm plate
DNA	10 μg	40 μg
dH₂O	450 μl	1.8ml
CaCl_2	50 μl	200 μl
2xBES	500 μl	2ml

Table 2.3: Preparation of DNA for CaCl_2 transfection.

2.4.5.2 Lipofectamine 2000 transfection

Transfection of cells in 60mm plates and smaller used Lipofectamine 2000 transfection reagent according to manufacturers protocol. Following optimisation, a 5:2 ratio of Lipofectamine 2000 (μl) to DNA (μg) was used, where 60mm plates were transfected with 5 μg DNA and 35mm plates with 2.5 μg .

Using the standard protocol, cells were seeded on day one and transfected on day two, and the media changed 4h post-transfection. Cells were lysed on day three.

For transfection of shRNA, the reverse transfection protocol was used in 35mm plates. Transfection mixture was added to plates prior to cell seeding on day one.

The media was then changed 4 hours post-transfection. If additional transfection of

other plasmids was required, standard transfection protocol was used on day three. Cells were lysed on day four.

2.4.6 Cell treatments

Cells were serum starved overnight, as indicated, by washing and subsequently placing the cells in DMEM supplemented with 1%(v/v) penicillin/streptomycin . Insulin treatment took place 30 minutes prior to lysis, using 10 μ g/ml. Rapamycin treatment (50nM) took place 1 hour prior to lysis unless otherwise stated. HIF1 α luciferase reporter assays required overnight treatment with both insulin and rapamycin if required. Treatment with 50 μ M MG132 took place 2 hours prior to lysis.

2.4.7 Standard cell lysis

Plates were placed on ice and washed with chilled PBS before resuspension in the appropriate lysis buffer with protease inhibitors (10 μ M leupeptin, 2 μ M antipain, 1mM benzamidine, 1 μ g/ml pepstatin, 0.1mM PMSF, 1mM Na₃VO₄ and 1mM DTT (not added prior to GST purification)). Cells were harvested by scraping and placed on ice for 20 minutes before centrifugation at 13000rpm for 8 minutes at 4°C. The supernatant was then used for subsequent analysis or experimentation.

2.4.8 Immunoprecipitation

Cell lysates were prepared as Section 2.4.7. The resulting supernatant was diluted to 1ml in the corresponding lysis buffer then rotated for 2h at 4°C with 0.4%(v/v) of the required antibody. Protein G Sepharose beads (40 μ l) were then added in a 50:50 slurry in the appropriate lysis buffer and incubated for 1h again at 4°C on a rotator. The beads were then washed 3 times in lysis buffer before eluting in sample buffer diluted to 1x in lysis buffer.

2.4.9 GST purification

Cells were lysed in Rheb Lysis buffer as described in Section 2.4.7. Following centrifugation, the supernatants were applied to a GST column, which had been prepared by placing 0.5ml Rheb Lysis buffer through the column and aspirating the buffer through. Columns were then incubated at 4°C for 2h, followed by three washes in chilled Rheb Lysis buffer and one wash in chilled Rheb Storage buffer.

Each wash consisted of applying lysis buffer to the column, inverting the tube three times, and aspirating the buffer away. Following the final wash, columns were subjected to pulse centrifugation at 4°C to remove all buffer. Proteins were then eluted in Rheb Storage buffer supplemented with 10mM glutathione.

2.4.10 Catch and release

HEK293 cells were lysed in NP40 lysis buffer and the Catch and Release v2.0 immunoprecipitation carried out as per manufacturer's protocol using α Tip41 antibody.

2.4.11 Cell fractionation

HEK293 cells were scraped in media into a universal tube prior to centrifugation at 2500rpm at 4°C for 10 minutes. The Qproteome Cell Compartment Kit was then followed as per manufacturer's instructions in order to generate nuclear, cytoplasmic, membrane and cytoskeletal fractions.

2.4.12 Cross-linking with DTBP

HEK293 cells were transfected with pRK5-Myc-Raptor and the required 'substrate' in 600mm plates as detailed in Section 2.4.5.2. Cells were lysed in 300 μ l Cross-Linking Buffer A supplemented with 0.5mg/ml DTBP and 0.3%(w/v) CHAPS at room temperature, and incubated for 30 minutes. The cross-linking reaction was subsequently quenched by adding 75 μ l 1M Tris-HCl pH7.4 and incubated on ice for 30 minutes. Lysates were then cleared by centrifugation at 13000rpm for 8 minutes at 4°C. Immunoprecipitation using α Myc antibody was then carried out as detailed in Section 2.4.8, with two washes taking place in Cross-Linking Buffer A supplemented with CHAPS and once in Cross-Linking Buffer A without CHAPS. DTBP was then cleaved from proteins by adding sample buffer diluted in Cross-Linking Buffer A supplemented with 250mM DTT.

2.4.13 *In vivo* radiolabelling

pDEST27-GST-Tip41 was transfected into 100mm plates as detailed in Section 2.4.5.1. Dr A. Tee then assisted in the remaining experimentation. On the day of lysis, cells were incubated in 5ml phosphate free medium containing 0.5mCi [γ -³²P]-

ATP for 3 hours. Cells were lysed in Radiolabelling Buffer A. GST-Tip41 was purified as detailed in Section 2.4.9, with two washes using Radiolabelling Buffer A and two using Radiolabelling Buffer B.

2.4.14 S6K1 assay

Cells were transfected with pRK7-HA-S6K1 (and other vectors to express proteins as required) in 60mm plates as detailed in Section 2.4.5.2. Cells were then lysed in Blenis Lysis buffer as in Section 2.4.7 and HA-S6K1 immunoprecipitated using α HA antibody as detailed in Section 2.4.8. Immunoprecipitates were washed once in Buffer A, followed by Buffer B and finally ST Buffer.

S6K1 kinase activity was measured towards a recombinant GST-S6 peptide encompassing the final 32 amino acids of the protein, with the assistance of Dr A. Tee. The recombinant GST peptide was purified as detailed in Section 2.4.9. Purified HA-S6K1 was split into two, with one sample used for radioactive assay and the other for control purposes. HA-S6K1 intended for radioactive assay was incubated with GST-S6 peptide in solution containing 20mM HEPES, 10mM $MgCl_2$, 50mM ATP, 5mCi [γ - ^{32}P]-ATP (radioactive samples only), 3ng/ml PKI at pH7.2 for 12 minutes at 30°C. Reactions were quenched with addition of sample buffer.

2.4.15 Luciferase reporter assay

Using the standard transfection protocol with Lipofectamine 2000 as in Section 2.4.5.2, HEK293 cells were transfected with HRE Luciferase Construct in a 2:1 ratio with pcDNA3.1-nV5-Tip41 (ie. 2 μ g Luciferase construct, 1 μ g expression vector) in triplicate. Negative control cells were transfected with empty vector in place of pcDNA3.1-nV5-Tip41. After overnight incubation in 1% O₂, cells were lysed in Blenis Lysis buffer as detailed in Section 2.4.7. A 20 μ l aliquot was taken of each sample (in triplicate) and placed in a 96 well plate. Luciferase activity was measured by luminosity, where 50 μ l Luciferase Reagent was injected into each well.

Luminescence was measured 10 seconds later.

Luminescence was adjusted to total protein levels using Bradford reagent. Total protein within each sample was measured against a standard curve produced using known concentrations of BSA. This was measured three times in each lysate, and

luminescence adjusted by dividing average luminescence by average total protein. Results are averaged from three separate experiments. Normal distribution was tested using the Anderson-Darling test, and equal variances were verified using Levene's test. A two-sample T-test was used to assess significant differences. Error bars represent standard deviation.

2.4.16 mTORC1 kinase assay

2.4.16.1 Purification of mTOR/Raptor complex

HEK293 cells in 100mm plates were transfected with pRK5-Myc-mTOR and pRK5-HA-Raptor as detailed in Section 2.4.5.1 and treated with insulin prior to lysis. One plate provided sufficient complexes for three assays. Cells were lysed in mTOR/Raptor Lysis buffer as detailed in Section 2.4.7. Purification with α Myc was carried out as detailed in Section 2.4.8. Following incubation with Protein G Sepharose 4 Fast Flow (GE Healthcare) beads, lysates were washed once in Low Salt Wash buffer, followed by two washes with High Salt Wash buffer and a final wash in HEPES KCl Wash buffer.

2.4.16.2 Purification of GST-Rheb

HEK293 cells were transfected with pRK7-GST-Rheb in 100mm plates as detailed in Section 2.4.5.1. Cells were then lysed as detailed in Section 2.4.7 using Rheb Lysis buffer, and GST-Rheb purified as in Section 2.4.9. Purified Rheb was loaded with GTP by combining 10 μ l GTP Loading buffer with 10 μ l purified Rheb and 2 μ l GTP γ S, and incubating for 5 minutes at 37°C with agitation. Radiolabelled assays contained 100 μ Ci [α -³²P]GTP in place of GTP γ S. The reaction was quenched by the addition of 20 μ l MgCl₂ Loading buffer.

2.4.16.3 Substrate purification

pcDNA₃-GST-4EBP1, pcDNA₃-GST-4EBP1(F114A) and pDEST27-GST-Tip41 were transfected into HEK293 cells (10mm plates) as in Section 2.4.5.1 and purified as in Section 2.4.9. Protein concentration was then quantified using Bradford reagent, measured against a BSA standard curve, to ensure equal amount of substrate was added to each assay.

2.4.16.4 Assay preparation

Purified mTOR/Raptor complexes were divided into the required number of samples and supernatant removed. Following this, 10 μ l 3x mTOR Kinase buffer and 5 μ l GTP-loaded Rheb was added in addition to 150ng of substrate. The assays were then equalised to 30 μ l with dH₂O. The FKBP12/rapamycin complex was generated by incubating 30mM rapamycin with FKBP12 for 5min in the dark, and was added to the reaction mixture 5 minutes before commencement. In order to start the reaction, Start buffer (10 μ l/reaction) was mixed with 500 μ M ATP (and 0.2 μ Ci [γ -³²P]ATP for radioactive kinase assays) and added to each sample, which were subsequently incubated at 30°C for 30 minutes with gentle agitation. The reaction was quenched with 13.3 μ l NuPAGE LDS Sample Buffer.

2.4.17 mTORC2 kinase assay

Method was followed as outlined in Sarbassov et al., 2005. GST-tagged Tip41 and 4EBP1 were purified separately as outlined in Section 2.4.9. HEK293 cells were transfected with pRK5-Myc-mTOR and pRK5-Myc-Rictor in 100mm plates as outlined in Section 2.4.5.1. Cells were lysed in Rictor Lysis buffer, and incubated on ice for 20 minutes prior to centrifugation at 13000rpm for 8min at 4°C.

Immunoprecipitation of Rictor using α Myc antibody was achieved using the protocol detailed in Section 2.4.8. Purified Rictor complexes were then resuspended in 15 μ l Rictor kinase buffer supplemented with 500ng inactive Akt, GST-4EBP1 or GST-Tip41 along with 500 μ M ATP and 0.2 μ Ci [γ -³²P]ATP. The FKBP12/rapamycin complex was generated by incubating 30mM rapamycin with FKBP12 for 5min in the dark, and was added to the reaction mixture 5 minutes before commencement. The reaction mixture was incubated at 37°C for 20min, and quenched by the addition of 8 μ l sample buffer.

2.4.18 SDS PAGE

For standard western blotting, precast gels were used according to manufacturer's protocol. Samples were prepared using NuPAGE LDS Sample Buffer (4X) and incubated at 70°C for 10 minutes.

To visualise mobility shift of 4EBP1, the 30% ProtoGel system was used to create a 12% gel as per manufacturer's protocol. Samples were prepared in Protein Loading

Buffer Blue (2X) before incubation at 95°C for 5 minutes. SDS PAGE Running buffer was used during electrophoresis.

2.4.19 Electrotransfer

Proteins within precast gels were transferred to PVDF transfer membrane in Transfer buffer for 2h at 25V. ProtoGels were transferred overnight at 25V and 4°C.

2.4.20 Western blot analysis

Following electrotransfer, the PVDF membrane was blocked in TBST with 5%(w/v) non-fat milk powder for 1h. The membrane was then incubated with primary antibody diluted in TBST with 2%(w/v) BSA overnight at 4°C. Membranes were subsequently washed 3 times in TBST followed by incubation with the appropriate secondary antibody diluted 1:10000 in TBST. After the incubation period, membranes were washed 4 times in TBST before 1 minute incubation in ECL detection reagents set up according to manufacturer's protocol. Proteins were visualised using FujiFilm Super RX and the Konica Minolta SRX-101A Film Processor. Image J (v1.44) software was used for densitometry analysis. Unless otherwise stated, densitometry was calculated by averaging absolute signal intensity between three separate experiments. These were then converted to relative intensities as a percentage using the highest signal intensity as 100% as detailed in 2.4.20. Error is indicative of standard deviation. All western blot results presented are representative of three separate experiments. Densitometry analysis similarly averaged data from three separate western blots.

2.4.21 Staining, fixing and drying polyacrylamide gels

Polyacrylamide gels were firstly fixed in 50%(v/v) methanol, 10%(v/v) acetic acid for 10 minutes on an orbital shaker, then stained using the Colloidal Blue Staining Kit as per manufacturer's protocol.

2.4.22 Mass Spectrometry

GST-Tip41 was purified and resolved by SDS PAGE. Purified proteins were identified with Colloidal Blue staining, and gel plugs (1.5 mm diameter) were manually excised and placed in a 96-well plate. Preparation and MS were then carried out by Cardiff University CBS Proteomics Service using the following protocol.

Peptides were recovered following trypsin digestion using a slightly modified version of the Shevchenko *et al.* (1996) method. Sequencing grade modified trypsin was used at 6.25 ng/ μ l in 25mM NH_4HCO_3 and incubated at 37°C for 3 hours. Finally the dried peptides were resuspended in 50%(v/v) acetonitrile in 0.1%(v/v) trifluoroacetic acid for MS analysis and an aliquot corresponding to 10% of the material (0.5 μ l) was spotted onto a 384 well MS plate. The samples were allowed to dry and the overlaid with α -cyano-4-hydroxycinnamic acid (CHCA) prepared by mixing 5mg matrix with 1ml of 50%(v/v) acetonitrile in 0.1%(v/v) TFA.

Mass spectrometry was performed using a MALDI TOF/TOF mass spectrometer with a 200 Hz solid state laser operating at a wavelength of 355nm (Medzihradszky *et al.*, 2000; Bienvenut *et al.*, 2002; Glückmann *et al.*, 2007; Brennan *et al.*, 2009). MALDI mass spectra and subsequent MS/MS spectra of the 8 most abundant MALDI peaks were obtained following routine calibration. Common trypsin autolysis peaks and matrix ion signals and precursors within 300 resolution of each other were excluded from the selection and the peaks were analysed with the strongest peak first. For positive-ion reflector mode spectra 800 laser shots were averaged (mass range 700-4000 Da; focus mass 2000). In MS/MS positive ion mode 4000 spectra were averaged with 1 kV collision energy (collision gas was air at a pressure of 1.6×10^{-6} Torr) and default calibration.

Combined PMF and MS/MS queries were performed using the MASCOT Database search engine v2.1 (Perkins *et al.*, 1999) embedded into Global Proteome Server (GPS) Explorer software v3.6 on the Swiss-Prot database. Searches were restricted to the human taxonomy with trypsin specificity (one missed cleavage allowed), the tolerances set for peptide identification searches at 50 ppm for MS and 0.3 Da for MS/MS. Cysteine modification by iodoacetamide was employed as a fixed modification with methionine oxidation as a variable modification. Search results were evaluated by manual inspection and conclusive identification confirmed if there was high quality tandem MS (good y-ion) data for ≥ 2 peptides (E value $p < 0.05$ for each peptide; overall $p < 0.0025$) or one peptide (only if E value was $p < 0.0001$).

2.4.23 Far western blotting

Cell lysates containing HA-Raptor were produced by CaCl_2 precipitation transfection in HEK293 cells as detailed in Section 2.4.5.1, followed by lysis in Raptor Lysis buffer and centrifugation (13000rpm, 8 minutes, 4°C). PVDF membrane was incubated with methanol for 1 minute and washed in TBST. 50ng of GST tagged purified protein was dotted onto the membranes, which were then blocked in TBST supplemented with 5%(w/v) non-fat milk powder for 1 hour. Membranes were then incubated with the appropriate lysate diluted in Raptor Lysis buffer supplemented with 5%(w/v) non-fat milk powder overnight on an orbital shaker at 4°C. The next day, the membranes were washed twice in TBST, then probed with αHA antibody for half an hour. The remainder of the protocol was as standard western blotting procedure as in Section 2.4.20.

2.4.24 Isoelectric focussing and SDS PAGE

HA-tagged Tip41 was produced using transfection as detailed in Section 2.4.5.2 and purified as detailed in Section 2.4.8 with αHA antibody. Samples were cleaned using the 2D Clean-Up Kit as detailed in manufacturer's protocol. Each sample was then resuspended in 1ml Rehydration buffer. From this 116 μl was removed for addition to Rehydration solution (116 μl protein sample in rehydration buffer, 1 μl bromophenol blue (1%(w/v)), 2 μl IPG buffer, 6.25 μl 1M DTT) to a total volume of 125 μl . HA-Tip41 in Rehydration solution was then subject to isoelectric focussing using a 7cm Immobiline Drystrip under the conditions detailed below.

Rehydration	12hours at 20oC
Step and hold	500V for 1h
Gradient	1000V for 2h
Step and hold	1000V for 1h
Gradient	8000V for 2 hours
Step and hold	8000V for 8h

Strips were then equilibrated. Firstly, strips were incubated in 5ml Reducing Solution (NuPAGE Sample Reducing Agent (10X) diluted in 1X sample buffer) for 15 minutes on a rocking incubator. This solution was then removed and replaced with 5ml Alkylating Solution (116mg iodoacetate dissolved in 5ml 1X sample buffer), and again incubated for 15 minutes. The strips were then placed in individual NuPAGE

Zoom Gels and subjected to electrophoresis detailed in Section 2.4.18, using MOPS Running Buffer. Proteins were then transferred to PVDF membrane as detailed in Section 2.4.19 and the western blot protocol followed as in Section 2.4.20.

2.4.25 Q-PCR

HEK293 cells were harvested and mRNA purified under standard mRNA handling precautions using the RNeasy Mini Kit according to manufacturer's protocol. Following purification, mRNA concentration was analysed. Contaminating genomic DNA (gDNA) was removed and purified mRNA converted to complimentary DNA (cDNA) using the QuantiTect Reverse Transcription Kit as per manufacturer's protocol. Samples were placed in a 96 well plate using the required primers and QuantiTect SYBR Green PCR Kit. The conditions for Q-PCR are detailed below.

Initial denaturation	95°C for 15min
Then 40 cycles of:	
Denaturation	94°C for 15s
Annealing	55°C for 30s
Extension	72°C for 40s

Levels of amplification product were analysed using the delta-delta-CT method and standardised to β -actin. Normal distribution was tested using the Anderson-Darling test, and equal variances were verified using Levene's test. A two-sample T-test was used to assess significant differences. Standard deviation was calculated and represented by error bars. Results are averaged from three separate experiments. Specificity of primers was determined by a dissociation step. Agarose gel electrophoresis was used to confirm that PCR products were the expected length as detailed by Sigma Aldrich Company Ltd.

3.1 Introduction

In budding yeast, two TOR complexes exist (Heitman et al., 1991). The rapamycin sensitive yeast TORC1 complexes are composed of TOR1 or TOR2 along with KOG1 and LST8 (Leowith et al., 2002, Wedaman et al., 2003). KOG1 mediates substrate binding allowing phosphorylation of TOR targets (Adami et al., 2007). Signalling by yeast TORC1 relies on the heavily regulated phosphatases Sit4 and Pph21/22. When yeast TORC1 is active, the negative regulatory phosphatase subunit Tap42 is phosphorylated and binds Sit4 and Pph21/22 and prevents interaction with substrates (Como & Arndt, 1996, Yan et al., 2006). Tap42 is regulated by Tip41. In the absence of stimulatory signals, Tip41 sequesters Tap42 and allows interaction of Sit4 and Pph21/22 with regulatory subunits resulting in dephosphorylation of TORC1 targets (Figure 1.2) (Jacinto et al., 2001).

In mammalian cells, the mTORC1 complex is composed of mTOR along with Raptor, mLST8 and others. Raptor is the mammalian homologue of KOG1, and similarly is essential for substrate recognition and phosphorylation by mTOR. Raptor contains an N-terminus RNC domain that is critical for substrate recognition, along with WD40 and HEAT repeats towards the C-terminus that mediate binding to mTOR (Kim et al., 2002). Activity of mTORC1 is partly controlled by phosphorylation of Raptor, where both activating and inhibitory phosphorylation events occur (Gwinn et al., 2008, Wang et al., 2009). Substrate phosphorylation by mTORC1 requires Raptor binding. The TOS motif partly mediates this and is essential for binding of the well-characterised mTORC1 substrates 4EBP1 and S6K1 with Raptor (Schalm et al., 2003, Wang et al., 2003).

As in budding yeast, phosphatase activity has also been shown as critical within mTORC1 signalling. PP2A has been identified as the phosphatase responsible for mTORC1 substrate dephosphorylation, as inhibition of PP2A prevents mTORC1 substrate dephosphorylation following treatment with rapamycin (Peterson et al., 1999, Bielinski & Mumby, 2007). In spite of this, the regulatory subunits involved remain poorly understood. Alpha4, the human homologue of Tap42, has been identified as a PP2Ac regulatory subunit towards mTORC1 substrates, although

whether Alpha4 is inhibitory or activating is unclear (Murata et al., 1997, Nanahoshi et al., 1998, Yamashita et al., 2005, Nien et al., 2007, Grech et al., 2008).

Meanwhile, little investigation into the role of Tip41 as a PP2A regulatory protein has been undertaken. Whilst *in vitro* analyses identified Tip41 as a negative PP2Ac regulator, the protein interactions of Tip41 with PP2Ac and Alpha4 have not been fully explored (McConnell et al., 2007). For instance, it is not known whether interactions between Tip41 and Alpha4 or PP2Ac are direct. As Tip41 contains a putative TOS motif, this places Tip41 as a promising candidate as a PP2Ac regulatory subunit towards mTORC1 substrates that is also regulated by mTORC1.

This chapter investigates the potential role of Tip41 in mTORC1 signalling. The results indicate that whilst Tip41 is a bona fide PP2Ac regulatory subunit, it is not regulated directly by mTORC1. Instead, Tip41 may regulate phosphatase activity upstream or parallel to mTORC1. Regulation of PP2A_{Tip41} is achieved by phosphorylation of Tip41, which does not affect binding to PP2A but may instead regulate substrate binding.

3.2 Results

3.2.1 Purification of Tip41 using polyclonal Tip41 antibody

In yeast, Tip41 binds the phosphatase inhibitory subunit Tap42 when TOR signalling is inactive allowing dephosphorylation of downstream substrates by the phosphatases Sit4 and Pph21/22. To identify potential members of a Tip41 complex within mammalian cells, endogenous Tip41 was purified from HEK293 cells using polyclonal α Tip41 antibody. To determine whether these interactions were dependent on the activity of mTOR, purification took place both with and without pre-treatment with insulin. Purified protein was resolved by SDS PAGE in duplicate and stained with colloidal blue or analysed by western blot (Figure 3.1A). Although Tip41 was successfully purified, as shown by western blot detection of the 32kDa protein in the immunoprecipitated sample, colloidal stain revealed a lot of non-specific protein bands that co-purified. All proteins were present in equal quantities both with and without insulin stimulation, indicating that interactions were not dependent on activity within the insulin signalling pathway.

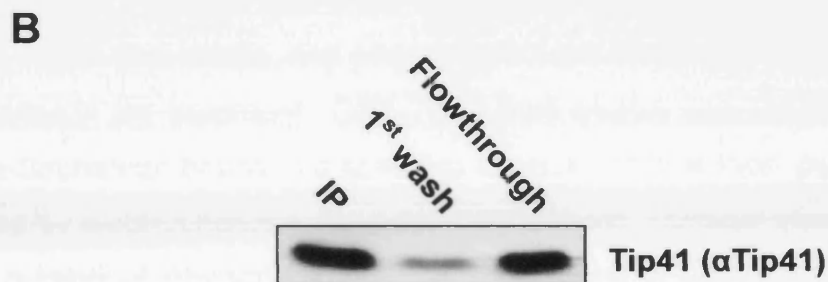
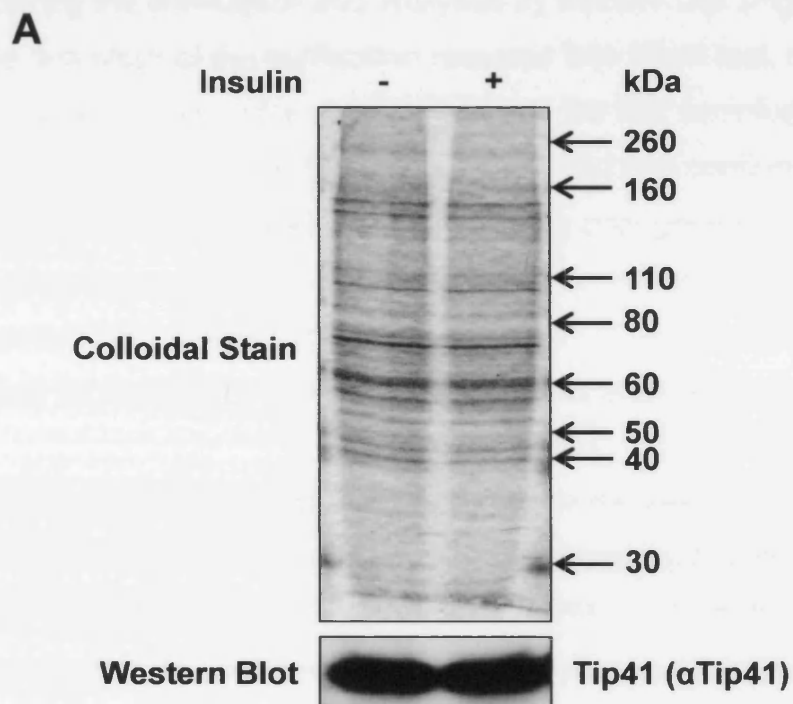


Figure 3.1: Purification of endogenous Tip41. **A.** Endogenous Tip41 was purified using αTip41 antibody. Prior to lysis cells were serum starved overnight, and stimulated with insulin as indicated. Samples were resolved by SDS PAGE and stained with colloidal blue to visualise purified protein. As many proteins were visible, the purification process was considered to be of poor quality and purification of endogenous Tip41 using this method was subsequently not used. **B.** Samples of the 1st wash and the flowthrough were taken during the purification and analysed by western blot. Much Tip41 was lost in the initial flowthrough (the solution lost after the first centrifugation step) indicating that a large amount of Tip41 was not purified. This confirmed ineffective purification of endogenous Tip41. All results are representative of 3 independent experiments.

For trouble shooting purposes, samples of the 1st wash and the flowthrough were also taken during the purification and analysed by western blot (Figure 3.1B). Although the first wash of the purification revealed little Tip41 lost, much Tip41 was lost in the initial flowthrough (the solution lost after the first centrifugation step). This indicates that a large amount of Tip41 was not purified and confirms ineffective purification of Tip41 using α Tip41 antibody to purify endogenous protein. Following this, it was clear that an alternative method of purification was required in order to identify potential Tip41 interacting proteins and deduce whether those interactions are dependent on the activity of the mTOR signalling pathway.

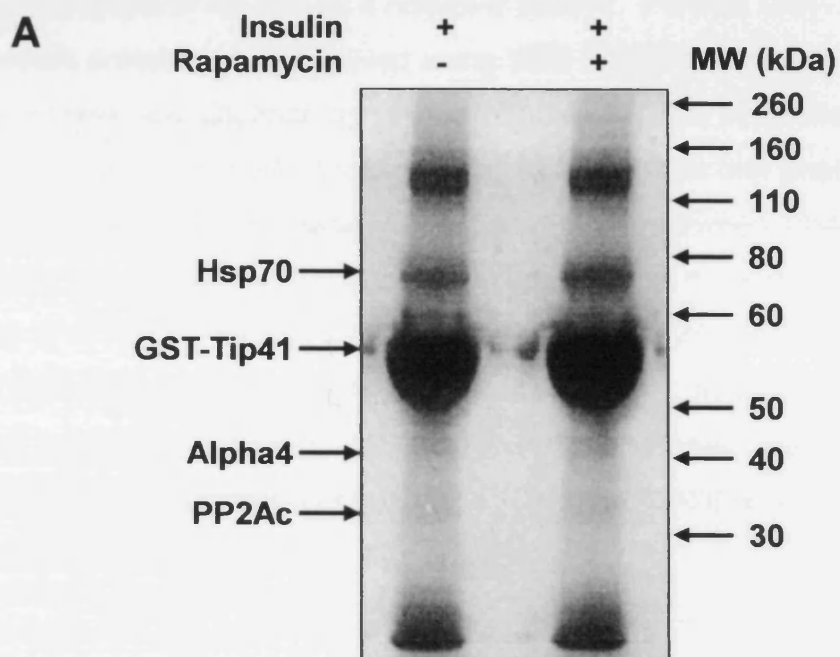
3.2.2 Tip41 interacts in complex with PP2Ac and Alpha4

To achieve higher quality purification, human *Tip41* was subcloned into an N-terminal GST-tagged vector. GST is of bacterial origin and consequently is not expressed endogenously in mammalian cells. This allows for high quality purification with little or no non-specific interactions when used to purify exogenous GST-tagged protein complexes. HEK293 cells were transfected with GST-tagged Tip41 cultured overnight in serum-free media, and prior to lysis were stimulated with insulin with or without rapamycin pre-treatment. GST-Tip41 from lysates was purified on Glutathione-Sepharose beads in a spin-trap column. After elution, purified sample was resolved by electrophoresis, fixed then stained with colloidal blue (Figure 3.2A). Although a number of interactions were identified, none of these were altered by rapamycin pre-treatment indicating that they may not be dependent on the activity of mTORC1. Regardless, samples were taken from each protein band and analysed by MALDI TOF/TOF mass spectrometry.

Although some of the protein bands could not be identified, mass spectrometry identified Heat Shock Protein 70 (Hsp70), Alpha4 and PP2Ac as potential Tip41 interacting proteins (Figure 3.2B). GST-Tip41 was also identified thus confirming expression and purification of the protein. Alpha4 and PP2Ac are human orthologues of Tap42 and Pph21/22 respectively, which suggests that a similar Tip41 complex exists in mammalian cells to that seen in yeast. Hsp70 on the other hand is a novel Tip41 interacting protein.

To confirm these interactions with Tip41, western blot was used. Following overnight culture in serum free media, cells expressing GST-Tip41 were lysed following pre-

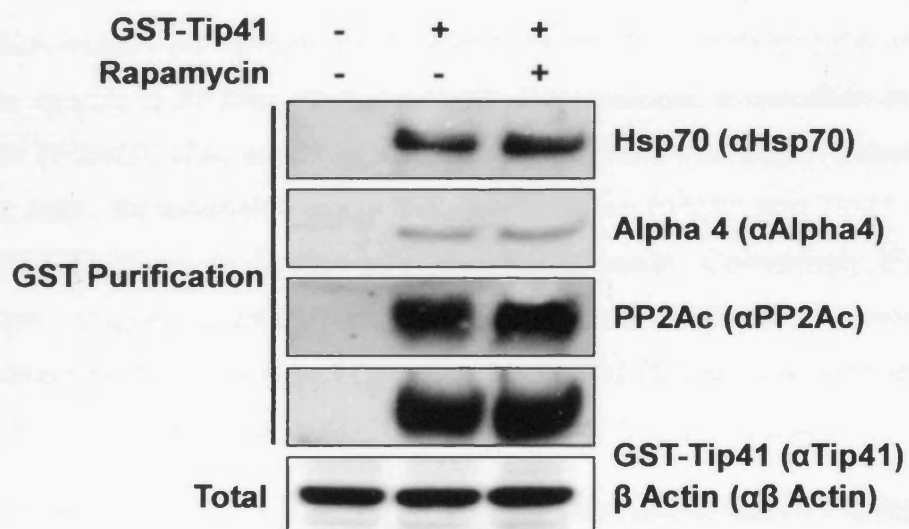
Figure 3.2: Tip41 interacts with PP2Ac, Alpha4 and Hsp70. **A.** HEK293 Cells transfected with pDEST27-GST-Tip41 were serum starved overnight and prior to lysis treated with insulin and rapamycin as indicated. Following purification of GST-Tip41 using a spin-trap column, samples were resolved by SDS PAGE and stained with colloidal blue. Proteins purified were visible as distinct bands. Gel plugs from each band were excised and used for MALDI TOF/TOF MS analysis. **B.** Analysis of the MS data revealed interaction with Alpha4, PP2Ac and Hsp70. The MS data was queried using Mascot. Table lists the E values (Expected) for the proteins identified. **C.** GST-Tip41 was purified as in **A** and samples resolved by SDS PAGE. Results identifying Tip41-interacting proteins from MS were confirmed by western blot. Results are representative of three independent experiments.



B

Protein Name	Accession No.	Number of Peptides	Mascot Score	E Values
Tip41	O75663	24	555	1.9×10^{-51}
Alpha4	P78318	17	145	1.9×10^{-10}
PP2Ac	P62714	22	320	6.2×10^{-28}
Hsp70	P08107	27	451	4.8×10^{-41}

C



treatment with rapamycin (as indicated) and stimulation with insulin. One set of cells were untransfected for use as a negative control. Purified GST-Tip41 and associated proteins were resolved using SDS PAGE followed by western blot (Figure 3.2C). Protein identification by mass spectrometry was confirmed by detection of the Hsp70, Alpha4 and PP2Ac proteins purified by western blot analysis. This raised the possibility of a conserved tertiary complex existing between Tip41, PP2Ac and Alpha4 in mammalian cells, although interactions did not appear to depend on the activity of mTORC1 as levels of these co-purified PP2Ac and Alpha4 proteins were equal both in the presence or absence of rapamycin. In addition, this work uncovers a novel interaction between Tip41 and Hsp70. Of interest, Hsp70 has been implicated in the regulation of the mTOR pathway, which will be further discussed in discussion.

3.2.3 Tip41 interacts directly with PP2Ac, but not with Alpha4

In yeast, Tip41 binds to Pph21/22 via Tap42. Following the identification of a possible tertiary complex between Tip41, PP2Ac (the human orthologue of Pph21/22) and Alpha4 (the human orthologue of Tap42) in mammalian cells, it was important to further characterise these interactions. As a single E42A point mutation of PP2Ac was found to reduce affinity for Alpha4, as much as 80%, this mutant was used to further investigate direct protein-protein interactions (Prickett & Brautigan, 2004). As less Alpha4 binds to the PP2Ac(E42A) mutant, the amount of Tip41 that co-purifies with the E42A mutant (in comparison to wild type PP2Ac) will determine whether Tip41 binds directly to PP2Ac, Alpha4 or both. For example, a reduction in Tip41 purified with PP2Ac(E42A) would indicate a direct protein interaction between Tip41 and Alpha4 only. By extension the interaction between PP2Ac and Tip41 in this model would take place via Alpha4 as is the case in yeast. Conversely, if equal levels of Tip41 co-purified with PP2Ac wild-type and mutant E42A, this would indicate a direct protein interaction between Tip41 and PP2Ac only, with the interaction between Alpha4 and Tip41 seen in Figure 3.2 taking place via PP2Ac.

HEK293 cells were transfected with GST-PP2Ac wild-type or GST-PP2Ac(E42A) mutated GST-PP2Ac, along with V5-Tip41. Cells transfected with V5-Tip41 along with empty vector was employed as a negative control. Following lysis, GST-PP2Ac was purified on Glutathione-Sepharose beads using a spin-trap column (Figure 3.3A).

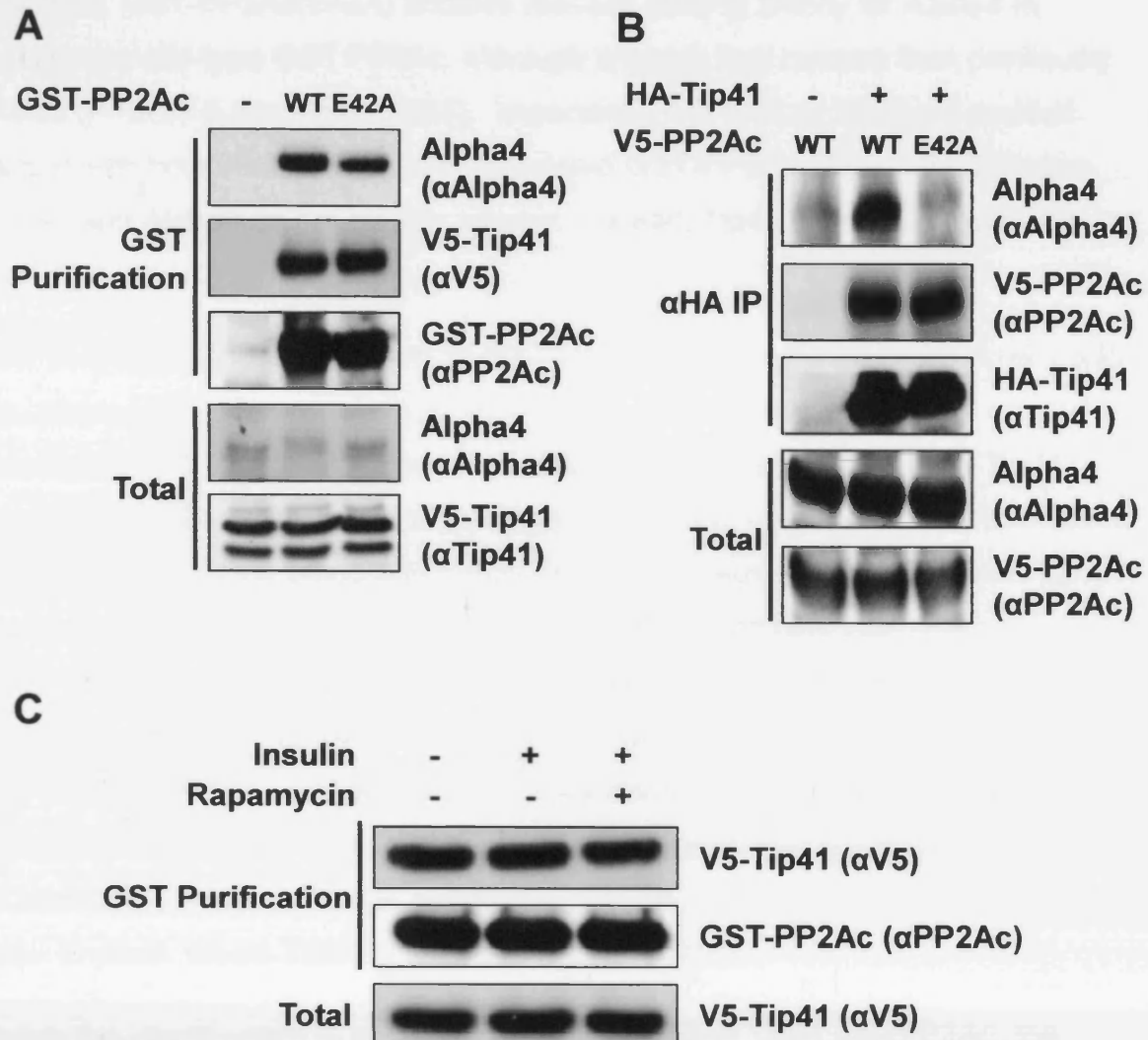


Figure 3.3: Tip41 interacts directly with PP2Ac. **A.** PP2A(E42A) interacts less effectively with PP2Ac. GST-PP2Ac wild type and E42A mutant were transiently expressed in HEK293 cells, purified and interaction with Tip41 and Alpha4 compared. Comparatively less Alpha4 purified with GST-PP2Ac(E42A) in comparison to wild type, indicating that mutant PP2Ac binds less effectively to Alpha4. Equal levels of Tip41 purified with both wild type and mutant PP2Ac. **B.** HA-Tip41 was co-transfected with V5-PP2Ac wild type and E42A mutant and expressed HA-Tip41 purified by immunoprecipitation. Levels of co-purified V5-PP2Ac(E42A) were equal to wild type, whereas comparatively less Alpha4 co-purified with HA-Tip41 and V5-PP2Ac(E42A). **C.** Interaction between PP2Ac and Tip41 was analysed in response to serum starvation, insulin stimulation and rapamycin treatment. GST-PP2Ac was co-expressed with V5-Tip41. Cells were cultured overnight in serum free media and treated with insulin and rapamycin prior to lysis as indicated. Levels of V5-Tip41 purified with GST-PP2Ac were unchanged by the cellular conditions indicated. Results are representative of three independent experiments.

As expected, GST-PP2Ac(E42A) showed reduced binding affinity for Alpha4 in comparison to wild-type GST PP2Ac, although this was less marked than previously described (Prickett & Brautigan, 2004). Importantly, the level of V5-Tip41 purified was equal with both wild-type and E42A mutated GST-PP2Ac. This data indicates that Tip41 and Alpha4 do not directly interact. Instead, Tip41 interacts directly with PP2Ac.

In order to confirm what was shown by GST purification, a reciprocal experiment was set up, where HA-Tip41 was purified with anti-HA antibodies from HEK293 cells and association of V5-PP2Ac was determined. Cells were transfected with HA-Tip41 along with V5-PP2Ac wild-type or E42A mutant. HEK293 cells were also transfected with V5-PP2Ac wild-type along with empty vector as a negative control. Less Alpha4 co-purified with the Tip41/PP2Ac(E42A) mutant protein complex than with Tip41/PP2Ac (Figure 3.3B). Both the wild-type and the E42A mutant V5-PP2Ac protein co-purified with HA-Tip41 to equivalent levels and again indicate that Tip41 directly binds to PP2Ac and not Alpha4. These interaction studies show that this phosphatase complex in mammalian cells exists as Tip41-PP2Ac-Alpha4 with no direct interaction between Alpha4 and Tip41. This is in contrast to the phosphatase complex in yeast, where Tip41 binds Pph21/22 via Tap42.

Following the identification of a direct interaction between Tip41 and PP2Ac, the nature of that interaction and the conditions under which it took place were of interest. As a PP2Ac interacting protein, it is possible that Tip41 acts as a regulatory subunit in mammalian cells. Regulatory PP2Ac subunits are thought to control substrate interactions and possibly mediate catalytic activity. As the regulation of PP2A is little understood, the possible control of PP2Ac_{Tip41} by intra-complex interactions was investigated. In order to determine whether the interaction was dependent on the activity of the mTORC1 signalling pathway, GST-PP2Ac was cotransfected with V5-Tip41 in HEK293 cells. Prior to lysis, whilst one set of cells was kept under serum-starved conditions, two sets were treated with insulin in the presence or absence of rapamycin pre-treatment. GST-PP2Ac was then purified on Glutathione-Sepharose beads using spin-trap columns (Figure 3.3C). V5-Tip41 co-purified with GST-PP2Ac to equal levels under all conditions. This indicates that the interaction between PP2Ac-Tip41 is not altered upon differences of mTORC1 signalling. If mTORC1 signalling affects PP2Ac-Tip41 phosphatase activity in cells, it is possible that this

phosphatase activity may instead be controlled by post-translational modification, cellular localisation, substrate targeting or a combination of these.

3.2.4 PP2Ac interacts with S6K1 in response to mTORC1 inhibition

PP2A is known to dephosphorylate mTORC1 substrates in response to rapamycin treatment (Schalm et al., 2005). Following identification of a direct interaction between Tip41 and PP2Ac, regulation in the context of the mTORC1 pathway was investigated. It is possible that Tip41 may regulate PP2Ac directly towards mTORC1 substrates. As Figure 3.3C shows no change in the interaction between PP2Ac and Tip41 following inhibition of mTORC1, the interaction between PP2Ac and S6K1 was of interest as regulation of PP2A/Tip41 may take place via substrate binding rather than alteration of interactions within the Tip41-PP2Ac-Alpha4 phosphatase complex. Interaction between PP2Ac and S6K1 was investigated. HEK293 cells were co-transfected with GST-PP2Ac along with HA-S6K1. A set of cells was transfected with HA-S6K1 along with empty vector and served as a negative control. Cells were cultured overnight in serum free media and prior to lysis, were treated with insulin in the presence or absence of rapamycin pre-treatment. GST-PP2Ac was then purified on Glutathione-Sepharose and the interaction of HA-S6K1 determined. Densitometry figures were calculated by averaging absolute signal intensity between three experiments, then converting to % relative intensity taking the highest intensity as 100% as detailed in 2.4.20. Error is indicative of standard deviation. Significance was calculated using a student's T test.

Binding between GST-PP2Ac and HA-S6K1 was identified under both mTORC1 stimulatory and inhibitory conditions, although the interaction was increased 2 fold by rapamycin ($p < 0.05$) (Figure 3.4). These data indicate that a PP2Ac complex binds S6K1, and this interaction is increased following inhibition of mTOR by treatment with rapamycin. In addition to Figure 3.3C, this shows that rather than interactions within the Tip41-PP2Ac-Alpha4 phosphatase complex being regulated by mTORC1, it is the interaction with substrates such as S6K1 that are instead modified in order to alter phosphorylation and therefore activity downstream of mTORC1.

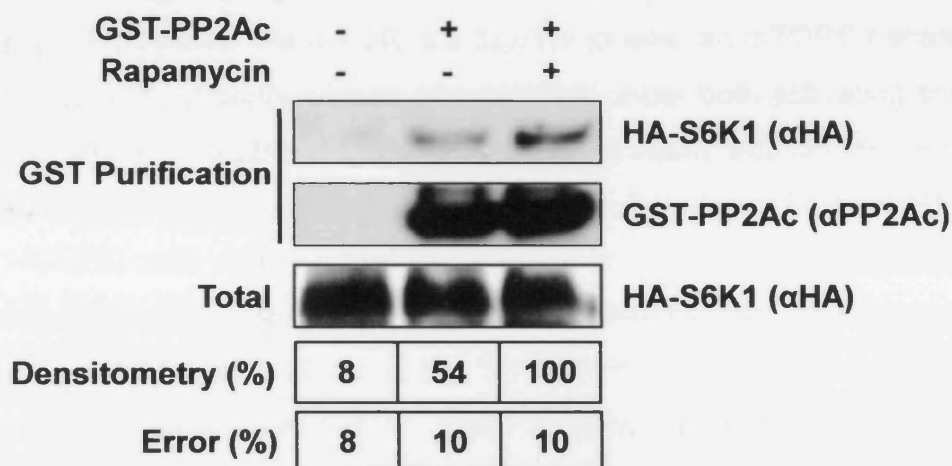


Figure 3.4: PP2Ac interaction with S6K1 is increased by rapamycin treatment. GST-PP2Ac was transiently expressed along with HA-S6K1, purified and levels of co-purified HA-S6K1 analysed by SDS PAGE and western blot. Whereas low levels of HA-S6K1 co-purified with GST-PP2Ac following insulin treatment, rapamycin increased the interaction between the two proteins. Densitometry analysis showed that rapamycin treatment almost doubled the amount of HA-S6K1 interacting with GST-PP2Ac in comparison to insulin stimulation ($p < 0.05$). Results are representative of three independent experiments. Densitometry figures were calculated by averaging absolute signal intensity between three experiments, then converting to % relative intensity taking the highest intensity as 100%. Error is representative of standard deviation. A T test was used to calculate significance.

3.2.5 Wortmannin but not staurosporine effectively inhibits mTORC1 *in vitro*

If Tip41 modulates PP2Ac activity towards substrates by altering substrate binding, rather than by altering binding with PP2Ac itself, it is important to understand how this regulation takes place. As mTOR is a Ser/Thr kinase, an mTORC1 assay was developed to quantify phosphorylation of substrates under both activating and inhibitory conditions. In addition, the specificity of the assay was determined using a F114A mutant 4EBP1 substrate that does not bind to Raptor within the mTORC1 complex. HEK293 cells were transfected with Myc-mTOR and HA-Raptor and purified using α Myc antibodies coupled to Protein G-Sepharose. The assay was then set up as in 'Materials and Methods'. GST-4EBP1 wild-type was used as a substrate along with an F114A mutant. This F114A mutation falls within the TOS motif of 4EBP1 at its extreme C-terminus, which is essential for Raptor binding and therefore phosphorylation by the mTORC1 complex. A duplicate assay using ERK was set up as a positive control, as ERK can also phosphorylate the Proline-directed Ser, and Thr residues within 4EBP1 at least *in vitro* (Schalm et al., 2005). After the mTORC1 assay, the samples were resolved by SDS PAGE and analysed by western blot to determine 4EBP1 phosphorylation. Analysis of 4EBP1 P-Thr36/45 shows that mTORC1 phosphorylated wild-type 4EBP1 but not the F114A mutant (Figure 3.5A). This shows that the TOS motif is essential for phosphorylation of 4EBP1 by mTORC1. As ERK phosphorylated both wild-type 4EBP1 and the F114A mutant equally, the requirement for the TOS motif is specific to mTORC1.

To identify an inhibitor that could be used in future assays, and to identify the concentration required for inhibition, an mTORC1 assay was set up with a panel of known mTORC1 inhibitors at differing concentrations. The mTORC1 assay was first set up as described in 'Materials and Methods', with GST-4EBP1 wild-type used as a substrate in all assays. Inhibitors were added immediately before commencement of the assay. These assays were then subjected to SDS PAGE and analysed by western blot (Figure 3.5B). 4EBP1 phosphorylation at Thr36/45 showed that the FKBP12/rapamycin complex effectively reduced mTORC1 activity within the assay as shown by a reduction in its phosphorylation. Wortmannin and LY294002 are both specific and competitive PI3K inhibitors that prevent ATP binding (Walker et al., 2000). Both wortmannin and LY294002 reduced mTORC1 activity when incubated with the assay at 20 μ M concentration, with wortmannin resulting in a slight decrease

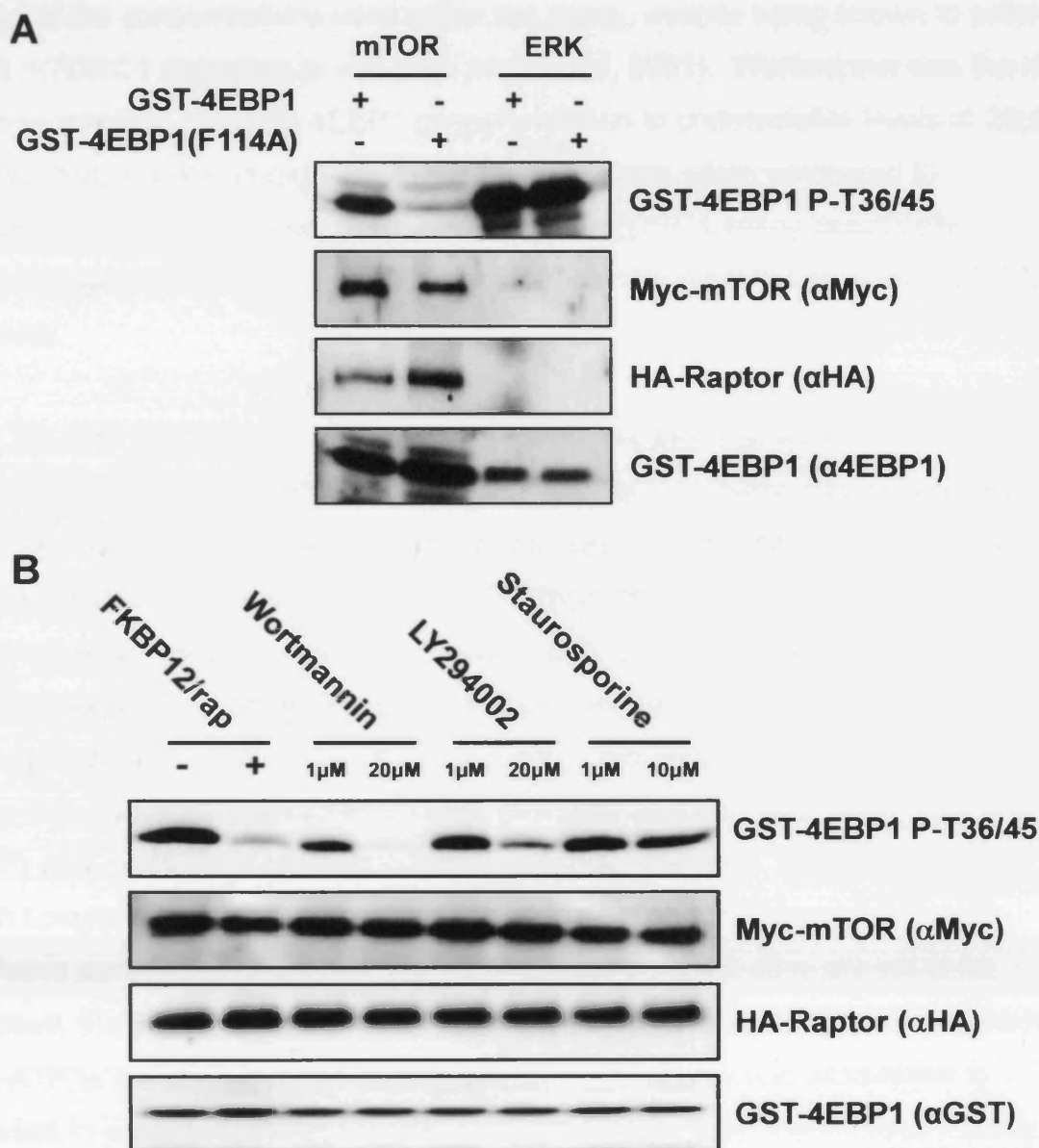


Figure 3.5: Optimisation of the mTORC1 kinase assay. **A.** The mTORC1 assay was optimised using the well-characterised substrate 4EBP1 as an indicator of successful purification of active complex. GST-4EBP1(F114A), corresponding to a mutation of the TOS motif, was phosphorylated less effectively than the wild type protein. ERK was used as a positive control, and phosphorylated wild type and F114A mutated 4EBP1 equally. **B.** In order to find an effective inhibitor, the assay was conducted in the presence of a number of known mTORC1 inhibitors at varying concentrations. Wortmannin at 20μM virtually abolished the ability of mTORC1 to phosphorylate GST-4EBP1, and was used in further assays. All results are representative of three independent experiments.

at 1 μ M. The broad spectrum kinase inhibitor staurosporine failed to inhibit mTORC1 activity at the concentrations used within the assay, despite being known to potently inhibit mTORC1 signalling *in vivo* (Tee and Proud, 2001). Wortmannin was the most effective inhibitor, reducing 4EBP1 phosphorylation to undetectable levels at 20 μ M, and resulting in lower phosphorylation of the substrate when compared to rapamycin/FKBP12. These data show that the mTORC1 assay specifically phosphorylates mTORC1 substrates and is effectively inhibited by known mTORC1 inhibitors.

3.2.6 Tip41 is not directly phosphorylated by mTORC1 *in vitro*

Figure 3.4 shows that the PP2A_{Tip41}-substrate tertiary complex may be regulated by substrate interactions that are specific to the activity of mTORC1. In yeast, Tip41 was shown to be phosphorylated in a TOR-dependent manner. Tip41 phosphorylation may be involved in regulating PP2Ac-substrate interaction in mammalian cells. To determine whether Tip41, as a potential PP2Ac regulatory subunit, is phosphorylated directly by mTORC1, the mTORC1 assay was used. The assay was prepared as described in the 'Materials and Methods'. Both GST-tagged 4EBP1 and Tip41 were used as substrates within the assay. 20 μ M wortmannin, which I previously confirmed as a potent inhibitor of mTOR (Figure 3.5B), was employed as a negative control. As Tip41 phosphorylation sites are yet to be identified, the assay relied on the incorporation of [³²P]-radiolabel into Tip41 from γ -[³²P]-ATP to identify substrate phosphorylation. The assay was assembled in duplicate to provide samples for total protein analysis. After the mTORC1 assay, the kinase assay reactions were resolved by SDS PAGE and after fixing, the gel was dried down and [³²P]-radiolabel incorporation determined by autoradiography (Figure 3.6). The duplicate assays containing cold ATP only were also resolved by SDS PAGE and then analysed by western blot. Autoradiography identified phosphorylation of 4EBP1 by mTORC1 which was inhibited by wortmannin as expected. Phosphorylation of Tip41 was not detected. This indicates that Tip41 is not directly phosphorylated by mTORC1 *in vitro* and suggests that Tip41 may be regulated by some other mechanism.

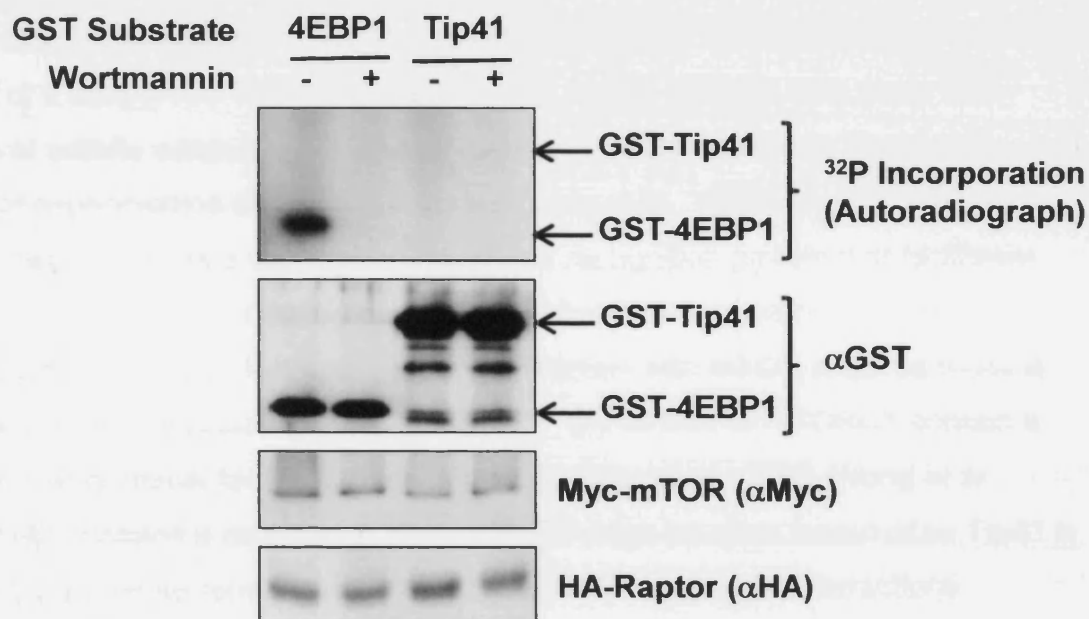


Figure 3.6: Tip41 is not a direct substrate of mTORC1. GST-Tip41 was used as a substrate in the mTORC1 kinase assay. As no phospho-antibodies are available for Tip41, the mTORC1 assay was supplemented with γ - ^{32}P ATP, and incorporation of ^{32}P into substrates quantified by autoradiography. GST-4EBP1 was used as a positive control. The purified mTORC1 complex robustly phosphorylated GST-4EBP1, which was inhibited by $20\mu\text{M}$ wortmannin. No phosphorylation of GST-Tip41 was observed, indicating that Tip41 is not a direct substrate of mTORC1. Results are representative of three independent experiments.

3.2.7 Tip41 may downregulate Raptor via proteasomal degradation

Phosphorylation of Tip41 was not detected within the *in vitro* mTORC1 assay. It is, however, still possible that mTORC1 might phosphorylate Tip41 *in vivo* due to the presence of a conserved TOS motif (Figure 1.5). As the assay is only an *in vitro* indication of activity within the purified complex, mTORC1 binding to Tip41 and potential phosphorylation should not be ruled out in cells. Within the mTORC1 complex, Raptor acts as a substrate binding and recognition protein that facilitates optimal substrate phosphorylation by mTOR. Substrate recognition by Raptor requires the RNC domain, whereas Raptor interaction with mTOR requires multiple interactions within the protein (Kim et al., 2002). Substrates of mTORC1 contain a TOS motif that is critical for Raptor interaction (Schalm et al., 2003, Wang et al., 2003). Tip41 contains a conserved TOS motif although whether mammalian Tip41 is an mTORC1 substrate remains to be discovered. To investigate interactions between Tip41 and mTORC1, co-immunoprecipitation between Tip41 and Raptor was performed. HEK293 cells were co-transfected with Myc-Raptor along with HA-tagged 4EBP1, Tip41 and S6K1. One set of cells was transfected with Myc-Raptor along with empty vector and serves as a negative control. After lysis, HA-tagged substrates were purified using α HA antibodies bound to Protein G-Sepharose beads. Samples were then resolved by SDS PAGE and analysed by western blot.

Whilst Myc-Raptor co-purified with HA-4EBP1, co-purification of Raptor was not enhanced when either HA-Tip41 or HA-S6K1 was purified from lysates (Figure 3.7A). This experiment indicates that the interaction between 4EBP1 and Raptor is robust, while Raptor interaction with S6K1, which is a well documented substrate of mTORC1 and contains a putative and well characterised TOS motif, is a much weaker interactor. This result is in line with other research groups who have also documented that they are unable to see an interaction between S6K1 and Raptor using immunoprecipitation techniques (Schalm et al., 2005). Due to limitation of this binding assay, the interaction of Tip41 with Raptor cannot be ruled out *in vivo*.

Of interest, densitometry analysis revealed that Raptor levels halved when co-expressed with HA-Tip41 ($p < 0.05$). Densitometry figures were calculated by averaging absolute signal intensity of totalMyc-Raptor between three experiments, then converting to % relative intensity taking the highest intensity as 100% as

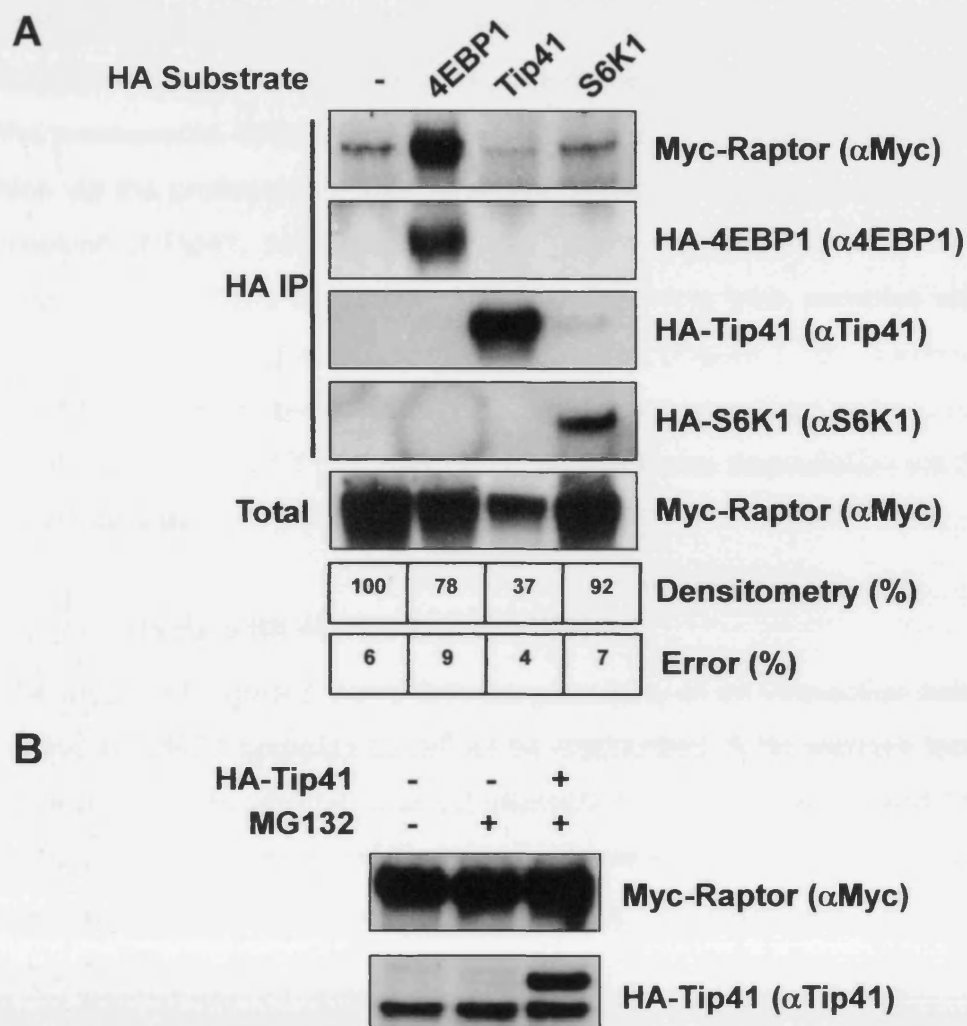


Figure 3.7: Raptor-substrate interaction analysis. **A.** Expressed Myc-Raptor was co-purified (α HA immunoprecipitation) with expressed HA-tagged 4EBP1, S6K1 or Tip41. Levels of Myc-Raptor in the immunoprecipitate were then analysed by SDS PAGE followed by western blot. Densitometry figures regarding total Myc-Raptor levels were calculated by averaging absolute signal intensity between three experiments, then converting to % relative intensity taking the highest intensity as 100%. A T test was then used to calculate significant differences between total Myc-Raptor when co-expressed with HA-Tip41 compared to when co-expressed with HA-4EBP1 or HA-S6K1. Error is indicative of standard deviation. Whilst HA-4EBP1 co-purified in complex with Raptor, concurrent with 4EBP1 as a substrate of mTORC1, HA-tagged S6K1 and Tip41 did not. Co-expression of Myc-Raptor with HA-Tip41 resulted in downregulation of Raptor protein. Densitometry analysis showed that levels were 37% of those seen in control cells ($p < 0.05$). **B.** To investigate the possibility that Tip41 overexpression was inducing proteasomal degradation of Raptor, HA-Tip41 was co-expressed with Myc-Raptor and cells treated with the proteasomal inhibitor MG132 2 hours prior to lysis. MG132 equalised levels of Myc-Raptor co-expressed with HA-Tip41. Results are representative of three independent experiments. Densitometry figures were calculated by averaging absolute signal intensity between three experiments, then converting to % relative intensity taking the highest intensity as 100%.

detailed in 2.4.20. Error is indicative of standard deviation, and a student's T test was used to ascertain significance. In order to check whether this was due to poor co-transfection or co-expression problems, or whether Tip41 may be destabilising Raptor, the proteasome inhibitor MG132 was used. If Tip41 leads to Raptor degradation via the proteasome, MG132 should equalise Raptor protein levels upon over-expression of Tip41. HA-Tip41 was again co-transfected with Myc-Raptor in the presence of MG132 two hours prior to lysis. Following lysis, samples were resolved by SDS PAGE and analysed by western blot (Figure 3.7B). Levels of Myc-Raptor in the presence of co-expressed HA-Tip41 were equalised by treatment with MG132. This indicates that Tip41 may be inducing Raptor degradation via the proteosomal pathway.

3.2.8 Raptor interacts with 4EBP1 but not Tip41

Despite the results of Figure 3.6 and 3.7, the possibility of an interaction between Tip41 and the mTORC1 complex could not be discounted. A far western technique was adopted to examine whether a direct interaction between Raptor and Tip41 was possible. To do this technique, GST-purified substrate was dotted onto PVDF membrane and incubated with cell lysate containing over-expressed HA-Raptor. Interaction between Raptor and the substrates was then determined with α HA antibodies. Firstly, this technique was optimised using a series of HA-Raptor mutants. Cell lysates were prepared containing over-expressed HA-Raptor wild-type or one of the six mutants. Mutants 1, 2, 3 and 4 all fall within the RNC domain, known to be involved in substrate binding. Mutant 7 contains a point mutation within an uncharacterised region of Raptor between the RNC domain and WD40 repeats. Mutant 9 contains a point mutation within the WD40 repeats. Lysate generated from cells transfected with empty vector was also used as a negative control. Purified GST-4EBP1 wild-type and F114A were dotted onto PVDF membrane and incubated overnight with the cell lysates. HA-Raptor interaction was then visualised using α HA antibodies followed by normal western blot protocol (Figure 3.8A).

The results show interaction between wild type HA Raptor and GST-4EBP1 wild-type, although no interaction was detected with GST-4EBP1(F114A). This is concurrent with results from Figures 3.5 and 3.7 and shows that the TOS signalling motif is essential for Raptor binding and therefore phosphorylation by mTOR. HA-Raptor

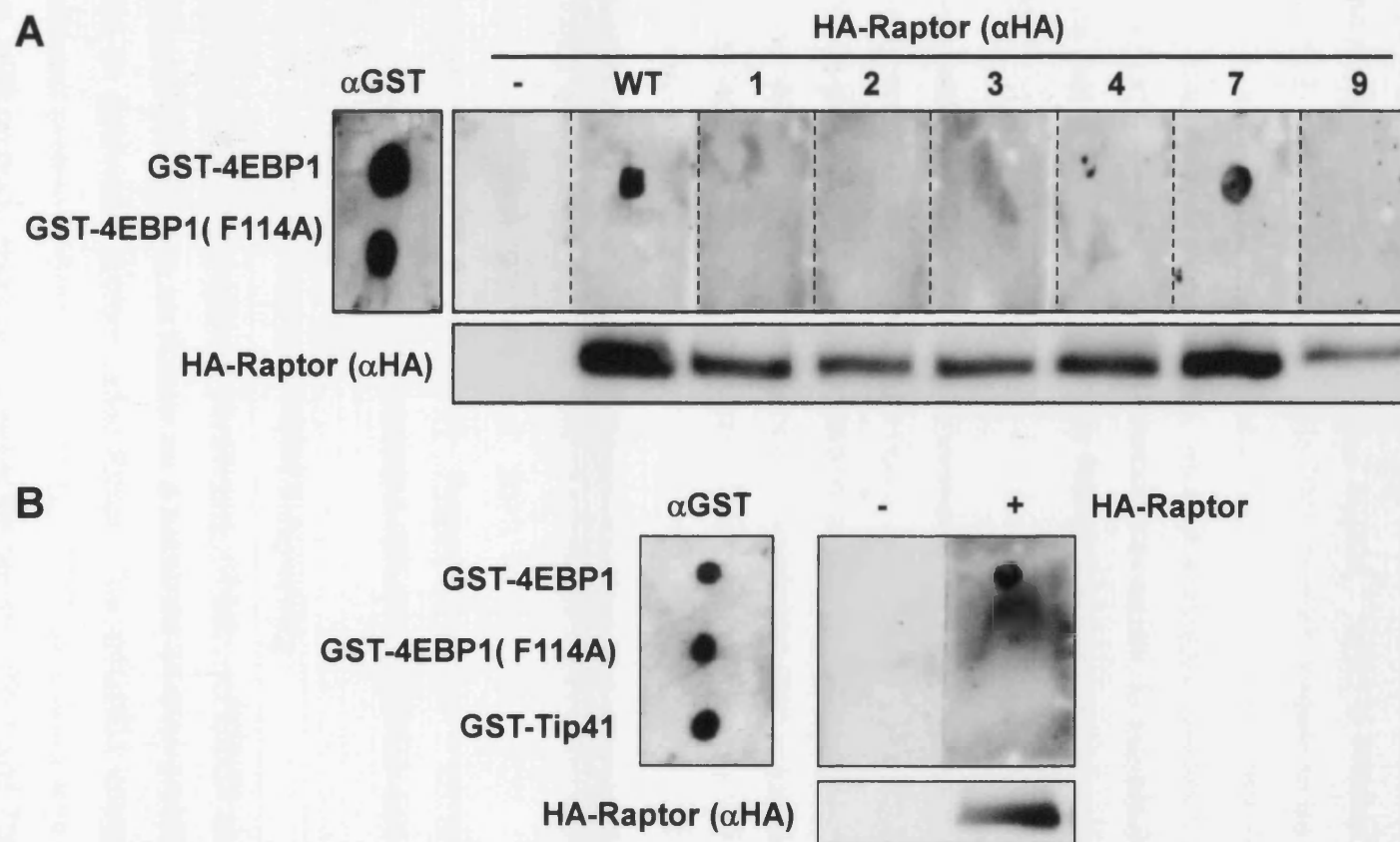


Figure 3.8: Multiple protein-protein interactions occur between Raptor and 4EBP1. **A.** A series of Raptor mutants containing point mutations within a number of domains were analysed for interaction with 4EBP1. Mutants 1 to 4 contain point mutations within the RNC domain. Mutant 4 contains a point mutation within an uncharacterised region of Raptor. Mutant 9 contains a point mutation within the WD40 repeats. Purified GST-4EBP1 wild type and F114A were dotted onto PVDF membrane and incubated in HEK293 cell lysate containing transiently expressed HA-Raptor wild-type or mutant. The presence of HA-Raptor bound to 4EBP1 was then analysed by western blot. HA-Raptor interacted with wild type 4EBP1, as did HA-Raptor mutant 7. GST-4EBP1(F114A) did not interact with wild-type or any mutant Raptor protein. **B.** The same technique was applied to analyse interaction between HA-Raptor (wild type only) and Tip41. GST-Tip41 was dotted onto PVDF membrane along with GST-4EBP1 wild type and F114A mutant, and incubated with HA-Raptor containing lysate. HA-Raptor did not interact with GST-Tip41, but as in A interaction was observed with wild-type 4EBP1. Results are representative of three independent experiments.

mutant 7 was also shown to interact with GST-4EBP1. This mutation falls within an uncharacterised region of Raptor between the HEAT and WD40 repeats, which does not appear to interfere with substrate binding (Kim et al., 2002). Binding of Raptor to mTOR requires multiple protein-protein interactions as only mutants 4 and 7 interact with mTOR (Kim et al., 2002). These data show that interaction with substrates also requires multiple interactions, as all but mutant 7 failed to interact with 4EBP1.

Mutants 1, 2, 3 and 4 all fall within the RNC domain, known to be involved in substrate binding. Thus the fact that point mutations within this domain abrogate substrate binding is of little surprise. Mutant 9 contains a point mutation within the WD40 repeats. Lack of binding of mutant 9 to 4EBP1 is therefore surprising and indicates that a range of points within Raptor are required for optimal substrate binding.

Following initial optimisation, the far western technique was used to identify possible interaction between HA-Raptor and Tip41. GST-4EBP1 wild-type, F114A and GST-Tip41 were dotted onto PVDF membrane and incubated overnight with cell lysate containing HA-Raptor. Lysate from cells transfected with empty vector was again used as a negative control. Membranes were then incubated with α -HA antibodies and visualised using the latter part of normal western blotting protocol. Figure 3.8B shows that HA-Raptor bound to wild-type but not F114A mutant 4EBP1 or Tip41. This shows that the TOS motif is critical for 4EBP1 binding to Raptor, in agreement with previous research (Schalm et al., 2003, Wang et al., 2003). This indicates that Raptor binds to 4EBP1 but not Tip41. Regulation of Tip41 therefore does not appear to be via phosphorylation by or interaction with the mTORC1 complex.

3.2.9 Tip41 is not involved in mTORC2 signalling

There are two known complexes containing mTOR - mTORC1 and mTORC2. Whereas mTORC1 relies on Raptor as a substrate binding protein, mTORC2 contains an equivalent protein called Rictor. The mTORC2 complex also contains an additional protein, mSin1. Although two TOR complexes also exist in yeast, there are two TOR proteins encoded by separate genes – *Tor1* and *Tor2*. As mammalian TOR is encoded by a single gene, functional redundancy may mean that Tip41 is a substrate of mTORC2. In addition, Tip41 purified with endogenous Hsp70 (Figure 3.2), a component of the mTORC2 complex (Martin et al., 2008). As Tip41 was

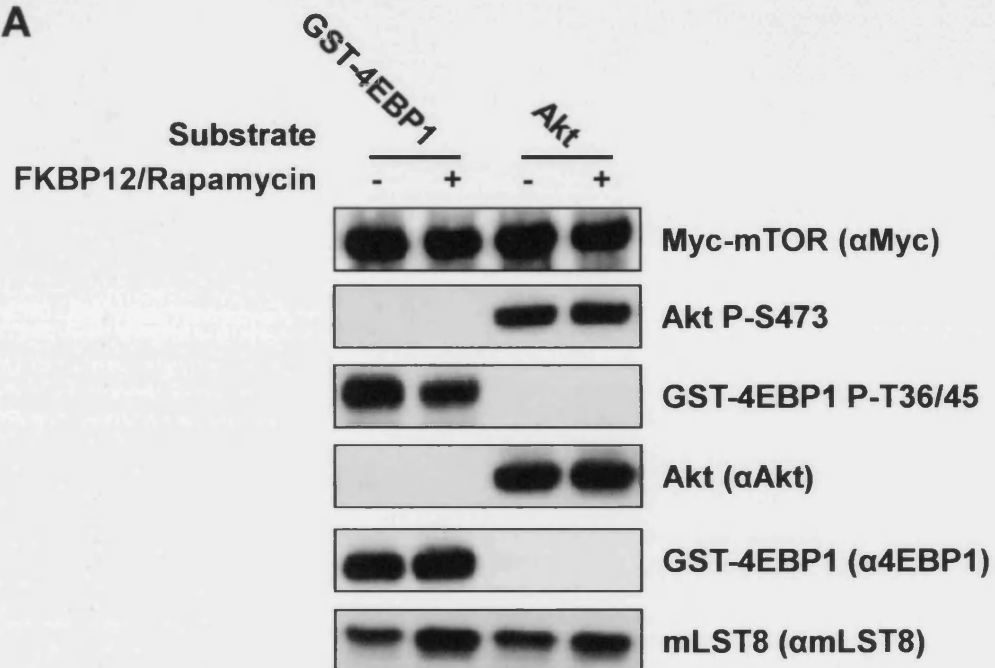
discounted as a substrate of the mTORC1 complex, the possibility of Tip41 as an mTORC2 substrate was investigated.

An *in vitro* mTORC2 kinase assay was optimised in order to do this. HEK293 cells were transfected with Myc-mTOR and Myc-Rictor and both proteins immunoprecipitated using α Myc antibodies. The mTORC2 assay was then set up as described in 'Materials and Methods'. Both purified GST-4EBP1 and Akt were used as substrates within the assay in the presence or absence of FKBP12/Rapamycin, providing both an mTORC1 and mTORC2 substrate, respectively. Following the mTORC2 assay, samples were resolved by SDS PAGE and analysed by western blot (Figure 3.9). Antibodies against 4EBP1 phosphorylation at Thr36/45 showed FKBP12/Rapamycin sensitive phosphorylation of the protein. This shows that some mTORC1 complex containing endogenous Raptor is purified in our assay. Phosphorylation of Akt at S473, the mTORC2 phosphorylation site, was also analysed. Purified mTORC2 phosphorylated Akt at this site and confirms the presence of this complex within the assay. This was not a FKBP12/Rapamycin sensitive phosphorylation event as expected, as mTORC2 is only inhibited in cells following rapamycin treatment at high concentrations. These data show that although some contaminating mTORC1 is purified within the assay, mTORC2 is present and able to phosphorylate the substrate Akt.

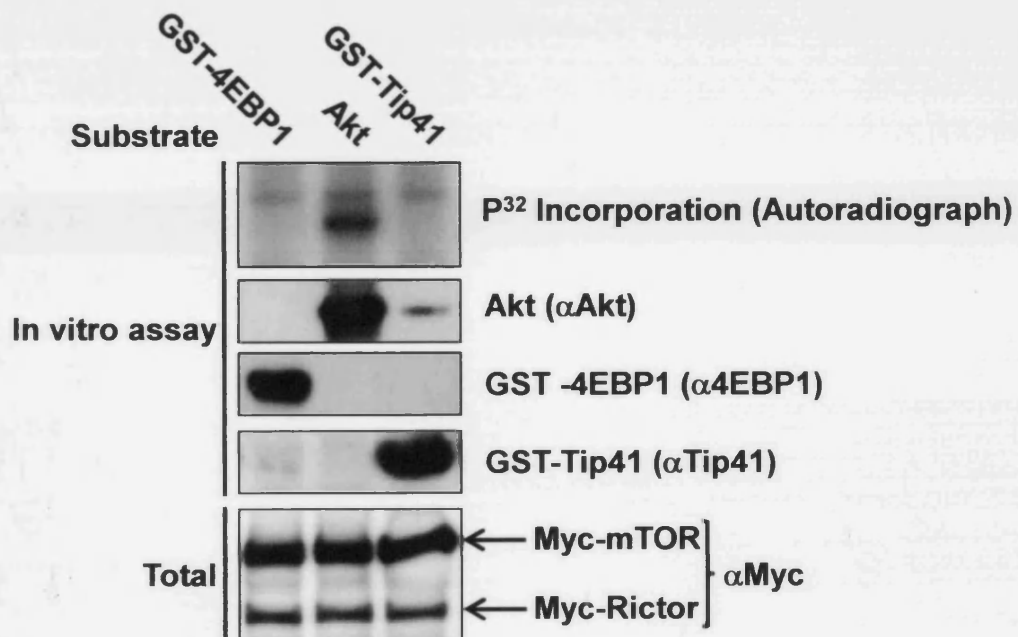
The assay was then used to investigate the possible phosphorylation of Tip41 by mTORC2 and was set up as described above and in the 'Materials and Methods'. Purified GST-4EBP1, inactive Akt and GST-Tip41 were used as substrates. As Tip41 phosphorylation sites are yet to be identified, the assay used γ -[32 P]-ATP to identify substrate phosphorylation. The assay was carried out in duplicate to also provide cold samples for total protein analysis. [32 P]-radiolabelled substrates were analysed after being resolved by SDS PAGE, fixed, dried down and subjected to autoradiography (Figure 3.9B). The mTORC2 assays carried containing only cold ATP were also resolved by SDS PAGE but were analysed by western blot. No 4EBP1 phosphorylation was detected within the mTORC2 assay indicating that there was little to no contamination of mTORC1 within the purified mTORC2. Strong phosphorylation of Akt indicated the presence of an active mTORC2 complex within this assay. No Tip41 phosphorylation was detected showing that Tip41 is not a direct substrate of mTORC2 under the conditions of this assay.

Figure 3.9: Tip41 is not a substrate for mTORC2. **A.** The mTORC2 kinase assay was optimised using Akt as a substrate. Control assays using GST-4EBP1 as substrate were also performed as an indicator of contamination of the assay with mTORC1. Akt was robustly phosphorylated in the assay, which was FKBP12/Rapamycin insensitive, indicating successful purification of active mTORC2. GST-4EBP1 was also phosphorylated in the assay, indicating a degree of contaminating mTORC1. **B.** The mTORC2 assay was used to analyse phosphorylation of Tip41 by mTORC2. As no Tip41 phospho-antibodies are available, the assay was supplemented with γ -[^{32}P]-ATP and substrate phosphorylation quantified by autoradiography. Akt phosphorylation was measured as a positive control. Whereas robust phosphorylation of Akt was observed, no ^{32}P was incorporated by GST-Tip41 indicating that the protein is not a substrate of mTORC2. In addition, no phosphorylation of 4EBP1 was observed indicating low levels of contaminating mTORC1. Results are representative of three independent experiments.

A



B



Although the results of Figure 3.9B discount the possibility of Tip41 as an mTORC2 substrate, it could act upstream of the complex *in vivo*. To investigate this V5-Tip41 was over-expressed in HEK293 cells and the phosphorylation of Akt at S473, the mTORC2 phosphorylation site, was visualised by SDS PAGE followed by western blot. Figure 3.10 shows that Akt phosphorylation at S473 was increased by insulin treatment but not affected by the over-expression of V5 Tip41. It therefore appears that Tip41 does not influence mTORC2 signalling. Combined with the results of Figure 3.9, Tip41 does not appear to act as a PP2Ac regulatory subunit in the mTORC2 signalling pathway. Of interest, analysis of total Tip41 levels revealed the possibility of post-translational modification of the protein, as evidenced on overexpression of V5-Tip41. Due to the small mobility shift apparent, it is possible that Tip41 is subject to phosphorylation.

3.2.10 Tip41 phosphorylation is negatively regulated by insulin signalling

Although Tip41 was not directly phosphorylated by mTORC1 or mTORC2 *in vitro*, phosphorylation of Tip41 by another kinase within the insulin signalling pathway was still possible. The phosphorylation of Tip41 was therefore investigated in response to insulin stimulation. Two methods were used to this end; *in vivo* [^{32}P]-radiolabelling and isoelectric focusing. The first investigation involved over-expression of GST-Tip41 in HEK293 cells followed by radiolabelling with [^{32}P]-orthophosphate. A set of cells was transfected with empty vector as a negative control. Prior to lysis, cells were maintained in phosphate free medium for 5 h supplemented with [^{32}P]-orthophosphate and stimulated with insulin with and without pre-treatment with rapamycin, as indicated. GST-Tip41 was then purified on Glutathione-Sepharose using spin-trap columns. Purified sample was then analysed by autoradiography after being resolved on SDS PAGE. Densitometry was calculated by averaging absolute signal intensity then converting to % setting the highest signal intensity at 100% as detailed in 2.4.20. Error is representative of standard deviation. Following confirmation of equal variances, p values were calculated using a student's T test. Figure 3.11A shows that GST-Tip41 is phosphorylated *in vivo*. Phosphorylation was maximal under serum-starved conditions and was reduced by insulin stimulation as shown by a reduction in signal intensity of approximately 20% ($p < 0.05$) under this condition. Rapamycin does not appear to have any effect on phosphorylation as no

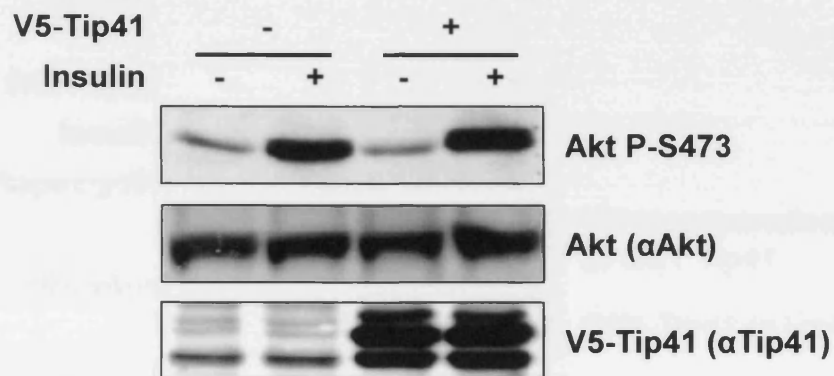


Figure 3.10: Tip41 does not act upstream of mTORC2. V5-Tip41 was transiently expressed in HEK293 cells and phosphorylation of Akt at S473, the mTORC2 sensitive site, analysed by SDS PAGE followed by western blot. The results showed that V5-Tip41 overexpression did not alter phosphorylation of Akt at S473. Results are representative of three independent experiments.

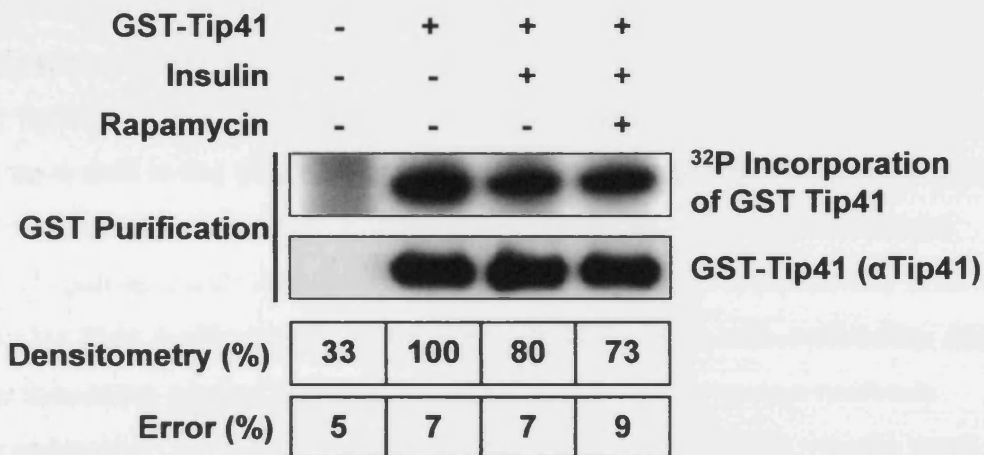
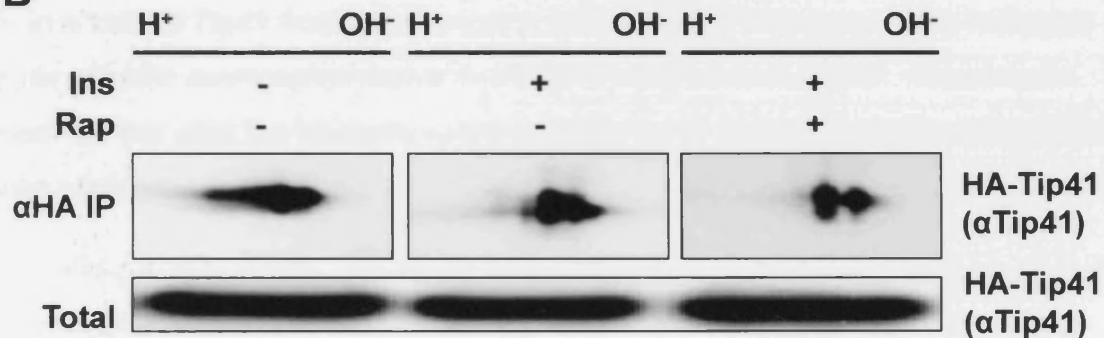
A**B**

Figure 3.11: Tip41 phosphorylation is sensitive to insulin stimulation. **A.** *In vivo* radiolabelling was used to analyse phosphorylation of overexpressed GST-Tip41. Cells were kept in phosphate-free media supplemented with γ -[^{32}P]-ATP for 5 hours prior to lysis. Autoradiography indicated that Tip41 is hyperphosphorylated under serum starved conditions, and phosphorylation reduced following insulin stimulation. Phosphorylation of Tip41 was not responsive to treatment with rapamycin. Densitometry figures were calculated by averaging absolute signal intensity between three experiments, then converting to % relative intensity taking the highest intensity as 100%. Error is indicative of standard deviation. A T test was then used to calculate p values. Analysis showed that insulin treatment reduced phosphorylation of Tip41 by 20% ($p=0.02$). **B.** Isoelectric focussing was also used to analyse phosphorylation of Tip41. Cells were transfected with HA-Tip41 and prior to lysis, cultured overnight in serum free media and treated with insulin and rapamycin as indicated. Expressed HA-Tip41 was immunoprecipitated (α HA) from cells and resolved by isoelectric focussing followed by SDS PAGE and western blot. These data also showed that Tip41 was hyperphosphorylated under serum starved conditions, and dephosphorylated in response to insulin, as shown by a shift towards the alkaline terminus. Again, rapamycin treatment did not alter apparent phosphorylation. As indicated by quantifying the number of migrating isoforms following maximal phosphorylation, Tip41 appears to undergo 4 phosphorylation events, two of which are sensitive to insulin stimulation. Results are representative of three independent experiments.

change in [^{32}P]-radiolabel incorporation was apparent in comparison to that following insulin stimulation alone ($p < 0.05$).

To confirm the phosphorylation of Tip41 *in vivo*, isoelectric focussing was used. Phosphorylation of proteins is detected by a shift towards the acidic (H^+) end of the isoelectric focussing gel. Similarly, protein dephosphorylation was detected as observed as a shift in the position of the Tip41 protein towards the alkaline (OH^-) end of the gel. HEK293 cells were transfected with HA-Tip41 and serum-starved overnight. Treatment with insulin and rapamycin took place immediately prior to lysis. HA-Tip41 was then purified using immunoprecipitation with αHA antibodies and isoelectric focussing carried out as described in the 'Materials and Methods'. Following separation by the second dimension using SDS PAGE, results were visualised by western blot analysis. Figure 3.11B shows that insulin treatment results in a shift of Tip41 from the H^+ to the OH^- terminal of the gel. This indicates an insulin specific dephosphorylation event occurring towards Tip41. Rapamycin treatment did not alter the phosphorylation status as no shift occurred in comparison to insulin stimulation alone.

Although difficult to quantify, isoelectric focusing suggests that there may be up to 4 phosphorylation sites within Tip41. Hyperphosphorylation, where the highest two phosphorylation sites are phosphorylated, appears to occur under serum-starved conditions. Two of these sites then appear to be dephosphorylated following treatment of cells with insulin, leaving two phosphorylation sites still present. This remains the case following rapamycin treatment. The results from Figure 3.11 show that Tip41 is regulated by phosphorylation. Hyperphosphorylation occurs under serum-starved conditions and insulin treatment results in dephosphorylation of possibly two of the four phosphorylation sites present within Tip41. This indicates that while Tip41 appears to be regulated by insulin signalling via phosphorylation, this regulation is not directly via mTORC1 as rapamycin has no effect on the phosphorylation of the protein. As phosphorylation of Tip41 is reduced on activation of the insulin signalling pathway, it appears that Tip41 is subject to an insulin specific dephosphorylation event.

3.3 Discussion

The results presented here show that Tip41 in mammalian cells stably binds PP2A independently of Alpha4, and may allosterically regulate PP2Ac activity towards substrates. Previous data show that Tip41 purifies as a protein complex with Alpha4 and PP2Ac, mimicking the Tip41-Tap42-Pph21/22 complex in yeast. Previous studies did not determine whether Tip41 bound PP2Ac directly or via Alpha4 (as in yeast) (Smetana & Zanchin, 2007). Purification of GST-Tip41 revealed a complex interaction with Alpha4 and PP2Ac (Figure 3.2). In order to define the exact interactions taking place between Alpha4, Tip41 and PP2Ac, GST-PP2Ac(E42A) mutant deficient in binding to Alpha4 was utilised (Prickett & Brautigan, 2004). Tip41 binds equally to wild-type and E42A mutant PP2Ac, indicating that Tip41 binds PP2Ac independently of Alpha4 (Figure 3.3). Whereas purification of GST-PP2Ac yielded little difference in Alpha4 interaction between WT and E42A, HA-Tip41 immunoprecipitation completely prevented Alpha4 interaction when mediated by V5-PP2Ac mutant E42A (Figure 3.3B – compare Alpha4 co-purified). This difference may provide further evidence for mutually exclusive binding of Alpha4 and Tip41 with PP2Ac.

Whereas immunoprecipitation of HA-Tip41 may provoke further loss of Alpha4 in comparison to GST-PP2Ac(E42A) due to indirect binding via PP2Ac, direct binding to GST-PP2Ac(E42A) allows the unstable interaction with Alpha4 to be maintained. Thus GST-PP2Ac(E42A) may bind Alpha4 less stably than wild-type, resulting in increased sensitivity of the interaction during immunoprecipitation indirectly with HA-Tip41. In yeast, Tip41 positively regulates Pph21/22 and Sit4 activity by removing the inhibitory subunit Tap42 in response to TORC1 signalling. During TORC1 inhibition, Tip41 sequesters the inhibitory subunit Tap42, resulting in activation of Pph21/22 and Sit4. Thus Tip41 is an indirect phosphatase activating protein (Jacinto et al., 2001). In contrast, Tip41 in human cells binds PP2Ac directly (Figure 3.3). Mammalian Tip41 may therefore be a regulatory subunit of PP2Ac.

Previous studies have shown that Tip41 negatively regulates PP2Ac activity *in vitro*, posing Tip41 as a potential negative regulatory subunit. As no A or B regulatory subunit was found in complex with PP2A_{Tip41} (Figure 3.2), Tip41 may act as a *bona fide* PP2Ac regulatory subunit. In contrast to the role of Tip41, the inhibition of

Alpha4 by PP2Ac has been extensively investigated. Alpha4 allosterically inhibits PP2Ac and binds independently of the regulatory A and B subunits (Chung et al., 1999, Prickett & Brautigan, 2004, Prickett & Brautigan, 2006). Tip41 may therefore have an analogous role towards PP2Ac, binding independently of A and B regulatory subunits to regulate activity of the phosphatase. As Tip41 may act as part of a complex with Alpha4 and PP2Ac, Tip41 and Alpha4 may have contrasting or compounding effects on PP2Ac. As both Alpha4 and Tip41 have been identified as PP2Ac inhibitors *in vitro*, both subunits could be acting in concert to inhibit PP2Ac (Chen et al., 1998, Nanahoshi et al., 1998, Nanahoshi et al., 1999, McConnell et al., 2007). In contrast, the role of Alpha4 as a PP2Ac inhibitor is not certain. For instance, studies have shown that Alpha4 can act as a positive PP2Ac regulatory subunit *in vivo* (Kong et al., 2004, Saleh et al., 2005, Nien et al., 2007, Prickett & Brautigan, 2007). Although Tip41 has been shown to inhibit PP2Ac activity *in vitro* (McConnell et al., 2007), this role may not translate *in vivo*. Owing to the unresolved regulatory mechanism of Alpha4 with PP2Ac (i.e., inhibitory or activating) a number of possibilities for the role of Tip41 exist, where it may also act as an activating or inhibitory PP2Ac subunit. For instance, Tip41 may inhibit whereas Alpha4 may activate PP2Ac activity, or vice versa. In contrast, both subunits may be inhibitory or activating towards PP2Ac. This requires further investigation.

In yeast, Tip41 regulates phosphatase activity downstream of TORC1. Therefore the possibility that Tip41 regulates PP2Ac downstream of mTORC1 was investigated. Raptor mediates mTOR substrate interaction, and Raptor binding is required for phosphorylation by mTOR. Binding within mTORC1 between Raptor and mTOR has been studied using a number of Raptor point mutations. The Raptor mutants were numbered 1, 2, 3, 4, 7 and 9. Raptor mutants 1, 2, 3, and 4 contain point mutations within the RNC domain critical for substrate recognition (Kim et al., 2002). Mutant 7 contains a point mutation in the uncharacterised region between the HEAT repeats and the WD40 repeats. Mutant 9 contains a point mutation within the fourth WD40 repeat (Kim et al., 2002). Thus the mutations encompass much of the structure of Raptor required for activity. Only mutants 4 and 7 were shown to bind mTOR, indicating that multiple interactions occur throughout Raptor sequence between mTOR and Raptor (Kim et al., 2002). In order to characterise Raptor-substrate binding, these mutants were analysed for their ability to bind 4EBP1. Only Raptor

mutant 7 interacted with 4EBP1, indicating that substrate interaction with Raptor also requires multiple protein-protein interactions (Figure 3.8A). As this mutation falls within the uncharacterised region of Raptor, this indicates that interaction takes place via the HEAT and WD40 repeats in addition to the RNC domain. Thus multiple points within Raptor are required for substrate binding.

Raptor substrate interaction requires a TOS motif within the substrate, which is a five amino acid sequence essential for Raptor binding (Schalm et al., 2003, Wang et al., 2003). The TOS motif within 4EBP1 is present at the C-terminus and comprises F₁₁₄EMDI. Whereas interaction between Raptor and wild-type 4EBP1 was observed, 4EBP1(F114A) did not bind Raptor (Figure 3.8). This highlights the importance of the TOS motif in Raptor-substrate interaction. This is in agreement with previous findings which identify the TOS motif as a requirement in regulating certain mTORC1 substrate phosphorylation events via binding to Raptor (Nojima et al., 2003, Schalm et al., 2003, Wang et al., 2003, Lee et al., 2008).

As Tip41 contains a putative TOS motif, its role as a potential mTORC1 substrate was investigated. Firstly, interaction between Raptor and Tip41 was investigated. No interaction between Tip41 and Raptor was observed in far western blot or on immunoprecipitation of Myc-Raptor (Figures 3.7 & 3.8). Interaction of Tip41 with Raptor cannot be completely ruled out as results in Figure 3.7 show that S6K1 did not interact with purified Myc-Raptor. This is in line with previous findings, where purified Raptor did not interact with S6K1 (Schalm et al., 2003). As S6K1 also contains a TOS motif and is a well-documented mTORC1 substrate, the possibility that Tip41 binds Raptor could not be completely ruled out.

In order to further investigate Tip41 as a potential mTORC1 substrate, an *in vitro* mTORC1 kinase assay was performed. Purified mTORC1 phosphorylated wild-type 4EBP1, but not 4EBP1(F114A) or Tip41 (Figure 3.5A). Combined with Figure 3.8, this agrees with previous data showing that Raptor interaction is essential for phosphorylation by mTORC1 as neither 4EBP1(F114A) or Tip41 co-purified with Raptor (Kim et al., 2002, Nojima et al., 2003, Schalm et al., 2003, Wang et al., 2003, Lee et al., 2008). The specificity of the TOS motif in mTORC1 signalling was shown by the ability of ERK to phosphorylate wild-type and 4EBP1(F114A) equally. This provides evidence that mutation of the TOS motif does not alter the structure of

4EBP1, but is specifically required for Raptor-mediated phosphorylation by mTOR. Tip41 was not phosphorylated by mTORC1 *in vitro*. Thus, although Tip41 contains a TOS motif, it is unable to be phosphorylated by mTORC1. This indicates that other protein-protein interactions mediate Raptor binding, and that although the TOS motif is required, it is not a key to phosphorylation by mTORC1. In support of this, 4EBP1 also contains a RAIP motif at the N terminus that is essential for Raptor binding (Lee et al., 2008). Thus multiple interactions mediate binding between Raptor and mTOR substrates. In combination with Figure 3.8, these data indicate that Tip41 is not a substrate of mTORC1. Regardless, Tip41 may act elsewhere upstream or downstream of mTORC1 to regulate PP2Ac activity.

In support of this, Tip41, possibly with PP2Ac, may alter Raptor stabilisation and therefore may act upstream or parallel to mTORC1. Over-expression of HA-Tip41 reduced levels of co-expressed Myc-Raptor which was equalised by treatment with the proteasome inhibitor MG132 (Figure 3.7). Tip41 may therefore promote Raptor degradation via the proteasome, which may be mediated by the phosphorylation status of Raptor. Following mTORC1 activation, Raptor is phosphorylated at S683 by mTOR which is required for phosphorylation of 4EBP1 and S6K1 (Wang et al., 2009). Inhibition of mTORC1 by AMPK is mediated in part by phosphorylation of Raptor at S722 and S792 which promote Raptor dissociation from mTORC1 and binding to 14-3-3 (Gwinn et al., 2008). Although some Raptor phosphorylation sites are required for mTORC1 activation, some are inhibitory and result in Raptor dissociation. Binding to 14-3-3 can protect proteins from proteasomal degradation (Li et al., 2002, Shumway et al., 2003, Cai et al., 2006). Thus, free Raptor may be prone to degradation, and it is conceivable that Tip41 alters the phosphorylation of Raptor, mediated by PP2Ac, resulting in dissociation from mTORC1 and proteasomal degradation. Again in this capacity it is possible that Tip41 acts as both a negative and positive PP2Ac regulatory subunit.

Following identification of a direct interaction between PP2Ac and Tip41 and the potential role of Tip41 as a regulatory subunit of PP2Ac, the mechanism of PP2Ac regulation was explored. PP2Ac can be regulated by a number of mechanisms including post-translational modification, regulatory subunit binding, and regulatory subunit phosphorylation. Analysis of PP2Ac purified with Tip41 revealed that the PP2A_{Tip41} interaction is not sensitive to rapamycin (Figure 3.3C), indicating that

PP2A is not regulated by Tip41 due to changes in interaction. This result indicates that if Tip41 acts as a PP2A regulatory subunit, other modes of modification are likely in order to allow regulation.

As interaction between PP2Ac and Tip41 is not regulated, the possibility that PP2A_{Tip41} is regulated by Tip41 phosphorylation was investigated. *In vivo* radio-labelling and 2D SDS PAGE both showed reduced phosphorylation of Tip41 on insulin stimulation, which was not sensitive to rapamycin (Figure 3.11). Although difficult to qualify, Tip41 appears to undergo four phosphorylation events on serum-starvation of cells (Figure 3.11B). On stimulation with insulin, two of these phosphorylation sites are removed. Dephosphorylation of Tip41 in response to insulin is unexpected as insulin signalling is associated with a general increase in phosphorylation events. Tip41 is not the only protein dephosphorylated in response to insulin signalling. In response to insulin, the translation initiation factor eIF2B is activated by dephosphorylation. This occurs by inhibition of the upstream kinase GSK3 by Akt-mediated phosphorylation. Akt phosphorylates and inactivates GSK3 leading to hypophosphorylation of eIF2B (Wang et al., 2001, Wang et al., 2002, Mariappan et al., 2008). This is required for progression from initiation to the elongation phase of translation. Thus Tip41 dephosphorylation in response to insulin may be caused by inactivating phosphorylation of an upstream kinase, such as GSK3, resulting in accumulation of hypophosphorylated Tip41. Phosphorylation of Tip41 may modify activity of the protein. Indeed phosphorylation of regulatory subunits is a documented mechanism of regulation of PP2A holoenzymes (Letourneux et al., 2006). Thus depending on its action as a PP2A activating or inhibitory subunit, Tip41 activity may be enhanced or hindered by phosphorylation on serum-starvation. As phosphorylation of Tip41 is not sensitive to rapamycin, Tip41 phosphorylation is regulated upstream of mTORC1. This is concurrent with Figure 3.3C which shows that Tip41 binding to PP2Ac is not sensitive to rapamycin. Therefore, mTORC1 does not directly regulate Tip41 phosphorylation.

Although PP2A_{Tip41} may act upstream or parallel to mTORC1, PP2A complexes appear to act in multiple points within the pathway. Figure 3.4 shows that PP2Ac binds directly to S6K1 in agreement with previous data showing that a PP2A complex directly dephosphorylates S6K1 (Peterson et al., 1999, Yamashita et al., 2005, Hahn et al., 2010). As the PP2Ac-S6K1 interaction increased markedly on

administration of rapamycin, it appears that PP2Ac activity is negatively regulated by mTORC1 and that interaction with S6K1 is the specific method of PP2A regulation downstream of mTORC1. Thus mTORC1, either directly or indirectly, may regulate a PP2A complex thus preventing interaction with S6K1. This agrees with a previously proposed model whereby mTORC1 binds and phosphorylates S6K1 thereby displacing PP2A. As the catalytically inactive S6K1 TOS mutant is rescued by phosphomimetic mutation of T389 and mutation of a putative PP2A binding motif, it was proposed that S6K1 activation requires removal of inhibitory phosphatase binding in addition to phosphorylation by mTORC1 (Schalm et al., 2005). The data in Figure 3.4 indicates a model where rapamycin inhibits mTORC1 thereby prevents binding to S6K1 allowing PP2A complex to bind and inhibit S6K1. Thus, active mTORC1 may displace PP2A allowing S6K1 phosphorylation. This also indicates that the basal state is PP2A in complex with S6K1, and activation of mTORC1 by upstream effectors acts to allow mTORC1 to remove this inhibitory basal state.

Binding of Tip41 to Hsp70 raised the interesting possibility that Tip41 is involved in mTORC2 signalling (Figure 3.2). Hsp70 is required for mTORC2 phosphorylation of Akt, and phosphorylation of Akt by mTORC2 is sensitive to heat shock (Martin et al., 2008, Oehler-Janne et al., 2008). Although the mTORC2 assay contained contaminating mTORC1, as shown by the ability to phosphorylate 4EBP1 (Figure 3.9A), as previous work showed mTORC1 did not phosphorylate Tip41 any phosphorylation seen in this assay could be assigned to mTORC2. As seen in Figure 3.9B, purified mTORC2 phosphorylated Akt but not Tip41. Tip41 is therefore not a substrate of mTORC2. Over-expression of Tip41 did not affect phosphorylation of Akt at S473, indicating that it does not lie upstream of the kinase (Figure 3.10). The role of Tip41 binding to Hsp70 therefore requires further investigation as it does not appear to involve regulation of mTORC2. Hsp70 has two potential roles within the insulin signalling pathway and contributes to type II diabetes potentially through both mechanisms. Firstly, Hsp70 inhibits insulin signalling by increasing stability of ENPP1, an inhibitor of insulin signalling (Chin et al., 2009, Zhou et al., 2009). Secondly, Hsp70 may activate insulin signalling by preventing misfolding of human amylin, associated with the pathology of type II diabetes (Chien et al., 2010). As Hsp70 is a phosphoprotein and is linked to insulin signalling, Tip41 may interact with and modify activity of the protein with regard to either of these two

activities by mediating interaction with PP2A thus the phosphorylation status of Hsp70 (Cvorovic et al., 1999). The role of Tip41-Hsp70 in insulin signalling requires further clarification.

In summary, Tip41 appears to act as an independent PP2Ac regulatory subunit and regulation of PP2A_{Tip41} may take place via phosphorylation of Tip41. PP2A_{Tip41} interaction is not regulated by insulin or rapamycin. However, it is possible that phosphorylation of Tip41 may alter allosteric regulation of PP2Ac or substrate binding. Phosphorylation of regulatory subunits is a recognised mechanism of PP2Ac regulation (Letourneux et al., 2006). Tip41 phosphorylation is negatively regulated by insulin and unresponsive to rapamycin, indicating that phosphorylation is mediated upstream of mTORC1 and by a kinase that is inhibited following insulin stimulation. In addition, as Tip41 does not interact with Raptor, nor is phosphorylated by mTORC1 *in vitro*, Tip41 may act upstream or parallel to mTORC1 to regulate phosphatase activity towards mTORC1 substrates.

CHAPTER 4 – TIP41 IN THE mTOR PATHWAY

4.1 Introduction

Activation of mTORC1 in response to growth factors is mediated by a PI3K/Akt cascade that begins with activation of an RTK. Following ligand binding, RTKs activate PI3K, either directly or via IRS-1, leading to accumulation of the lipid second messenger PIP₃ (Chung et al., 1994, Mendez et al., 1996, Maehama & Dixon, 1998, Aoki et al., 2001). This provides a docking site at the plasma membrane for proteins containing a PH domain, such as Akt and PDK1 (Engelmen et al., 2006).

Recruitment of Akt and PDK1 to the membrane allows PDK1-mediated phosphorylation of Akt at T308 (Alessi et al., 1997). Full activation requires additional phosphorylation at S473 by mTORC2. Activated Akt then phosphorylates a number of downstream targets including TSC2, PRAS40 and mTOR (reviewed in Manning & Cantley, 2007). Phosphorylation of TSC2 results in inhibition of GAP activity towards Rheb resulting in accumulation of GTP-Rheb (Inoki et al., 2002). This allows activation of mTORC1 and results in phosphorylation of S6K1 and 4EBP1, and activation of HIF1.

Activation of mTORC1 results in attenuation of upstream signalling via a negative feedback loop. When activated, mTOR and S6K1 phosphorylate IRS-1 at S636/639 and S1101, respectively (Veileux et al., 2010). This prevents interaction with PI3K and depletes IRS-1 protein levels. This renders the PI3K/Akt pathway unresponsive to upstream signals. An additional feedback mechanism exists triggered by chronic mTORC1 inactivation. Inhibition of mTORC1 by rapamycin results in upregulation of Akt through an unidentified mechanism (Wang et al., 2008, Chen et al., 2010). Although the upstream activation of mTORC1 is relatively well understood, the inhibitory actions of phosphatases require further study. PP2A has been implicated in the pathway but the regulatory subunits involved have not been conclusively identified. As Tip41 has been identified as a PP2A regulatory subunit acting downstream of TORC1 in budding yeast (Jacinto et al., 2001), the potential role in mTORC1 signalling has been investigated in this chapter. These data show that Tip41 inhibits mTORC1 signalling. In contrast to *in vitro* assays implicating Tip41 as an inhibitor of PP2A, this work indicates that Tip41 activates PP2Ac. As Tip41 over-

expression was not able to reduce S6K1 phosphorylation in *TSC2*^{-/-} MEFs, it appears to act upstream of mTORC1, with *TSC2* identified as a potential substrate.

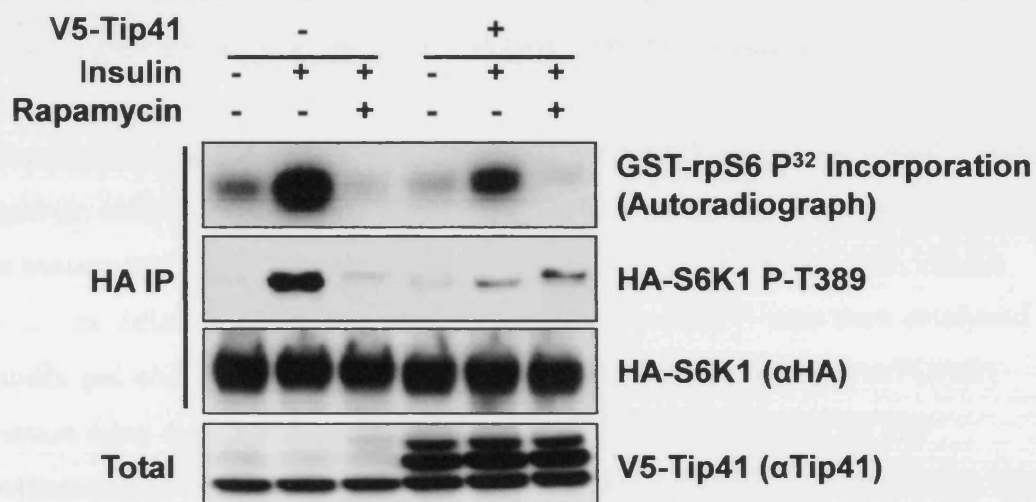
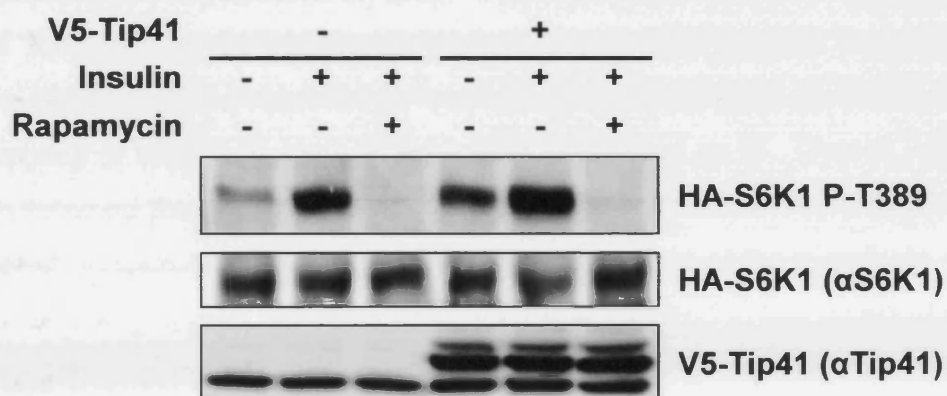
4.2 Results

4.2.1 Over-expression of Tip41 can inhibit or activate mTORC1

To analyse the possible role of Tip41 within the mTORC1 signalling pathway in more detail, Tip41 was over-expressed in HEK293 cells and the effect on the activity of co-expressed HA-S6K1 evaluated. V5-Tip41 was co-transfected with HA-S6K1 in HEK293 cells and serum-starved overnight. Prior to lysis, cells were treated with insulin and rapamycin as indicated. Where the HA-S6K1 assay was performed, HA-S6K1 was then purified from lysates by α HA immunoprecipitation, and assays carried out as detailed in the 'Materials and Methods'. The activity of HA-S6K1 towards the substrate GST-rpS6 was evaluated by incorporation of [³²P] using autoradiography. Where the HA-S6K1 assay was not performed, HA-S6K1 phosphorylation was visualised by SDS-PAGE followed by western blot using phospho-S6K1 antibodies. Figure 4.1A indicates low activity of HA-S6K1 under serum-starved conditions as shown by low levels of GST-rpS6 phosphorylation within the assay. This was then increased by insulin treatment and subsequently reduced by rapamycin. Over-expression of V5-Tip41 reduced HA-S6K1 activity towards GST-rpS6, which is particularly apparent following insulin stimulation, as shown by a reduction of [³²P]-radiolabel incorporation into rpS6 substrate. A portion of purified HA-S6K1 was retained for analysis by western blot. Phosphorylation of HA-S6K1 at T389 reflected the result seen within the assay. HA-S6K1 phosphorylation was low following serum-starvation, increased by insulin stimulation and subsequently reduced by rapamycin inhibition as expected. Over-expression of V5-Tip41 reduced phosphorylation of HA-S6K1, which was apparent following insulin stimulation.

While over-expression of Tip41 resulted in a decrease in the activity of mTORC1 substrates, an increase in activity was occasionally observed. V5-Tip41 was co-expressed with HA-S6K1 in HEK293 cells and the phosphorylation of HA-S6K1 observed by SDS PAGE followed by western blot (Figure 4.1B). Phosphorylation of HA-S6K1 at T389 was increased under both starved and insulin stimulated conditions in the presence of V5-Tip41 in comparison to control cells expressing HA-

Figure 4.1: Tip41 overexpression can either enhance or inhibit phosphorylation of S6K1. **A.** V5-Tip41 was co-expressed with HA-S6K1 in HEK293 cells. Following overnight serum starvation, cells were treated with insulin and rapamycin as indicated. HA-S6K1 was then purified by immunoprecipitation (α HA) and an S6K1 assay performed. Incorporation of 32 P into the GST-rpS6 substrate indicated that overexpression of Tip41 reduced activity of S6K1 towards rpS6 in comparison to control cells expressing HA-S6K1 only. Western blot analysis of S6K1 phosphorylation at T389 was concurrent with this result, as overexpression of Tip41 also reduced phosphorylation of S6K1 at this site. **B.** V5-Tip41 was coexpressed with HA-S6K1. Prior to lysis, cells were cultured overnight in serum free media and treated with insulin and rapamycin as indicated. Phosphorylation of S6K1 was analysed by western blot. The results show that overexpression of Tip41 enhanced phosphorylation of S6K1 at T389 in comparison to control cells under serum starved and insulin treated conditions. Rapamycin abrogated the increase in S6K1 phosphorylation caused by overexpression of Tip41

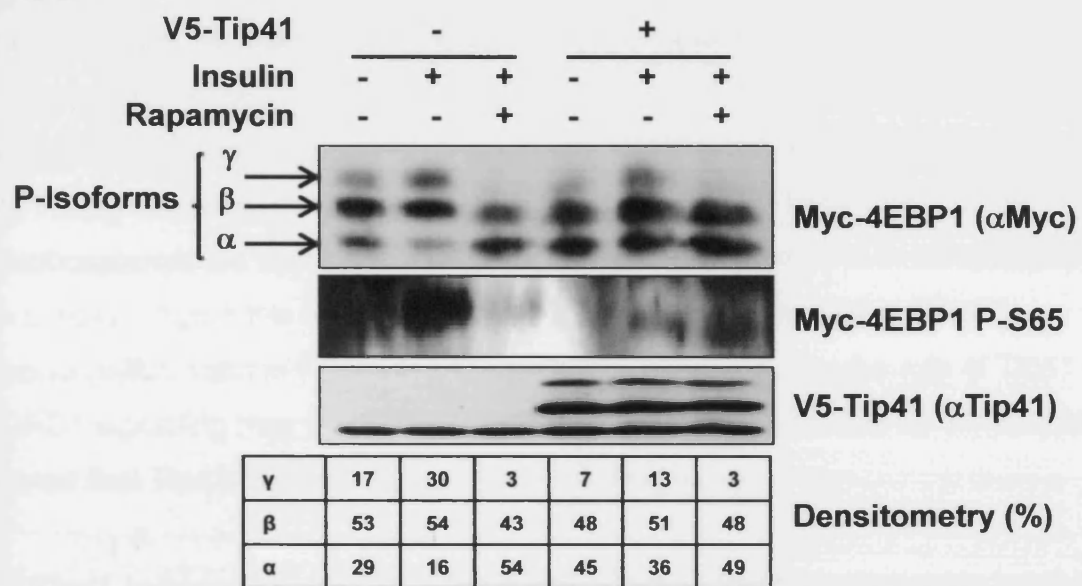
A**B**

S6K1 only. Rapamycin treatment completely ablated HA-S6K1 phosphorylation in both sample sets. This result was in complete contrast to the effect seen in Figure 4.1A where V5-Tip41 expression reduced HA-S6K1 phosphorylation.

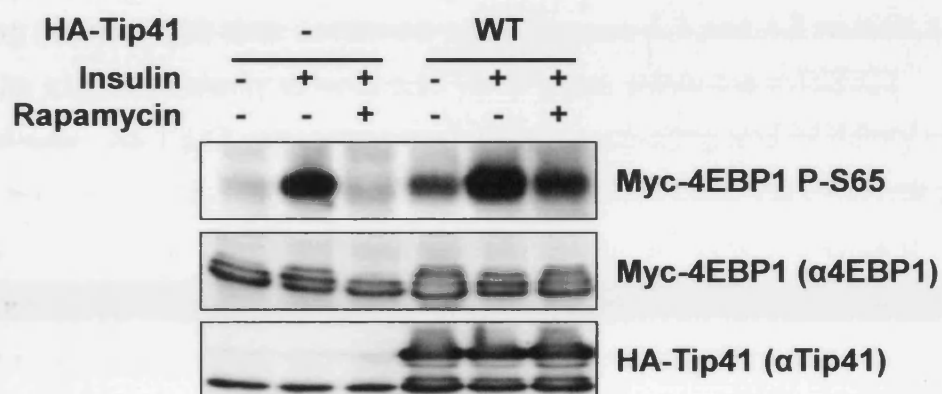
The phosphorylation of another well characterised mTORC1 substrate, 4EBP1, was also analysed on over-expression of Tip41. V5-Tip41 was co-transfected with Myc-4EBP1 and serum-starved overnight. Prior to lysis, cells were treated with insulin and rapamycin as detailed. The phosphorylation of Myc-4EBP1 was then analysed using a mobility gel shift assay (Figure 4.2A). 4EBP1 contains multiple mTORC1 phosphorylation sites and resolves as three phospho-isoforms based on the phosphorylation status of the protein. The bottom band (labelled 'α') represents the least phosphorylated species of 4EBP1, while the top band (labelled 'γ') represents the most phosphorylated species of 4EBP1. Densitometry was used to determine the ratio of these different phosphorylated isoforms of 4EBP1 within the assay. Total signal intensity was calculated for each condition and taken as 100%, with the relative intensity of 4EBP1 in each isoform then calculated as %. Overall, V5-Tip41 expression reduced the phosphorylation of Myc-4EBP1 as shown by a reduction in the γ-resolved isoform of Myc-4EBP1 and an accumulation of the α-isoform. In the absence of over-expressed Tip41, serum-starvation resulted in low levels of Myc-4EBP1 phosphorylation, with 29% resolving as the lowest phosphorylated α-isoform and 17% resolving as the highest phosphorylated γ-isoform. V5-Tip41 expression resulted in a further increase in the α-isoform (45%) and a reduction in the γ-form (7%) indicating a reduction in 4EBP1 phosphorylation. Insulin stimulation in the absence of V5-Tip41 resulted in a 30% shift to the highest phosphorylated γ-isoform. V5-Tip41 expression reduced the γ-isoform band to 13% with a resulting accumulation of Myc-4EBP1 to the α isoform (36%). In the control cells, rapamycin treatment reduced Myc-4EBP1 phosphorylation, as observed by 54% of 4EBP1 resolving as the α-isoform and only 3% in the γ-isoform. This was largely unchanged by V5-Tip41 expression. Tip41 therefore appears to act as an inhibitor within the mTORC1 signalling pathway in a manner analogous to rapamycin, with over-expression resulting in a decrease in phosphorylation of mTORC1 substrates.

Concurrent with the dual effect of Tip41 overexpression on the activation or inhibition of HA-S6K1, expression of Tip41 also on occasion enhanced 4EBP1

A



B



phosphorylation. In this instance, HA-Tip41 was co-expressed with Myc-4EBP1 and the effect on 4EBP1 phosphorylation observed by SDS PAGE followed by western blot and mobility gel shift assay (Figure 4.2B). An observed increase in 4EBP1 phosphorylation at S65 resulted from expression of HA-Tip41 under starved, insulin and rapamycin treated conditions in comparison to control cells expressing Myc-4EBP1 only. Mobility gel shift assay also showed a shift in Myc4EBP1 to the γ hyperphosphorylated isoform of 4EBP1 under all three conditions in comparison to control cells. Again this is in direct contrast to the reduction of Myc-4EBP1 phosphorylation seen in Figure 4.2A. Therefore it appears that the role of Tip41 in mTORC1 signalling may be more complex than previously thought as these data indicated that Tip41 can also activate mTORC1 signalling.

4.2.2 Tip41 induces an mTORC1 feedback loop, indicative of chronic inhibition, via upregulation of Akt

The conflicting nature of the data contained within Figures 4.1 and 4.2 reveals a requirement for improved clarity to what role Tip41 plays within the mTORC1 signalling pathway. As Tip41 expression had both an activating and inhibitory role towards mTORC1 substrates, the possibility that the protein could be influencing one of the feedback loops was considered. Signalling through mTORC1 is subject to control by a number of regulatory feedback loops that could occur upon prolonged Tip41 expression. Following activation of mTORC1 signalling, IRS1 is phosphorylated at a number of Ser and Thr residues, including S636 and S639, resulting in its destabilisation, degradation or relocalisation (Veilleux et al., 2010). In addition, the transcription of IRS1 is reduced (Zhande et al., 2002, Greene et al., 2003). As IRS1 is critical in transmitting signals from the IR to PI3K, a reduction in levels attenuates signalling to Akt and therefore the TSC1/2 complex. Regulatory feedback loops also exist to counteract chronic inhibition of mTORC1 signalling. For instance, rapamycin treatment increases Akt phosphorylation and this feedback may be responsible for the poor efficacy of mTORC1 inhibitors in clinical trials (Javle et al., 2010).

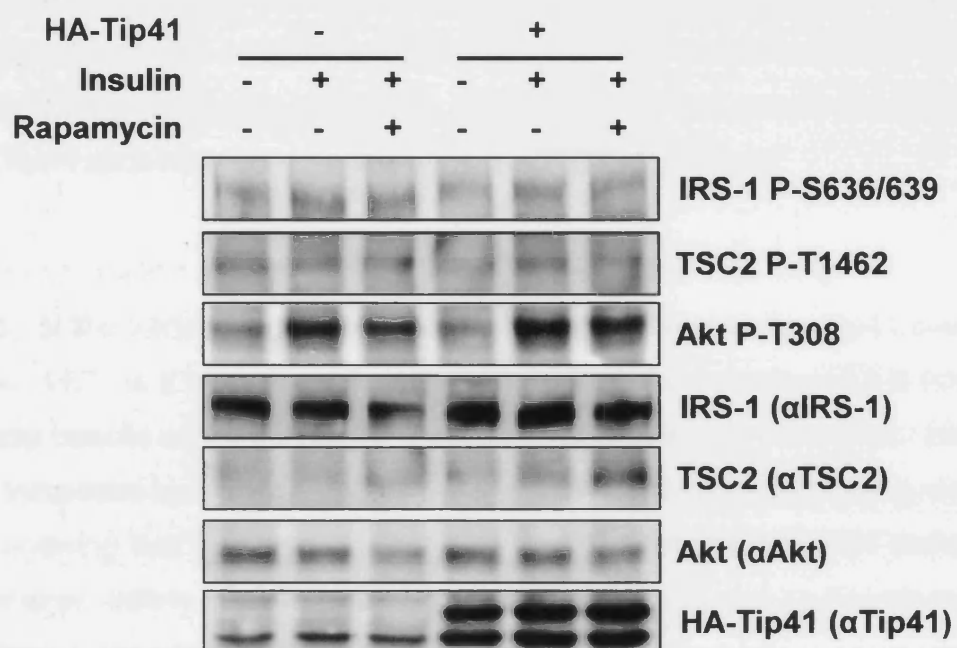
To examine whether these feedback loops are active, the phosphorylation of proteins upstream of mTORC1 can be investigated. If feedback involving IRS1 destabilisation occurs, a reduction in IRS1 phosphorylation would be observed.

Feedback following mTORC1 inhibition would result in increased phosphorylation of Akt.

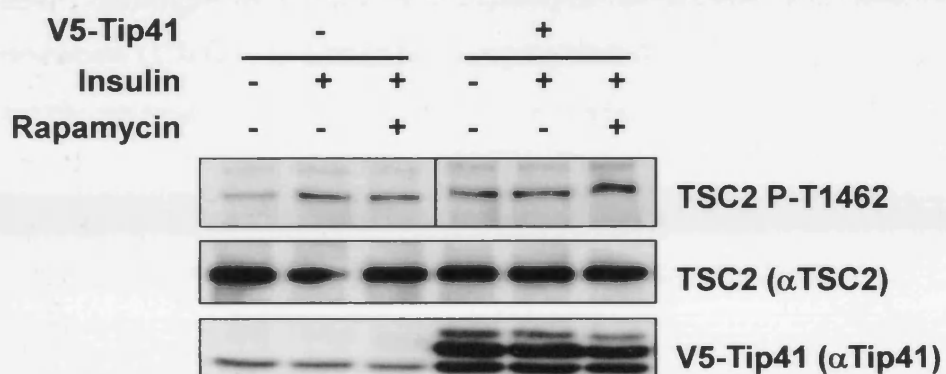
To investigate the true effect of Tip41 over-expression within the mTORC1 signalling pathway, it was important to deduce whether any of the feedback loops was activated. In theory, an initial increase in mTORC1 signalling resulting from V5-Tip41 expression could result in activation of the IRS-1 phosphorylation feedback loop ending with an apparent reduction in mTORC1 signalling seen in Figures 4.1A and 4.2A. Samples from each of the three repetitions of Figure 4.1A, where a reduction in HA-S6K1 phosphorylation was observed, were used to analyse whether the negative regulatory feedback loop was activated by the presence of V5-Tip41. Phosphorylation of IRS-1, TSC2 and Akt were analysed after V5-Tip41 expression in comparison to control cells expressing HA-S6K1 only (Figure 4.3A). IRS-1 phosphorylation at S636/639 was unaffected by the presence of V5-Tip41, as was TSC2 phosphorylation at T1462 and Akt phosphorylation at T308. This indicated that the negative feedback loop had not been activated.

Next the possibility that the feedback loop involving Akt had been activated in Figure 4.1B and 4.2B was investigated. In this instance, the increase in the phosphorylation of mTORC1 substrates could be explained by V5-Tip41 expression initially reducing activity within the mTORC1 pathway resulting in activation of a regulatory feedback loop via upregulation of Akt. This may then feed into the mTORC1 pathway causing an apparent increase in the phosphorylation of mTORC1 substrates. Samples from each of the three repetitions of Figure 4.1B where V5-Tip41 expression resulted in an increase in HA-S6K1 phosphorylation were analysed for activation of an upstream feedback loop by investigating TSC2 phosphorylation at T1462. As TSC2 is a direct substrate of Akt, it follows that increased Akt activity would increase its phosphorylation. Densitometry figures were calculated by averaging absolute signal intensity of TSC2 phosphorylation at T1462 between three experiments, then converting to % relative intensity taking the highest intensity as 100% as detailed in 2.4.20. Error is indicative of standard deviation, and significance was calculated using a T test. Western blot showed that indeed an increase in TSC2 phosphorylation was apparent after expression of V5-Tip41 as compared to control cells expressing HA-S6K1 only ($p < 0.05$) (Figure 4.3B). This indicates that the

A



B



Densitometry (%)	38	70	62	83	100	98
Error (%)	7	9	11	13	15	12

regulatory feedback loop was repressed in these samples as observed by an increase in Akt activity. Combined with the results of Figure 4.3A, this shows that the true effect of Tip41 over-expression is a reduction in mTORC1 signalling. In certain experiments, this could also lead increased TSC2 phosphorylation via an increase in Akt activity, which then feeds into mTORC1. The results therefore implicate Tip41 as a negative regulator of the mTORC1 pathway.

4.2.3 Overexpression of Tip41 leads to inhibition of HIF1 activity

The activity of the transcription factor HIF1 was also analysed after Tip41 over-expression. HIF1 is a less well characterised mTORC1 substrate and it is not yet clear exactly how its regulation within the mTORC1 pathway is controlled. HIF1 activity is increased by insulin treatment and conversely reduced following rapamycin inhibition showing that HIF1 is a downstream target within the mTORC1 pathway (Laughner et al., 2001). To analyse HIF1 activity, V5-Tip41 was co-transfected with a HIF luciferase reporter construct. Cells were cultured overnight in serum free media, treated overnight with insulin and rapamycin as detailed and were kept under hypoxic conditions (1% O₂) to stabilise endogenous HIF1. A luciferase reporter assay was then carried out to quantify the accumulation of luciferase and consequently the transcriptional activity of HIF1 in each sample (Figure 4.4). Raw data was converted to % Luciferase Activity by setting 100% as the luciferase activity detected in the absence of V5-Tip41 under insulin stimulated conditions.

Under serum-starved conditions, HIF1 activity was 72% in comparison to insulin stimulation. Expression of V5-Tip41 reduced activity to 56% and 75%, respectively. Rapamycin treatment in control cells reduced activity to 69% which was further reduced to 52% by V5-Tip41 expression. These data show that Tip41 reduces HIF1 activity ($p < 0.05$) and is concurrent with Figures 4.1A and 4.2A with Tip41 as an inhibitor of mTORC1 signalling. Error bars are indicative of standard deviation. Overall, Tip41 over-expression reduces the activity of mTORC1 substrates. This indicates a role of Tip41 as an inhibitor within the mTORC1 signalling pathway, which may be via a role as a PP2Ac regulatory subunit.

4.2.4 Inactive mutants of Tip41 are underexpressed in comparison to wild-type protein

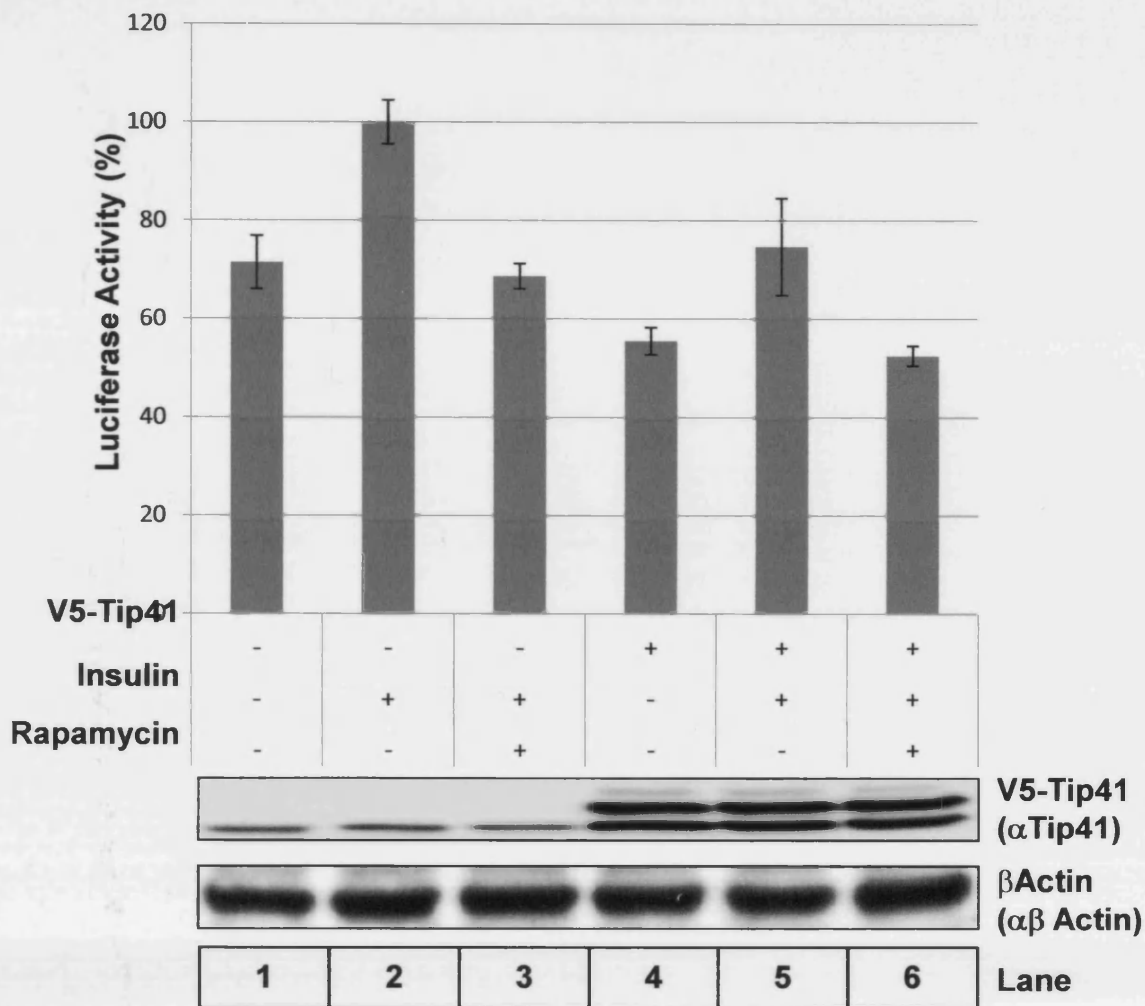


Figure 4.4: Tip41 negatively regulates HIF1 activity. HIF1 activity was measured using a HIF1 luciferase reporter construct, which was co-transfected with V5-Tip41. Cells transfected with the reported construct along with empty vector were used as a control. Cells were serum starved overnight, along with overnight treatment with insulin and rapamycin (as indicated) in 1% oxygen. Chemiluminescence was used as an indicator of luciferase activity, and by extension the transcriptional activity of HIF1. These data show that overexpression of Tip41 reduced HIF1 activity under all conditions tested, in comparison to control cells. Comparison between lanes 1 and 4, 2 and 5, and 3 and 6 all showed a significant difference with $p < 0.05$. Error bars are representative of standard deviation. Results are indicative of three independent experiments.

The TOS motif is essential for substrate binding and phosphorylation by mTORC1 and mutation of the motif prevents substrate binding and phosphorylation by the complex (Figures 3.5 and 3.8). Tip41 contains a TOS motif that is conserved between yeast and the mammalian orthologue of the protein (Figure 1.5). Although results of Figure 3.6, 3.7 and 3.8 do not indicate a role of Tip41 as a substrate of mTORC1, this possibility still cannot be ruled out conclusively. A HA-Tip41 construct with a mutation contained within the TOS motif was produced by myself by site directed mutagenesis resulting in HA-Tip41(F156A). If Tip41 is indeed a substrate of mTORC1, mutation of the TOS motif may result in an ineffective protein that would not affect phosphorylation of mTORC1 targets.

In order to see whether the F156A mutation would alter the reduction of mTORC1 substrate phosphorylation seen in Figure 4.1A, HA-Tip41 wild-type and F156A mutant were co-expressed with HA-S6K1. HA-Tip41 wild type and F156A mutant were expressed in HEK293 cells with HA-S6K1. Cells expressing HA-S6K1 only were used as a control. Cells were cultured overnight prior to lysis and treated with insulin and rapamycin as indicated. Immunoprecipitation using α HA antibodies was used to purify HA-S6K1 for use in an S6K1 assay as described in 'Materials and Methods'. HA-S6K1 activity was analysed by 32 P incorporation of the substrate GST-rpS6 followed by autoradiography. The results show that HA-Tip41 wild-type expression reduces HA-S6K1 activity, concurrent with results in Figure 4.1A, as indicated by a reduction in phosphorylation of GST-rpS6 in comparison to control samples (Figure 4.5A). HA-Tip41 F156A had no effect on S6K1 activity within the assay as indicated by equal levels of GST-rpS6 phosphorylation in comparison with control samples. Analysis of total protein levels by western blot indicated that HA-Tip41(F156A) expression was lower than expression of wild-type HA-Tip41. Therefore, although expression of HA-Tip41(F156A) shows no impact on the activity of HA-S6K1, this may be due to a lower expression level of the protein.

In order to try and rectify this problem, HA-Tip41 wild-type and F156A mutant were expressed in the presence of the proteasomal inhibitor MG132. As reduced HA-Tip41(F156A) levels may be caused by proteasomal degradation, MG132 treatment could result in equal levels in comparison to wild-type HA-Tip41. The results show that levels of HA-Tip41(F156A) were not improved by treatment with MG132, and

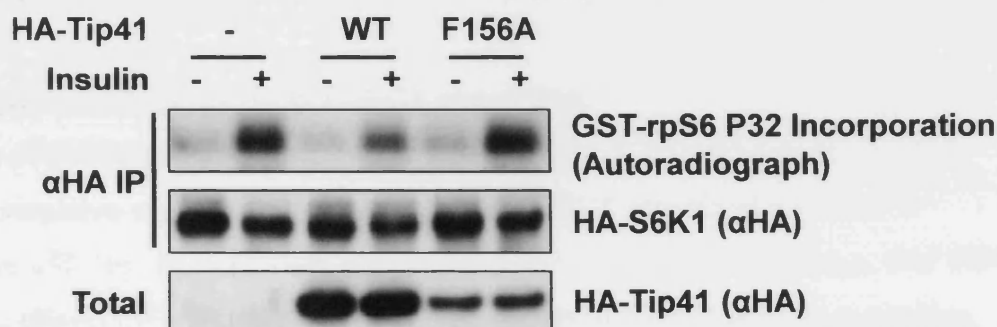
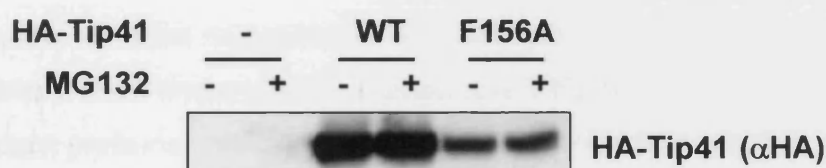
A**B**

Figure 4.5: The Tip41 TOS mutant expressed at lower levels than HA-Tip41 wild type. **A.** HA-Tip41 wild type and TOS mutant F156A were overexpressed in HEK293 cells with HA-S6K1. Cells were serum starved overnight and treated with insulin and rapamycin prior to lysis as indicated. HA-S6K1 was then purified (α HA) and used in an S6K1 assay. Activity of S6K1 was measured against the GST-rpS6 substrate by incorporation of 32 P and subsequent autoradiography. The data show that overexpression of HA-Tip41 reduced activity of S6K1 as measured by phosphorylation of rpS6 substrate, whilst overexpression of HA-Tip41(F156A) had no effect in comparison to control cells. Analysis of total Tip41 levels showed that HA-Tip41(F156A) was expressed at lower levels in comparison to wild type HA-Tip41. **B.** In order to try and equalise levels of HA-Tip41 wild type and F156A mutant, cells expressing the proteins were treated with the proteasomal inhibitor MG132 prior to lysis. Treatment with MG132 had no effect on the varying expression levels of wild type and F156A mutant HA-Tip41, indicating that HA-Tip41(F156A) is not subject to proteasomal degradation. Results are representative of three independent experiments.

were still considerably lower than expressed levels of HA-Tip41 wild-type (Figure 4.5B). This indicates that the reduction in HA-Tip41(F156A) levels is not caused by protein instability or targeting of the protein for proteasomal degradation.

4.2.5 Analysis of PP2A_{Tip41} in mTORC1 signalling

The results of over-expression studies (Figures 4.1A , 4.2A and 4.4) implicating Tip41 as a negative regulatory protein within mTORC1 signalling pathway in combination with results indicating a direct interaction between PP2A_{Tip41} and S6K1 (Figure 3.4) raised the intriguing question as to whether Tip41 acts as a positive regulatory PP2Ac subunit within the mTORC1 pathway. To investigate this possibility, site-directed mutagenesis was used to create three Tip41 mutants that had previously been shown not to interact with PP2Ac (Smetana & Zanchin, 2007). These mutant proteins could, in theory, be used to confirm whether the effect of Tip41 in mTORC1 was due to interaction with PP2Ac. GST-PP2Ac purification was then used to investigate whether the mutants did not interact with PP2Ac. GST-PP2Ac was co-expressed with V5-Tip41 wild-type along with each of the three mutants; D71L, Y79H and M196V. These mutants have previously been shown as defective in binding to PP2Ac (Smetana & Zanchin, 2007). Following GST purification interaction was analysed by western blot.

Figure 4.6A shows that although mutant D71L did not co-purify with GST-PP2Ac, mutants Y79H and M196V both interacted with PP2Ac. Levels of purified Alpha4 were also analysed and showed no change on co-purification of GST-PP2Ac and V5-Tip41(D71L). This backs up data from Figure 3.2 and 3.3 indicating a direct interaction between PP2A_{Alpha4} and PP2A_{Tip41} but not between Alpha4 and Tip41. Analysis of total protein however showed that V5-Tip41(D71L) was expressed at a lower level than wild-type and mutants Y79H and M196V. Regardless, the lack of co-purified V5-Tip41(D71L) still indicates a protein deficient in PP2Ac binding as almost none was present after purification.

If the reduction seen in mTORC1 signalling (Figures 4.1A, 4.2A and 4.4) on over-expression of Tip41 was as a result of Tip41 acting as a positive regulatory subunit of PP2Ac, then mutant D71L would be predicted to have no effect on phosphorylation of mTORC1 substrates. Wild-type V5-Tip41 was co-expressed with

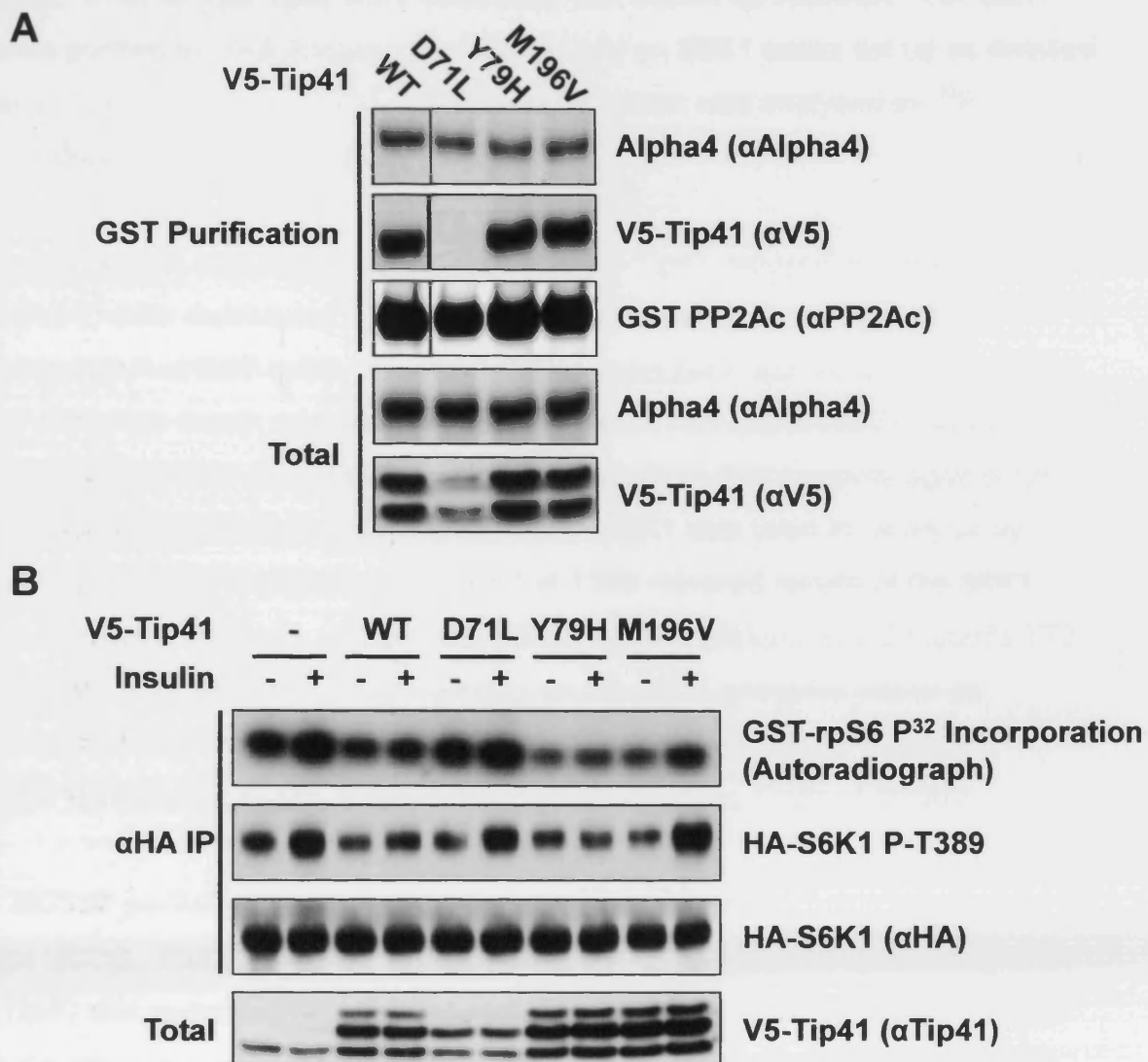


Figure 4.6: A Tip41 mutant deficient in PP2Ac binding is expressed at a lower level than wild type counterparts. **A.** GST-PP2Ac was co-expressed with V5-Tip41 wild type, mutant D71L, Y79H or M196V. Following lysis, GST-PP2Ac was purified and levels of co-purified V5-Tip41 analysed by western blot. Whilst V5-Tip41 wild type, Y79H and M196V interacted equally with GST-PP2Ac, V5-Tip41(D71L) interacted with PP2Ac less effectively. Analysis of total protein levels showed that V5-Tip41(D71L) was expressed at a lower level than V5-Tip41 wild type. **B.** Co-expression of V5-Tip41 wild type, D71L, Y79H and M196V mutants with HA-S6K1 was used to measure the result of Tip41 mutant expression on mTORC1 activity. HA-S6K1 was purified (αHA) and activity towards the substrate GST-rpS6 measured by ³²P incorporation via autoradiography. The results showed that overexpression of wild type V5-Tip41, along with V5-Tip41(Y79H) and V5-Tip41(M196V) resulted in reduced phosphorylation of GST-rpS6 in comparison to control samples whilst expression of V5-Tip41(D71L) inhibited activity of HA-S6K1. These results were reflected on analysis of HA-S6K1 phosphorylation at T389. Analysis of total protein again showed reduced expression of V5-Tip41(D71L) in comparison to wild type V5-Tip41. results are representative of three independent experiments.

HA-S6K1 along with the three mutants (D71L, Y79H and M196V) and serum-starved overnight. Prior to lysis, cells were stimulated with insulin as indicated. HA-S6K1 was then purified by α HA immunoprecipitation and an S6K1 assay set up as detailed in 'Materials and Methods'. GST-rpS6 phosphorylation was analysed by ^{32}P incorporation by autoradiography.

Figure 4.6B shows that expression of wild-type V5-Tip41 reduced HA-S6K1 activity compared to cells expressing HA-S6K1 only as indicated by a reduction in phosphorylation of GST-rpS6. V5-Tip41(D71L) expression did not affect HA-S6K1 activity within the assay, whereas the two mutants (Y79H and M196V) that were able to interact with PP2Ac also resulted in a reduction of HA-S6K1 activity against GST-rpS6. HA-S6K1A portion of retained purified HA-S6K1 was used for analysis by western blot. Phosphorylation of HA-S6K1 at T389 mirrored results of the S6K1 assay. Wild-type V5-Tip41 reduced HA-S6K1 phosphorylation, as did mutants Y79H and M196V. V5-Tip41(D71L) had no effect on HA-S6K1 phosphorylation as compared to samples where HA-S6K1 was expressed in isolation. Again V5-Tip41(D71L) was expressed at lower levels than wild-type, Y79H or M196V.

4.2.6 MG132 partially rescued expression of Tip41(D71L)

As Tip41(D71L) mutant showed reduced expression in cells in comparison to wild-type Tip41 and mutants Y79H and M196V, attempts were made to equalise levels using the proteasomal inhibitor MG132. This was based on the principle that V5-Tip41(D71L) may be less stable than other V5-Tip41 constructs and therefore targeted for proteasomal degradation. V5-Tip41 wild-type, along with mutants D71L, Y79H and M196V, were transiently expressed in HEK293 cells. Prior to lysis cells were cultured overnight in serum free media and treated with insulin and rapamycin as indicated. MG132 treatment took place 2h prior to lysis. Figure 4.7 shows that levels of V5-Tip41(D71L) are increased following treatment with MG132 in comparison to levels seen in previous experiments (Figure 4.6). Although an increase was seen on MG132 treatment, the recovery of V5-Tip41(D71L) expression in comparison to other V5-Tip41 constructs was not complete. As MG132 treatment increased levels of V5-Tip41(D71L) protein, this indicates that the protein is targeted for proteasomal degradation, possibly due to instability or due to lack of function of the protein. This finding may therefore point to a possibility of dysfunctional Tip41

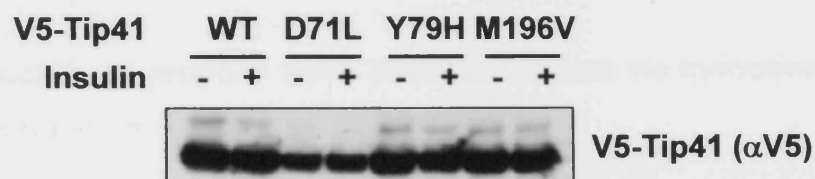


Figure 4.7: MG132 partially rescues levels of V5-Tip41(D71L) in comparison to wild type levels. In order to try to increase levels of V5-Tip41(D71L), V5-Tip41 wild type, D71L, Y79H and M196V were expressed in HEK293 cells. Cells were serum starved overnight and treated with insulin (where indicated) and the proteasomal inhibitor MG132 prior to lysis. Whilst treatment with MG132 slightly improved levels of V5-Tip41(D71L), levels were not completely equalised. Results are representative of three independent experiments.

being targeted for proteasomal degradation to remove dysfunctional Tip41 from the cell.

4.2.7 Tip41 knockdown reduces S6K1 phosphorylation via induction of a negative feedback loop

As over-expression of Tip41 resulted in a reduction in mTORC1 signalling, with the occasional inhibition of the negative feedback loop to IRS-1, the effect of reducing endogenous Tip41 levels within the cell using shRNA was investigated. A panel of shRNA vectors was tested to find the most effective clone for knockdown of Tip41. Firstly, the amount of time between transfection of cells with shRNA and actual knockdown taking place was optimised. Cells were seeded in triplicate on day one, transfected on day 2 and lysed on either day 3, 4 or 5. Control cells were transfected with non-coding shRNA. This showed that effective knockdown of Tip41 occurred 72h post transfection within cells lysed on day 5 (data not shown). To minimise the time between cell seeding and lysis, the reverse transfection procedure was attempted. Cells were seeded and transfected in one step on day 1, with cell lysis occurring on day 4. This resulted in improved cell condition prior to lysis. Levels of Tip41 were analysed by western blot. Figure 4.8A shows that following reverse transfection and cell lysis on day 4, clone 1 was the most effective at reducing Tip41 levels. This clone was then used in all future shRNA experimentation.

Q-PCR was then used to quantify the extent of Tip41 knockdown. Cells were cultured overnight in serum free media prior to lysis. Q-PCR procedure and analysis was undertaken as detailed in 'Materials and Methods'. Figure 4.8B shows that Tip41 mRNA levels following knockdown were reduced in comparison to control cells transfected with non-coding shRNA ($p < 0.05$) and confirms the result from the western blot seen in Figure 4.8A. Error bars are indicative of standard deviation. Thus Tip41 shRNA reduced Tip41 mRNA levels leading to a subsequent reduction in Tip41 protein levels as expected.

As Tip41 over-expression resulted in a reduction in mTORC1 substrate phosphorylation, the effect of Tip41 reduction in cells was also investigated. Following shRNA transfection on day 1 (targeted or non-coding), cells were transfected on day 3 with HA-S6K1 and serum starved overnight. Prior to lysis on

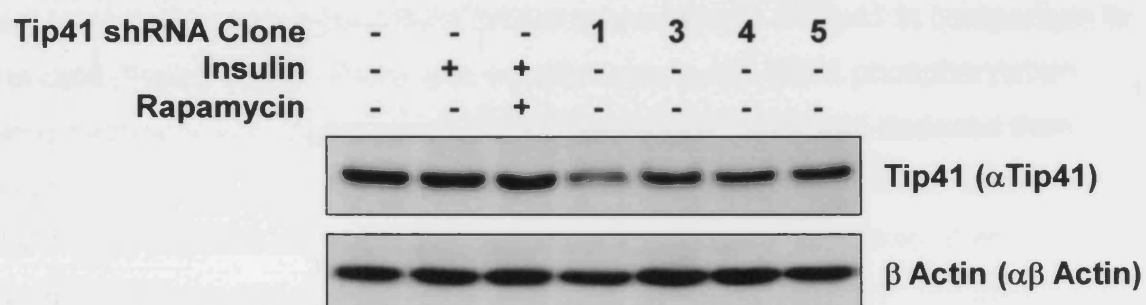
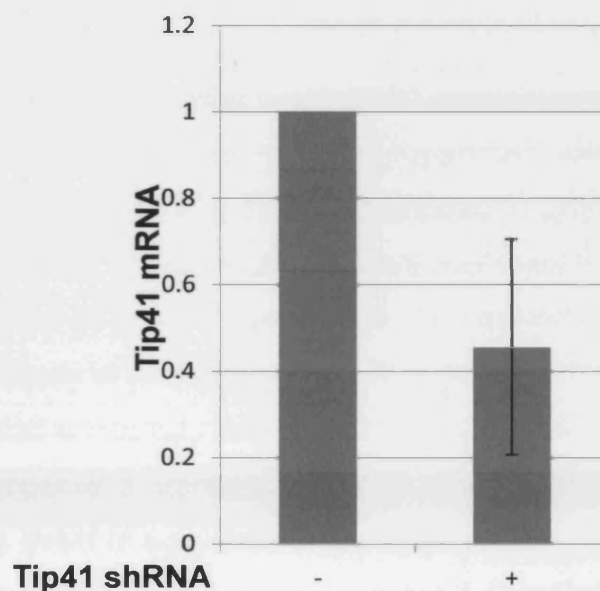
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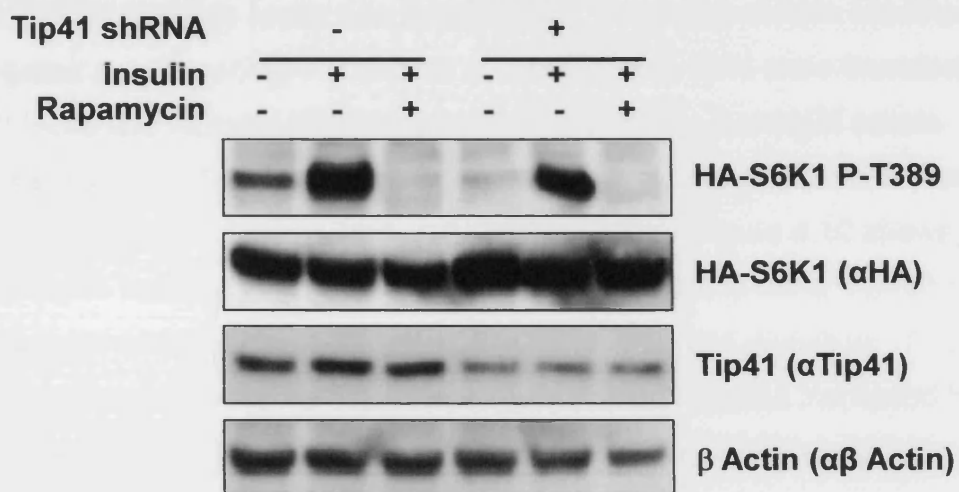
Figure 4.8: Optimisation of Tip41 knockdown using shRNA. **A.** HEK293 cells were transfected with a panel of four different Tip41 shRNA vectors and levels of Tip41 analysed in comparison to cells transfected with non-coding shRNA by western blot. Levels of Tip41 indicated that shRNA clone 1 was the most effective at reduced levels of Tip41. **B.** In order to confirm knockdown of Tip41 by shRNA clone 1, Q-PCR was used, using primers directed towards Tip41. Analysis showed that shRNA lowered Tip41 levels to approximately 50% of those seen in control cells. Error bars are representative of standard deviation. Results are representative of three independent experiments.

day 4, cells were treated with insulin and rapamycin as indicated. HA-S6K1 phosphorylation was then analysed by SDS PAGE followed by western blot. Phosphorylation of HA-S6K1 at T389 was markedly reduced under both serum-starved and insulin treated conditions following knockdown of Tip41 in comparison to control cells (Figure 4.9A). There was no difference in HA-S6K1 phosphorylation following treatment with rapamycin in either cell sample. As it was deduced from Figures 4.1A, 4.2A, 4.3A and 4.4 that Tip41 acts as an inhibitory protein within the mTORC1 pathway, this was quite an unexpected result as knockdown of an inhibitory protein would be expected to increase mTORC1 substrate phosphorylation. Instead the reverse result opened up the possibility of a feedback loop acting as seen in Figure 4.3.

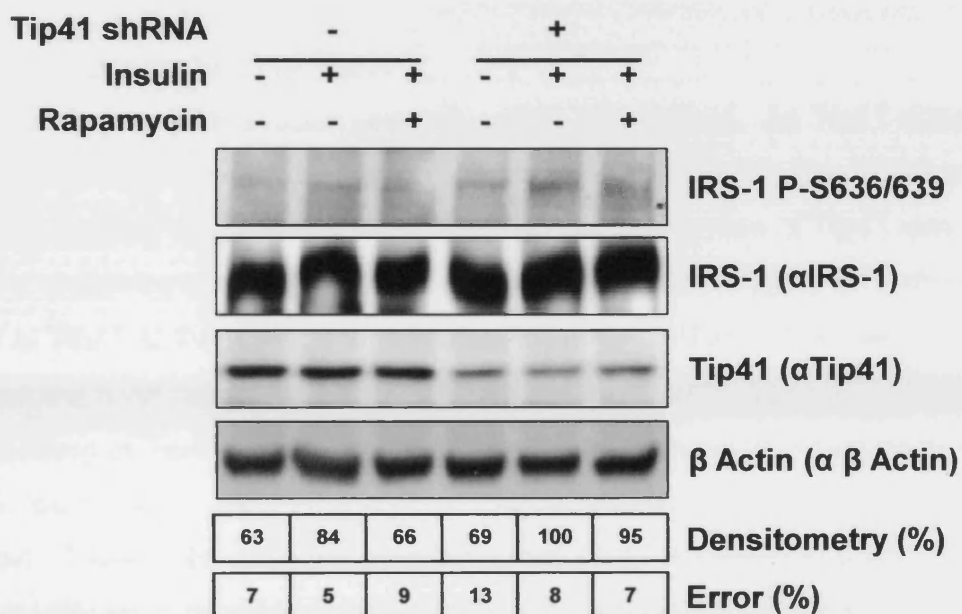
Following the unexpected reduction in HA-S6K1 phosphorylation after knockdown of Tip41 seen in Figure 4.9A, samples from this experiment were used to investigate the possibility that a feedback loop had been initiated in cells where Tip41 levels were reduced by shRNA. Western blot analysis was used to evaluate the phosphorylation status of IRS-1. Densitometry figures were calculated by averaging absolute signal intensity of phosphorylated IRS1 between three experiments, then converting to % relative intensity taking the highest intensity as 100% as detailed in 2.4.20. Error is indicative of standard deviation and a T test was used to ascertain significance. In the event of activation of the negative feedback loop, IRS-1 phosphorylation at S636/639 is increased via direct phosphorylation by mTORC1 (Veileux et al., 2010). Figure 4.9B shows that knockdown of Tip41 resulted in an increase in IRS-1 phosphorylation concurrent with activation of the negative feedback loop ($p < 0.05$). Therefore the apparent reduction in HA-S6K1 phosphorylation seen on Tip41 knockdown is actually due to initiation of a feedback loop. Thus initially, knockdown of Tip41 must result in an increase in mTORC1 activity which phosphorylates IRS-1 resulting in reduced signalling through the insulin signalling pathway through to mTORC1. The result is an apparent reduction in HA-S6K1 phosphorylation on Tip41 knockdown. This result therefore confirms that Tip41 acts as an inhibitory protein within the mTORC1 signalling pathway.

4.2.8 Tip41 knockdown increases activity of HIF1

A



B



As Tip41 over-expression resulted in a reduction in the activity of HIF1 as shown using a HIF1 luciferase reporter element (Figure 4.4), the effect of Tip41 knockdown on activity of the transcription factor was investigated. Following shRNA transfection on day 1 (targeted or non-coding in the case of control cells), cells were transfected with the HIF1 luciferase reporter element on day 3 followed by overnight serum-starvation under hypoxic (1% O₂) conditions. Following cell lysis a luciferase assay was carried out as described in the 'Materials and Methods'. Figure 4.10 shows that knockdown of Tip41 resulted in an 8 fold increase in HIF1 activity using the HIF1 luciferase assay ($p < 0.05$). Error bars are indicative of standard deviation. This is concurrent with the result in Figure 4.4 where Tip41 over-expression increased HIF1 activity in the same assay. Tip41 therefore appears to act as a PP2Ac regulatory subunit reducing activity of HIF1.

4.2.9 A novel nuclear isoform of Tip41

The transcription factor HIF1 is found primarily within the nucleus. As Tip41 appears to play a role in the regulation of HIF1 activity (Figures 4.4 and 4.10) the presence of Tip41 within the nucleus was investigated. The cellular localisation of Tip41 was analysed in the presence and absence of rapamycin treatment. Figure 4.11 shows the presence of Tip41 at the expected molecular weight (~32kDa) within the cytoplasm, and the level remained unchanged by treatment with rapamycin. In addition, an isoform of Tip41 resolving at a higher molecular weight (~39kDa) was present within the nucleus. Again the level of this remained unchanged by treatment with rapamycin. These data raise the possibility that Tip41 is subject to post-translational modification resulting in translocation to the nucleus where it may control the activity of HIF1. It also shows that Tip41 is not shuttled to the nucleus or cell membrane as a method of regulation following treatment with rapamycin.

4.2.10 Tip41 acts upstream of mTORC1

Tip41 appears to act as an inhibitory protein within the mTORC1 pathway (Figures 4.1A, 4.2A, 4.3A, 4.4, 4.9 and 4.10), possibly as a positive regulatory subunit of PP2Ac (Figures 3.2 and 3.3). Activation of upstream pathways that positively regulate mTORC1 results in inhibition of the TSC1/2 complex. This results in the accumulation of GTP-Rheb and the subsequent activation of mTORC1. Figures 3.10 and 4.3 shows that over-expression of Tip41 in the absence of activation of any

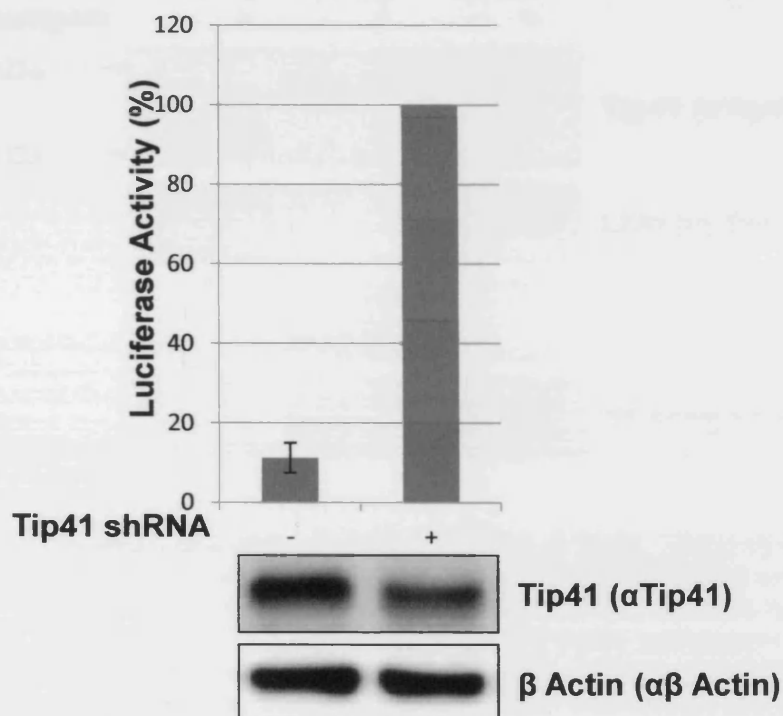


Figure 4.10: Knockdown of Tip41 substantially increases activity of HIF1. After initial transfection with Tip41 shRNA plasmid, the HIF1 luciferase reporter was transfected into HEK293 cells. Prior to lysis cells were placed in 1% O₂ overnight in serum free media. HIF1 transcriptional activity was measured indirectly via accumulated luciferase activity. The results, combining data from three distinct experiments, showed that knockdown of Tip41 significantly increased activity of HIF1 ($p < 0.05$), almost 10 fold, in comparison to control cells that were transfected with non-coding shRNA. Error bars are indicative of standard deviation. Results are representative of three independent experiments.

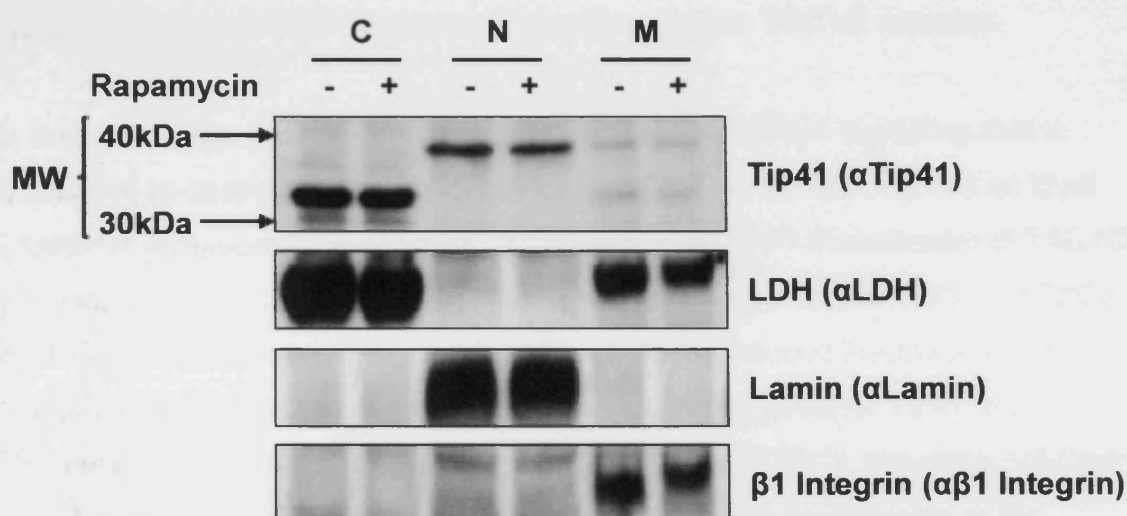


Figure 4.11: Discovery of a distinct nuclear isoform of Tip41. Untransfected cells were cultured in full serum media and treated with rapamycin prior to lysis where indicated. Cytoplasmic (C), nuclear (N) and membrane (M) fractions were isolated by use of a kit. The location of Tip41 was analysed by SDS PAGE and western blot. The results showed that cytoplasmic Tip41 is of the expected molecular weight of approximately 32kDa, whereas a nuclear fraction of Tip41 was isolated at a molecular weight of approximately 8kDa more. The distribution of cytoplasmic and nuclear Tip41 was unaffected by treatment with rapamycin. Results are representative of three independent experiments.

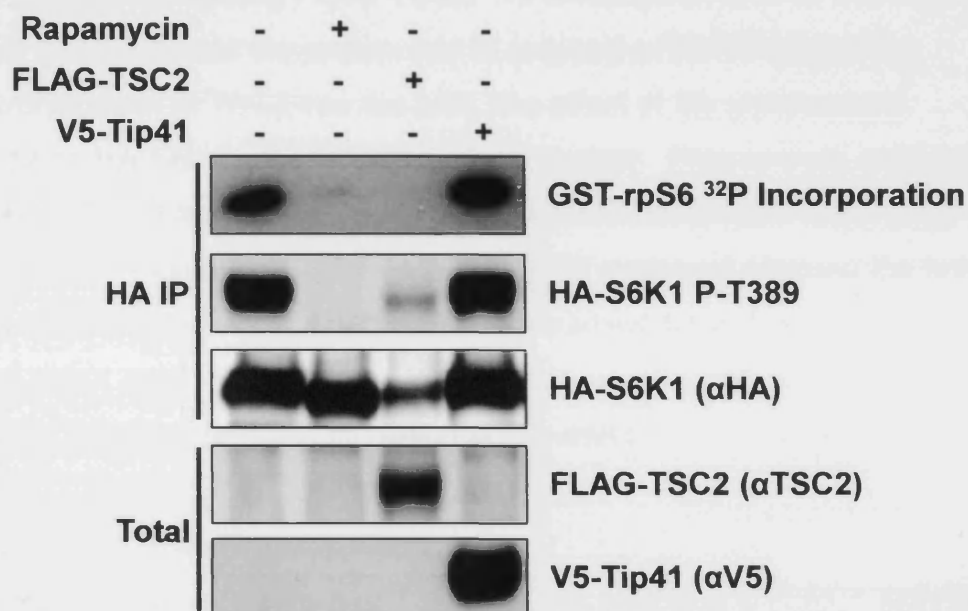
feedback loops does not alter phosphorylation of Akt or TSC2. This indicates a role of Tip41 within the mTORC1 complex downstream of the TSC1/2 complex.

Cells deficient in the *TSC2* gene display hyperactive mTORC1 signalling that is unresponsive to upstream regulatory pathways. For this reason they are an ideal candidate for investigating a possible inhibitory role of Tip41 downstream of TSC1/2, as the cells are unresponsive to feedback loops involving phosphorylation of TSC2. As such *TSC2*^{-/-} cells are, in theory, immune to Tip41-induced feedback loops when analysing targets downstream of TSC1/2. To investigate whether Tip41 acts downstream of the TSC1/2 complex in order to inhibit mTORC1 signalling, V5-Tip41 was expressed in *TSC2*^{-/-} MEF cells. If Tip41 indeed acts downstream of the TSC1/2 complex, over-expression of Tip41 in *TSC2*^{-/-} MEF cells should reduce phosphorylation of mTORC1 substrates. V5-Tip41 was expressed in *TSC2*^{-/-} MEFs along with HA-S6K1. Following lysis, HA-S6K1 was purified from cell lysates using α HA immunoprecipitation and an S6K1 assay carried out as detailed in the 'Materials and Methods'. HA-S6K1 activity was visualised by ³²P incorporation of the substrate GST-rpS6.

Figure 4.12A shows that *TSC2*^{-/-} MEF cells have a high basal level of HA-S6K1 activity displayed as high levels of GST-rpS6 phosphorylation. Rapamycin is a direct mTORC1 inhibitor and consequently treatment with the drug reduced HA-S6K1 activity in comparison to untreated cells. Cells over-expressing FLAG-TSC2 also showed reduced HA-S6K1 activity in comparison to control cells. Expression of V5-Tip41 had no effect on HA-S6K1 activity as no change in GST-rpS6 phosphorylation was apparent in comparison to *TSC2*^{-/-} MEF cells expressing HA-S6K1 only. These data show that Tip41 over-expression is unable to reduce HA-S6K1 activity in cells devoid of mTORC1 inhibition by TSC2.

Excess purified HA-S6K1 was retained for analysis by western blot. Phosphorylation of HA-S6K1 at T389 mirrored results seen in the S6K1 assay. High basal levels of HA-S6K1 phosphorylation were observed in the *TSC2*^{-/-} MEF cells expressing HA-S6K1 only. This was reduced by treatment with rapamycin and expression of FLAG-TSC2. V5-Tip41 expression had no effect on HA-S6K1 phosphorylation at T389 in comparison to control cells expressing HA-S6K1 only.

A



B

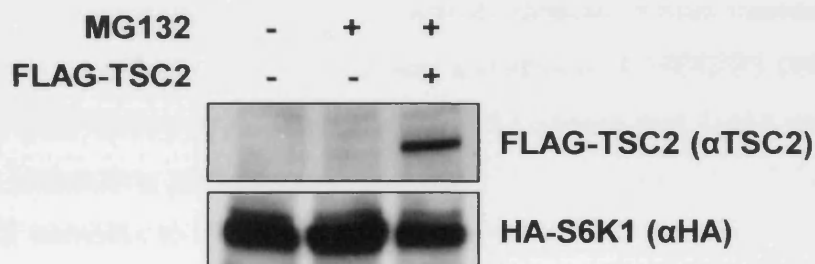


Figure 4.12: Constitutive activation of Rheb confers resistance to the inhibitory action of Tip41. A. V5-Tip41 and HA-S6K1 were expressed in *TSC2*^{-/-} MEFs, and activity of mTORC1 analysed using an HA-S6K1 assay with GST-rpS6 as substrate. The results showed that HA-S6K1 activity, measured by ³²P incorporation of GST-rpS6, is high in *TSC2*^{-/-} MEFs and is sensitive to rapamycin and expression of TSC2. Expression of V5-Tip41 did not induce inhibition of HA-S6K1 activity in these cells. Analysis of HA-S6K1 phosphorylation mirrored these results. Analysis of total HA-S6K1 protein showed that expression of FLAG-TSC2 reduced levels of HA-S6K1. **B.** In order to determine whether expression of FLAG-TSC2 caused destabilisation of HA-S6K1, HA-S6K1 and FLAG-TSC2 were expressed in *TSC2*^{-/-} MEFs in the presence of the proteasomal inhibitor MG132 prior to lysis. The results show that MG132 treatment equalised levels of HA-S6K1 present when co-expressed with TSC2. Results are representative of three independent experiments.

Analysis of total HA-S6K1 levels within the assay showed reduced levels of the protein within cells co-expressing FLAG-TSC2. To investigate whether this may be due to reduced stabilisation of the protein due to reduced mTORC1 signalling caused by reintroduction of TSC2 into the cells, the effect of the proteasomal inhibitor MG132 on HA-S6K1 expression was investigated. Prior to lysis, cells were treated with MG132 for 2 hours. *TSC2*^{-/-} MEF cells were transfected with FLAG-TSC2 and HA-S6K1. Figure 4.12B shows that MG132 treatment rescued the levels of HA-S6K1 in cells expressing FLAG-TSC2 in comparison to control cells expressing HA-S6K1 only. This indicates that TSC2 over-expression in *TSC2*^{-/-} MEF cells results in proteasomal degradation of HA-S6K1.

4.2.11 Tip41 does not interact with TSC2

Figure 4.12 indicated the possibility that Tip41 may be inhibiting mTORC1 activity by acting on the TSC1/2 complex in a way that does not alter TSC2 phosphorylation at T1462. If Tip41 indeed influences TSC1/2 activity directly, it may interact with TSC2. To investigate this possibility, GST-TSC2 was expressed in HEK293 cells and purified using a GST spin-trap column. Figure 4.13 shows that Tip41 was not detected as an interacting protein of GST-TSC2. This indicates that if Tip41 activates the TSC1-TSC2 complex to inhibit mTORC1 signalling, this may not occur by direct interaction with TSC2. Alternatively, interaction between Tip41 and TSC2 may be transient.

4.2.12 Tip41 does not inhibit mTORC1 via the NF- κ B or ATM/ATR signalling pathways

Previously published data indicate a role of Tip41 within both the ATM/ATR and NF- κ B signalling pathways. Mutation of *ATM* in humans leads to the autosomal recessive disease A-T. Following ionising radiation, the resulting double and single strand DNA breaks cause activation of ATM and ATR kinases respectively. This results in initiation of a signalling cascade that includes substrates such as the checkpoint kinases Chk1 and Chk2, culminating in cell cycle arrest. Activation of mTORC1 signalling has been observed in the thymocytes of *ATM*^{-/-} mice resulting in c-Myc deregulation and spontaneous DNA synthesis (Barlow et al., 1996 & Kuang et al., 2009). In addition, a number of proteins within the IGF-1 pathway, including

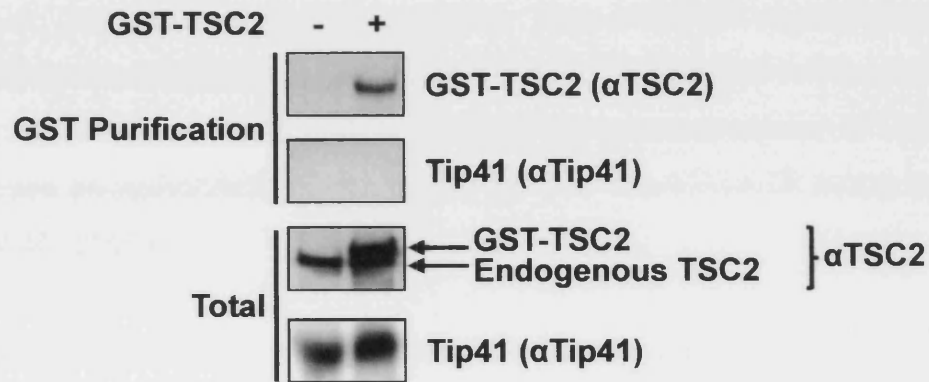


Figure 4.13: GST-TSC2 did not co-purify Tip41. GST-TSC2 was expressed in HEK293 cells, purified and analysed by western blot for interaction with endogenous Tip41. The data show that Tip41 did not co-purify with TSC2. Results are representative of three independent experiments.

TSC1 and 4EBP1, undergo phosphorylation events in response to ionising radiation (Matsuoka et al., 2007 & Braunstein et al., 2009). Thus mTORC1 signalling appears to be regulated by the ATM/ATR signalling cascade and is involved in the phenotype of A-T. Using ATM/ATR substrate phospho-antibodies, over-expression of Tip41 was found to increase phosphorylation of an unidentified 32kDa ATM/ATR substrate (McConnell et al., 2007).

To rule out that Tip41 mediated inhibition of mTORC1 signalling occurs via activation of an ATM/ATR substrate, activity of the kinases on over-expression of Tip41 was investigated. Prior to lysis, cells were cultured overnight in serum free media and treated with insulin as indicated. V5-Tip41 was expressed in HEK293 cells and ATM/ATR substrate phosphorylation analysed by western blot. Figure 4.14 shows that expression of V5-Tip41 had no effect on the phosphorylation of any ATM/ATR substrates in our hands. This indicates that the inhibition of mTORC1 signalling caused by over-expression of Tip41 in HEK293 cells is not due to any influence on the ATM/ATR signalling pathway.

A role of Tip41 has also been observed within the NF- κ B signalling pathway. Binding of the cytokine IL-1 β to its cognate receptor IL-1R results in activation of IKK. This requires TAK1 along with a number of TAB (TAK1 activating) proteins. Prickett et al (2008) found that Tip41 acts as a novel TAB protein, TAB4, by inducing autophosphorylation and activation of TAK1. This results in activation of TAK1 and subsequently IKK β . Active IKK β phosphorylates the inhibitory protein I κ B, resulting in its degradation by the 26S proteasome, and thus activates NF- κ B. However, over-expression of Tip41 alone had no effect on the pathway, and activation of IKK β required co-expression with TAB1 (Prickett et al., 2008). Activated IKK β inhibits insulin signalling in response to TNF α via phosphorylation of IRS-1 at S312 (Gao et al., 2002). In contrast, IKK β also phosphorylates TSC1 resulting in activation of mTORC1, and phosphorylation of IRS-1 at S307 and S636/639 via a negative feedback loop (Lee et al., 2008).

In order to determine whether inhibition of mTORC1 signalling by Tip41 may be caused by activation of NF- κ B signalling, the phosphorylation of IKK β at S176/180

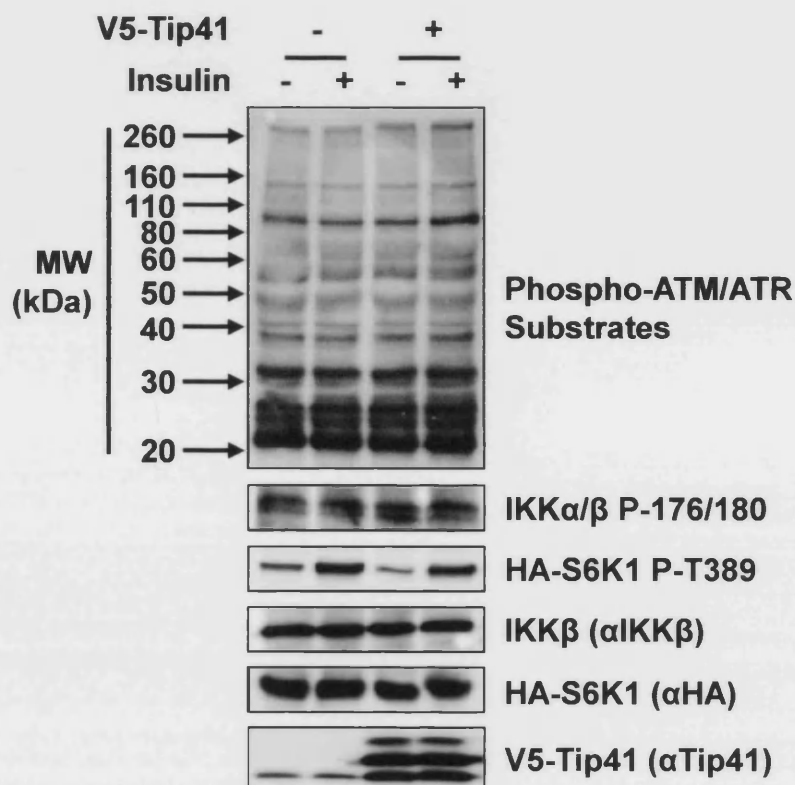


Figure 4.14: Tip41 does not alter activity of the ATM/ATR kinases or IKK β . As a role of Tip41 in signalling to NF- κ B and ATM/ATR, activity within these pathways was analysed on overexpression of Tip41 in order to ensure that inhibition of mTORC1 by Tip41 was specific to the mTORC1 pathway. HEK293 cells transiently expressing V5-Tip41 were cultured overnight in serum free media and treated with insulin prior to lysis as indicated. Analysis using the ATM/ATR substrate phosphorylation antibody showed no alteration on expression of V5-Tip41 in comparison to control cells. In addition, analysis of IKK β phosphorylation, which lies upstream of NF- κ B, showed no difference on expression of V5-Tip41 in comparison to control cells. Results are representative of three independent experiments.

was investigated by western blot (Figure 4.14). Expression of V5-Tip41 did not increase phosphorylation of IKK β . This shows that the inhibition of mTORC1 by Tip41 is specific to Tip41 action within the mTORC1 pathway. In conclusion these data show that the inhibition of mTORC1 signalling by Tip41 is specific to the pathway and is not due to the influence of Tip41 on certain other pathways within the cell.

4.3 Discussion

These data implicate Tip41 as an inhibitor of mTORC1 signalling. The true effect of over-expression of Tip41 leads to inhibition of S6K1 at T389 and activity towards the substrate rpS6 (Figure 4.1A). Phosphorylation of 4EBP1 is also reduced on expression of V5-Tip41 (Figure 4.2A). Four mTORC1 responsive phosphorylation sites exist within 4EBP1; T37, T46, S65 and T70. Phosphorylation of T37 and T46 require amino acids, and act as priming sites for S65 and T70 which are responsive to other upstream stimuli such as insulin and growth factors (Gingras et al., 2001). The numerous phosphorylation sites within 4EBP1 give rise to three isoforms when resolved by gel electrophoresis, with the α isoform being hypophosphorylated and the γ -isoform hyperphosphorylated. Over-expression of Tip41 resulted in accumulation of the α -isoform of 4EBP1 following serum-starvation and insulin stimulation (Figure 4.2A). On over-expression of Tip41, 36% of 4EBP1 was present in the α -isoform as compared to 16% in control cells following insulin stimulation. Tip41 overexpression also inhibited phosphorylation of 4EBP1 at S65. These data implicated Tip41 as an inhibitor towards phosphorylation of the two best characterised mTORC1 substrates. Transcriptional activity of HIF1 is also activated by mTORC1 (Laughner et al., 2001). Analysis of HIF1 activity on over-expression of V5-Tip41 also showed reduced activity using a HIF1 luciferase assay (Figure 4.2) ($p < 0.05$). As HIF1 activity is activated by mTORC1, this is concurrent with Tip41 acting as a general inhibitor of mTORC1 activity.

As an upstream regulator of these mTORC1 substrates, Tip41 may have diverse consequences in the cell. 4EBP1 is an inhibitor of cap-dependant translation by preventing interaction of eIF4G with eIF4E. Phosphorylation of 4EBP1 results in removal from eIF4E and allows interaction with eIF4G (Brunn et al., 1997, Schalm et

al., 2003, Eguchi et al., 2006). S6K1 has numerous positive effects on cap-dependent translation, including phosphorylation of ribosomal protein S6 and eIF4B (Shahbazian et al., 2006). Thus inhibition of phosphorylation of these proteins by Tip41 may result in a global inhibition of cap-dependent translation. HIF1 controls expression of genes involved in angiogenesis and glycolysis in response to hypoxia (reviewed in Semenza, 2001). Tip41 may also act to inhibit expression of these genes and thus inhibit the hypoxic response mediated by mTORC1. The placement of Tip41 as an inhibitor of mTORC1 signalling is analogous to the role of Tip41 in yeast. Yeast Tip41 indirectly inhibits TORC1 by sequestering the Pph21/22 and Sit4 negative regulatory subunit Tap42. When TORC1 is inactive, Tip41 sequesters Tap42 allowing dephosphorylation of substrates by Sit4 and Pph21/22 and inhibition of downstream signalling. Thus Tip41 could indirectly enhance the dephosphorylation of TORC1 substrates (Jacinto et al., 2001).

Studies *in vitro* have identified Tip41 as a negative regulator of PP2Ac (McConnell et al., 2007). In contrast, inhibition of mTORC1 by Tip41 over-expression places Tip41 as a positive regulatory subunit of PP2Ac. A similar situation exists regarding Alpha4. Studies *in vitro* implicate Alpha4 as a PP2Ac inhibitor (Chung et al., 1999, Prickett & Brautigan, 2004, Prickett & Brautigan, 2006), whereas Alpha4 over-expression leads to both a reduction and increase in 4EPB1 and S6K1 phosphorylation (Nanahoshi et al., 1998, Nien et al., 2007, Grech et al., 2008). Other studies place Alpha4 as a positive PP2Ac regulatory subunit *in vivo* (Prickett & Brautigan, 2007, McConnell et al., 2010). Therefore, the regulation of PP2Ac by Alpha4 is not clear cut. Tip41 may act similarly, resulting in PP2Ac inhibition *in vitro* but mediating dephosphorylation of substrates *in vivo*. Tip41 may therefore act as a positive regulatory PP2Ac subunit within the mTORC1 pathway.

In some instances Tip41 over-expression resulted in increased phosphorylation of S6K1 (Figure 4.1B) and 4EPB1 (Figure 4.2B). This prompted further investigation into the possibility that Tip41 over-expression was initiating feedback loops. On stimulation of mTORC1, negative feedback loops attenuate activation preventing aberrant signalling. The principle method of negative feedback is via serine phosphorylation of IRS-1 at S636/639 and S1101 by mTORC1 and S6K1 (Veileux et al., 2010). This results in depletion of IRS-1 by ubiquitination-mediated proteasomal

degradation (Zhande et al., 2002, Greene et al., 2003). Chronic inactivation of mTORC1 conversely results in upregulation of Akt via an IRS-1/PI3K independent mechanism (Wang et al., 2008, Chen et al., 2010). Akt phosphorylates TSC2 at a number of serine residues to inhibit GAP activity, including T1462 (Dan et al., 2002, Inoki et al., 2003, Potter et al., 2003). It was necessary to decipher the true effect of Tip41 over-expression in relation to mTORC1. In addition to the possibility of Tip41 activating mTORC1 by inhibiting PP2A, Tip41 expression could in theory be causing upregulation of Akt induced by chronic inhibition. Conversely, Tip41 inhibition of mTORC1 substrate phosphorylation could be due to activation of PP2A or a negative feedback loop-induced downregulation of IRS-1 filtering down to mTORC1 by chronic activation of mTORC1.

In samples where Tip41 over-expression resulted in increased phosphorylation of S6K1 and 4EBP1, TSC2 phosphorylation at T1462 was enhanced, indicative of upregulated Akt (Figure 4.3B). Analysis of samples where a reduction in mTORC1 substrate phosphorylation was observed showed no change to phosphorylation of IRS-1 at S636/639, TSC2 at T1462 or Akt at T308 in comparison to control cells indicating no induction of negative feedback (Figure 4.3A). Thus the direct effect of Tip41 over-expression is to inhibit mTORC1 signalling, and any observed increase in phosphorylation of mTORC1 substrates was due to the upregulation of Akt as an indirect consequence of elevating the negative feedback mechanism from mTORC1/S6K1 to IRS-1. This indirect consequence fed into heightened mTORC1 by phosphorylation of TSC2 on residue T1462 by Akt.

Similarly, knockdown studies using shRNA targeted to Tip41 resulted in inhibition of S6K1 phosphorylation at T389 (Figure 4.9A). This is in contrast to the expected outcome of increased S6K1 phosphorylation due to removal of inhibitory Tip41 activity towards mTORC1. Given the long duration of Tip41 knockdown by reverse transfection (4 days post-transfection), it is very likely that the cells have adapted accordingly by down-regulating the PI3K/Akt pathway. Analysis of IRS-1 phosphorylation revealed a slight increase in IRS-1 phosphorylation at S636/639, indicating activation of the negative feedback loop via S6K1 and mTORC1. The poor effect of Tip41 knockdown on S6K1 (and IRS-1) phosphorylation could be due to the incomplete knockdown of Tip41 by shRNA of approximately 50% (Figure 4.9B).

Alteration of Tip41 protein levels within the cell clearly renders mTORC1 sensitive to feedback mechanisms. The opposing outcome of Tip41 overexpression on mTORC1 signalling was probably caused by slight variations in the time between transfection and lysis. This ultimately would lead to variations in the time that Tip41 was overexpressed. Although every attempt was made to maintain consistency in my work, the exact time of transfection and lysis was in hindsight critical. Tip41 overexpression over a slightly longer timescale would in theory create opportunity for the cells to respond by instigating feedback via upregulation of Akt. Therefore if the work was to be repeated, I would ensure that cells were transfected and lysed at precisely the same time in each experiment to ensure consistency. To determine whether this theory is correct, and Tip41 overexpression over a longer timescale would promote induction of feedback via upregulation of Akt, a transfection to lysis time course experiment could be performed. Multiple experiments as in Figure 4.1 would be set up simultaneously, with single experiments lysed at precisely 45h, 48h and 51h post-transfection. Analysis of HA-S6K1 phosphorylation would, if this theory is correct, show initial inhibition in cells lysed 45h post-transfection, migrating to enhanced phosphorylation at 51h. Analysis of TSC2 phosphorylation at T1462 would also provide a marker for feedback induction. This would then also provide confidence in further experimentation, where the exact time of lysis would be known to ensure consistent results.

In contrast to the effect on S6K1 phosphorylation, knockdown of Tip41 resulted in a 10 fold increase in activity of HIF1 ($p < 0.05$) (Figure 4.10). Tip41 regulation of HIF1 activity therefore appears more sensitive to Tip41 knockdown than S6K1, which indicates that Tip41 regulation of mTORC1 may be more central in regulation of the hypoxic response downstream of the kinase. In addition, HIF1 was not sensitive to the induction of feedback loops initiated by modification of Tip41 levels within the cell.

Tip41 regulation of HIF1 may be explained by the discovery of a higher resolving nuclear form of Tip41 (Figure 4.11). Nuclear Tip41 has an apparent molecular weight of approximately 39kDa as opposed to 32kDa for the cytoplasmic form. Thus nuclear Tip41 may be subject to post-translational modification resulting in the apparent shift in molecular weight. Although Tip41 is a phosphoprotein (Figure 3.11),

the jump in apparent molecular weight is too large to be explained by phosphorylation.

Ubiquitin is an 8kDa protein used as a post-translational modification used to modify protein stability, protein-protein interactions, activity and subcellular localisation. Both mono- and polyubiquitination can occur, mediated by an E3 ligase and resulting in the transfer of one or multiple ubiquitin molecules respectively. Ubiquitin molecules are linked by Lysine residues within both itself and the target protein. The shift in molecular weight may therefore be caused by monoubiquitination of Tip41 which may result in nuclear translocation and ability to regulate HIF1 activity. By a similar mechanism, the transcription factor FOXO4 is regulated by ubiquitination. Ubiquitination of FOXO4 by Mdm2 leads to nuclear translocation and transcriptional activation (Kubbutat et al., 1997, Lai et al., 2001, van der Horst et al., 2006, Brenkman et al., 2008).

A nuclear complex of PP2A_{Alpha4} with mTORC1 has been implicated in activation of STAT1 (Fielhaber et al., 2009). Indeed post-translational modification of Alpha4 with O-linked β -N-acetylglucosamine is required for nuclear translocation (Dauphinee et al., 2005). Thus nuclear translocation for the activity of PP2A_{Alpha4} with mTORC1 has been documented. Tip41 could similarly translocate with PP2A to the nucleus following modification by ubiquitination where it regulates the activity of HIF1. It would be of interest to investigate the effect of Tip41 on other mTORC1 regulated transcription factors including STAT3 and YY1, and see whether Tip41 has increased effect on transcriptional rather than translational regulation by mTORC1. Tip41 is known to bind ubiquitinated proteins (Prickett et al., 2008). It is therefore conceivable that ubiquitination of Tip41 provides a platform for protein-protein interactions, where Tip41 forms a complex with other proteins to mediate substrate interaction, which all bind via ubiquitin. Ubiquitination of Tip41 may therefore allow binding of transcription factors or other proteins involved in the inhibition of transcription factors. In summary, the possibility of ubiquitination of Tip41 may cause nuclear translocation or may result from location in the nucleus to mediate complex interaction involved in regulation of transcription factors such as HIF1.

Of interest in this regard would be the activity of a Tip41 mutant deficient in PP2A binding. In contrast to previously published work, mutation of Y79H and M196V retained ability to bind PP2A (Figure 4.6A) (Smetana et al., 2007). Although V5-Tip41(D71L) did not bind PP2A (Figure 4.6A), it also had a reduced expression that was not equalised by inhibition of the proteasome by MG132 (Figure 4.7). Thus although Tip41(D71L) had no effect on S6K1 phosphorylation, in contrast to inhibition of S6K1 phosphorylation by wild-type V5-Tip41, this could be explained by the reduced expression of the mutant. An attempt to equalise expression of wild-type V5-Tip41 and V5-Tip41(D71L) resulted in very low expression levels and no effect on S6K1 phosphorylation in comparison to control cells. Similar can be said of the Tip41 TOS mutant F156A. Expression of HA-Tip41 (F156A) resulted in inability to inhibit phosphorylation of rpS6 in an S6K1 assay in comparison to inhibition by wild-type HA-Tip41 (Figure 4.5A). Despite this, HA-Tip41(F156A) levels were reduced in comparison to wild-type HA-Tip41, which was not equalised by treatment with MG132 (Figure 4.5B). Failure of these experiments renders us unable to determine that the effects of Tip41 within the pathway are definitely as a result of Tip41 binding to PP2A.

On discovery that Tip41 inhibits phosphorylation or activation of mTORC1 substrates, the point of action of Tip41 within the pathway was investigated. Data on investigation of the negative feedback loop (Figure 4.3A) showed that Tip41 did not affect phosphorylation of Akt at T308 (Figure 4.3A), S473 (Figure 3.11) or TSC2 phosphorylation at T1462 (Figure 4.3A). As the two phosphorylation sites on Akt were unaffected by Tip41 over-expression, an effect on Akt or upstream of Akt could quite confidently be ruled out. TSC2 has a number of phosphorylation sites in addition to T1462, so although over-expression of Tip41 did not alter phosphorylation of this site, this does not rule out PP2A_{Tip41}-mediated dephosphorylation of other sites. Activity of Tip41 at or downstream of TSC2 was therefore investigated (Li et al., 2002, Shumway et al., 2003, Cai et al., 2006).

Phosphorylation of TSC2 at a number of phosphorylation sites including S1462 results in inhibition of GAP activity allowing the accumulation of GTP-Rheb, activating mTORC1 (Tee et al., 2001(a), Inoki et al., 2002). Tip41 could inhibit mTORC1 through PP2A-mediated dephosphorylation of these TSC2

phosphorylation sites resulting in activation of TSC1/2. To investigate this Tip41 was expressed in *TSC2*^{-/-} MEFs. *TSC2*^{-/-} MEFs have overactive mTORC1. If Tip41 acts downstream of TSC2, over-expression in *TSC2*^{-/-} MEFs would abrogate S6K1 phosphorylation. These cells were of particular use owing to the feedback induction caused by Tip4 overexpression. Owing to their genetic failings, *TSC2*^{-/-} MEFs are resistant to Tip41-induced feedback via phosphorylation of TSC2, in theory, when analysing targets downstream of TSC1/2. Overexpression of Tip41 in *TSC2*^{-/-} MEFs resulted in no reduction in phosphorylation of S6K1 at T389 (Figure 4.12A). As Akt lies directly upstream of TSC2 and Tip41 over-expression didn't alter Akt phosphorylation, this provides evidence that Tip41 may act directly at the point of TSC2. If Tip41 were to act as a PP2A regulatory subunit towards TSC2, you would expect to see binding between TSC2 and Tip41. As no interaction between the two proteins was observed (Figure 4.13), Tip41 may act in a parallel pathway to ensure inhibition of mTORC1 substrates, possibly by mediating activity of PP2A. Alternatively PP2A_{Tip41} interaction with TSC2 may be transient and therefore was not detected. Thus the possibility that PP2A_{Tip41} dephosphorylates inhibitory TSC2 phosphorylation events in order to inhibit mTORC1 remains.

Tip41 has been shown to act within the ATM/ATR cascade and within signalling to NF-κB. ATM is known to inhibit phosphorylation of 4EBP1 and therefore potentially inhibits upstream of mTORC1 (Barlow et al., 1996, Matsuoka et al., 2007, Braunstein et al., 2009). Tip41 was shown to activate phosphorylation of an unidentified 33kDa ATM/ATR substrate (McConnell et al., 2007). This result was not reproduced here (Figure 4.14) indicating that Tip41 is not acting within the ATM/ATR pathway to inhibit mTORC1. Tip41 is also known as TAB4 involved in activation of NF-κB (Prickett et al., 2008). As signalling modules to NF-κB are involved in mTORC1 activation, the role of Tip41 as TAB4 was unlikely to inhibit mTORC1 signalling (Hartley & Cooper, 2002, Lee et al., 2008). In addition, the effect of over-expressed Tip41 alone did not activate phosphorylation of TAK1, and required co-expression of TAB1 (Prickett et al., 2008). In agreement with published findings, Tip41 did not increase phosphorylation of IKK (Figure 4.14), indicating that in our cells Tip41 expression is not activating NF-κB signalling.

Although Tip41 may mediate dephosphorylation of inhibitory TSC2 phosphorylation events by PP2Ac, it may also act to inhibit a parallel pathway feeding into mTORC1. Activation of mTORC1 also occurs in response to amino acids by various mechanisms, in addition to via PA and MAPK. Amino acid activation of mTORC1 relies on localisation of mTORC1 complex to membranes and is achieved by hVps34, the Rag GTPases and RalA (Gulati et al., 2008, Maehama et al., 2008, Sancak et al., 2008). Localisation to membranes allows interaction with Rheb and subsequent activation of the complex. Tip41 may therefore inhibit any of these processes in order to reduce phosphorylation of mTORC1 substrates. Tip41 is unlikely to inhibit PA-induced mTORC1 activity as this occurs downstream of Rheb, and results indicate that Tip41 acts upstream of Rheb (Figure 4.12). In addition to Akt, TSC2 is also a target of MAPK. In response to MAPK activation, RSK phosphorylates TSC2 at S1798 which removes the inhibitory effect of TSC2 allowing activation of mTORC1 (Roux et al., 2004). Tip41 could therefore act via MAPK to remove MAPK inhibitory phosphorylation sites of TSC2.

In summary, Tip41 inhibits mTORC1 activity upstream or at the point of the TSC1/2 complex. Alteration of cellular Tip41 levels enhances cell sensitivity to the induction of feedback loops via IRS-1 downregulation or activation of Akt. In addition, a specific nuclear isoform of Tip41 exists, possibly modified with ubiquitin, which may specifically regulate activity of HIF1. Interestingly, it appears that mTORC1-regulated activity of HIF1 is resistant to induction of negative feedback loops.

CHAPTER 5 – OTHER PP2A COMPLEXES AND mTOR

5.1 Introduction

Historically, DNA tumour viruses have been used to investigate phosphatase regulation within the cell. For example, the SV40 small T antigen enhances cell survival by inhibiting PP2Ac binding with the regulatory B subunit (reviewed in Branton & Roopchand, 2001). Mechanisms such as this are used by viruses to overcome checkpoints to enable replication. The adenoviral proteins E4ORF1 and E4ORF4 activate mTORC1. Whilst E4ORF1 activates PI3K, E4ORF4 inhibits PP2A_{Bα} by inhibition of substrate recognition (O'Shea et al., 2005(a)). Thus PP2A_{Bα} was identified as a phosphatase acting to inhibit mTORC1 activity. As E4ORF4 has no effect on Rheb loading, but S6K1 activation induced by the protein is sensitive to rapamycin, it was proposed that PP2A_{Bα} may act in a parallel pathway to growth factor stimulation of mTORC1.

As the mammalian functional homologue of Sit4, PP6c is well placed as a potential phosphatase acting within the mTORC1 pathway (Bastians & Ponstingl, 1996). Although a member of the PP2A family, PP6c has separate regulatory subunits including PP6RPs and Ankrd proteins and is therefore subject to distinct regulatory mechanisms (Stefansson et al., 2008). Sit4 controls phosphorylation of substrates downstream of TORC1. Despite this, the role of PP6c with regards to mTORC1 has not been investigated.

In this chapter the role of PP2A_{Bα} and PP6c in relation to mTORC1 signalling is investigated. The data show that PP2A_{Bα} acts upstream of mTORC1 but is able to overcome Rheb-induced activation of the kinase. In addition, mTORC1 may control PP2A_{Bα} directly by ubiquitination via the CUL4-DDB1 ligase, providing a feedback mechanism for activation of mTORC1. As PP6c unexpectedly appears to activate mTORC1, the potential role of the phosphatase in relation to mTORC1 is discussed.

5.2 Results

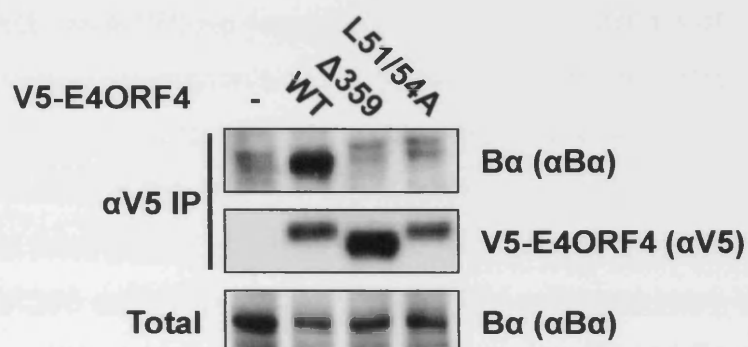
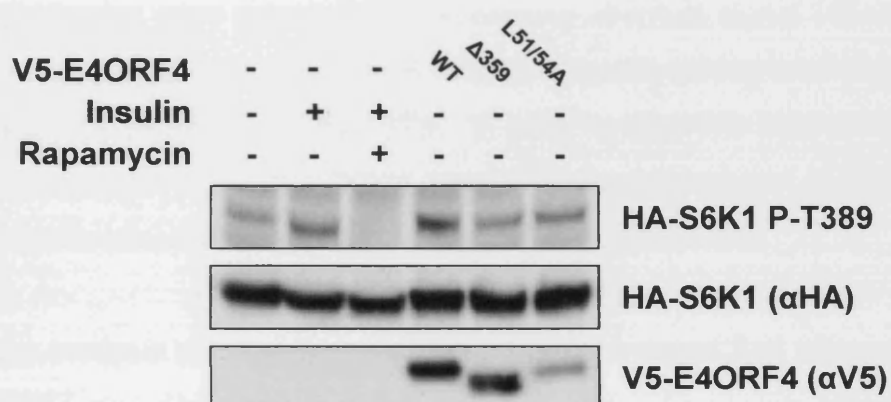
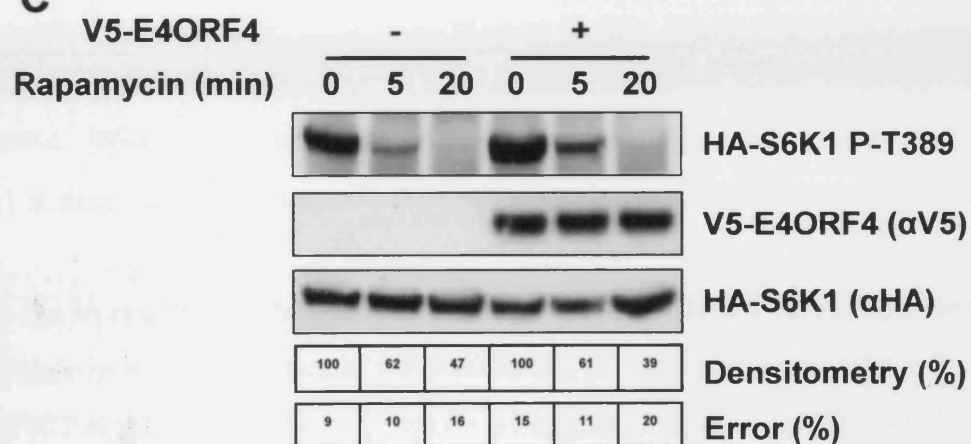
5.2.1 E4ORF4 activates mTORC1 signalling by sequestering the B α regulatory subunit of PP2Ac

The adenoviral protein E4ORF4 enhances mTORC1 signalling to S6K1 and 4EBP1 by sequestering the PP2Ac regulatory B α subunit to the nucleus (O'Shea et al., 2005(a)). In addition to wild-type E4ORF4, two mutants of E4ORF4 deficient in B α interaction, E4ORF4(L51/54A) (point mutant) and E4ORF4(Δ 359) (truncated) were obtained as a kind gift from Dr. D. Stokoe and cloned into V5-tagged vectors. In order to verify that wild-type E4ORF4 protein binds the B α regulatory subunit of PP2Ac, V5-E4ORF4 was expressed in HEK293 cells and purified using α V5 immunoprecipitation. V5-E4ORF4(Δ 359) and L51/54A mutant were also expressed and purified. Interaction with B α was then analysed by western blot. Figure 5.1A shows that V5-E4ORF4 co-purified with B α thus confirming interaction between the two proteins. Neither of the V5-E4ORF4 mutants, Δ 359 or L51/54A, co-purified with B α showing that these mutants are indeed deficient in binding to the regulatory subunit of PP2Ac.

In order to verify that E4ORF4 increases mTORC1 substrate phosphorylation via B α subunit interaction, the ability of wild-type V5-E4ORF4 to activate T389 phosphorylation of S6K1 was compared to the Δ 359 and L51/54A binding mutants. HEK293 cells expressing the three V5-E4ORF4 protein constructs were cultured in serum free medium overnight prior to lysis and treated with insulin and rapamycin as indicated. Figure 5.1B shows that over-expression of V5-E4ORF4 wild-type had increased levels of HA-S6K1 phosphorylation at T389 in comparison to serum-starved (and insulin treated) control cells expressing HA-S6K1 only. This shows that V5-E4ORF4 enhances mTORC1 signalling towards S6K1. Neither of the B α -binding mutants of E4ORF (Δ 359 or L51/54A) altered HA-S6K1 phosphorylation in comparison to serum-starved control cells. This data indicates that the increase in mTORC1 signalling towards S6K1 by E4ORF4 over-expression is dependent on E4ORF4 binding to the B α subunit.

Previous studies revealed that E4ORF4 interacts with the B α subunit (O'Shea et al., 2005(a)), and this data confirms that this interaction is necessary for heightened

Figure 5.1: E4ORF4 activates mTORC1 by inhibition of PP2A_{Bα}. **A.** In order to verify that V5-E4ORF4 mutants Δ359 and L51/54A were deficient in Bα interaction, pcDNA3.1-nV5-E4ORF4 wild type and the mutants Δ359 and L51/54A were transfected into HEK293 cells and the expressed proteins immunoprecipitated (αV5) and analysed for co-purification of Bα. The results showed that mutation of L51/54A and Δ359 mutation of V5-E4ORF4 prevented interaction with Bα. **B.** To qualify that the activation of mTORC1 by E4ORF4 was as a result of Bα inhibition, V5-E4ORF4 wild type and mutants were co-expressed in HEK293 cells with HA-S6K1 and the activity of HA-S6K1 analysed. Cells were cultured overnight in serum free media and prior to lysis treated with insulin and rapamycin as indicated. The results showed that V5-E4ORF4(Δ359) and L51/54A were unable to enhance phosphorylation of HA-S6K1, whereas wild type V5-E4ORF4 enhanced phosphorylation of HA-S6K1 above the level as seen with insulin stimulation. **C.** A rapamycin time-course experiment was used to assay whether E4ORF4 could inhibit the ability of rapamycin to promote mTORC1 substrate dephosphorylation, and by extension whether PP2A_{Bα} was responsible for mTORC1 substrate dephosphorylation downstream of the kinase. Cells were cultured overnight in serum free media and treated with insulin. Rapamycin treatment occurred 0, 5 or 20 minutes prior to lysis, and activity of co-expressed HA-S6K1 analysed as a measure of mTORC1 activity. Maximal HA-S6K1 phosphorylation was standardised for densitometry analysis both with and without V5-E4ORF4 expression to take into consideration the initial increase in HA-S6K1 phosphorylation caused by expression of V5-E4ORF4. Intensity was calculated by averaging data from three independent experiments and taking HA-S6K1 phosphorylation prior to rapamycin treatment (with or without E4ORF4 expression) as 100%. Significance was calculated using a T test. Error is indicative of standard deviation. Densitometry analysis of HA-S6K1 phosphorylation showed that expression of V5-E4ORF4 did not significantly alter the ability of rapamycin to promote mTORC1 substrate dephosphorylation. Results are representative of three independent experiments.

A**B****C**

phosphorylation of S6K1 (Schalm et al., 2005). Rapamycin is thought to activate a phosphatase activity that specifically dephosphorylates mTORC1 substrates, such as S6K1. If B α acts as a PP2Ac regulatory subunit downstream of mTORC1, E4ORF4 may prevent rapamycin's ability to induce rapid dephosphorylation of mTORC1 substrates. Therefore, I wanted to determine whether E4ORF4 over-expression could reduce the ability of rapamycin to dephosphorylate S6K1. To do this, a rapamycin time course experiment was performed upon E4ORF4 over-expression. HEK293 cells expressing V5-E4ORF4 wild-type were treated with rapamycin for 5 or 20 min following stimulation with insulin and the phosphorylation of co-expressed HA-S6K1 compared to control cells expressing HA-S6K1 only. Densitometry figures were calculated by averaging absolute signal intensity between three experiments, then converting to % relative intensity taking intensity following 0 minutes of rapamycin treatment as 100%. In order to take into account the initial enhancement of S6K1 phosphorylation caused by overexpression of E4ORF4, 100% was taken standardised between experimental and control cells.

Densitometry analysis of HA-S6K1 phosphorylation revealed that although E4ORF4 enhanced S6K1 phosphorylation, rapamycin inhibition was equally efficient in comparison to control cells, with no significant difference seen between experimental and control cells at each time point (Figure 5.1C). Following 5 min rapamycin treatment, S6K1 phosphorylation was at 60% maximal levels both with and without E4ORF4. This provides evidence that the rapamycin induced dephosphorylation of S6K1 is dominant with regards to E4ORF4 over-expression.

5.2.2 B α is regulated by ubiquitination in an mTORC1 specific manner

A number of studies implicate PP2Ac as the principle phosphatase within the mTORC1 signalling pathway (Begum & Ragola, 1996, Peterson et al., 1999, Schlam et al., 2005, Bielinski & Mumby, 2007, Liu et al., 2010). Regulatory subunits of PP2Ac are known to control substrate recognition, localisation or kinetic activity. It is therefore essential to identify the subunit regulating PP2Ac along with the method of regulation in the context of mTORC1 signalling. The results of Figure 5.1 suggest that B α regulates PP2Ac activity towards the mTORC1 substrate, S6K1. Regulation of PP2Ac activity may take place via interaction between the catalytic subunit and

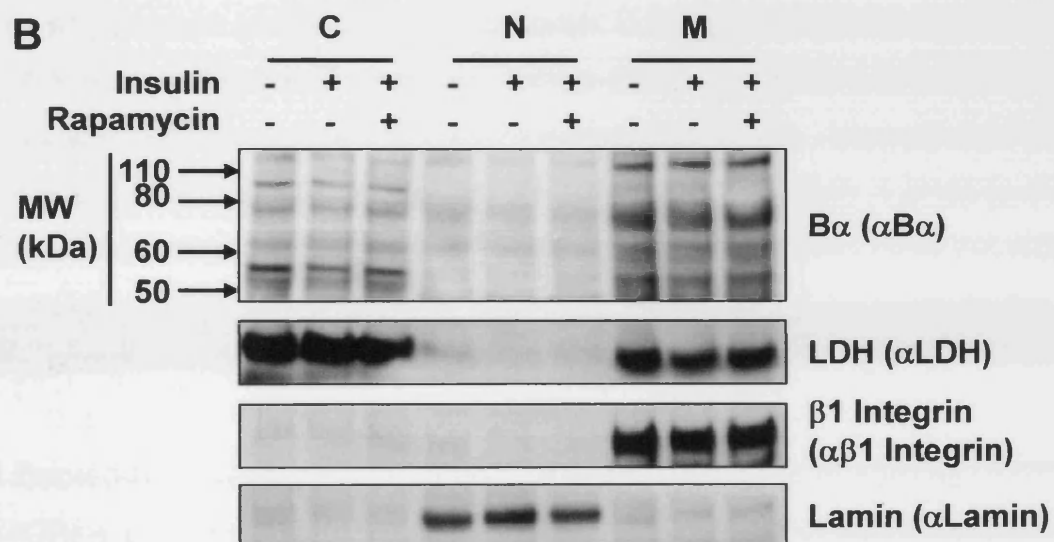
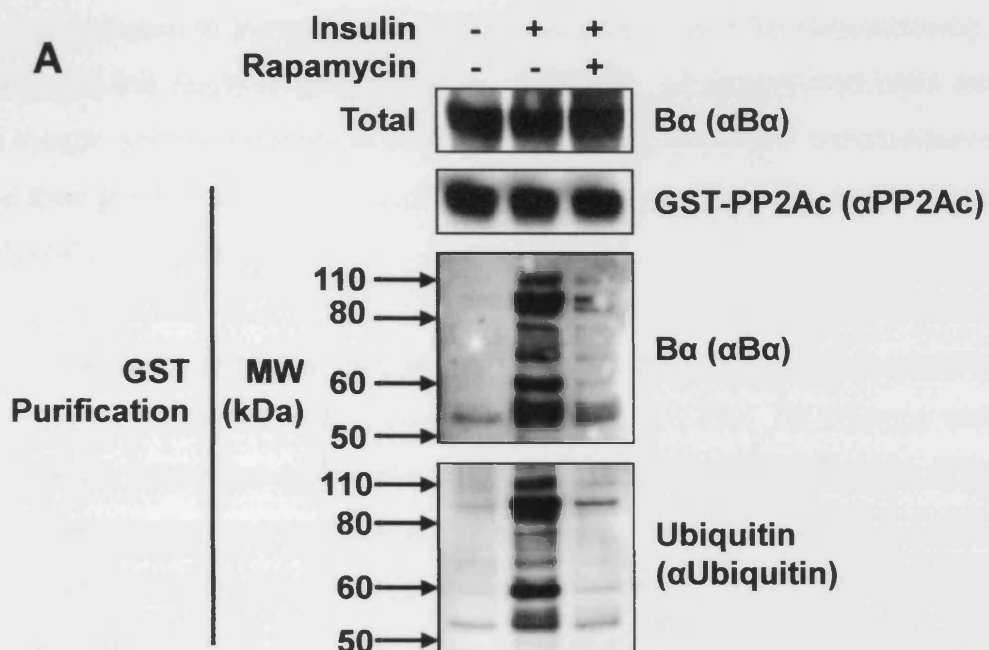
the B α regulatory subunit. It is therefore possible that under conditions when mTORC1 is inactive, PP2Ac binding to the regulatory subunit involved in targeting to mTORC1 substrates may increase.

To determine whether interaction between PP2Ac and the B α regulatory subunit was modulated, GST-PP2Ac was transfected into HEK293 cells and purified following serum-starvation, insulin stimulation and rapamycin treatment, as indicated.

Purification of GST-PP2Ac was then achieved using a GST spin-trap column. The levels of co-purified endogenous B α were then evaluated by western blot. Figure 5.2A shows that B α purified with GST-PP2Ac under all three conditions. However, following insulin treatment, purified B α resolved at a number of different molecular weights in comparison to the blot seen on serum-starvation, and this effect was almost completely ablated following treatment of cells with rapamycin. Due to the shift in the apparent molecular weight, with bands seen at approximately 8.5kDa intervals, it was possible that the B α subunit was post translationally modified by ubiquitination. Following re-analysis of purified sample by western blot using α -ubiquitin, a near identical pattern was observed of ubiquitinated protein bands. This data reveals that the apparent shift in B α molecular weight observed in complex with GST-PP2Ac following insulin stimulation was due to ubiquitination of the B α regulatory subunit.

As B α co-purified with GST-PP2Ac under all conditions, interaction between the two proteins does not appear to be a method for regulation. Instead, ubiquitination of the B α subunit appears to be used to control activity of the complex, and may be used to reduce activity of PP2A_{B α} under conditions when phosphatase activity is not required. In this instance, ubiquitination does not appear to result in proteasomal degradation of B α , as levels of the protein seen in total lysate are equal under all conditions of purification.

As ubiquitination did not appear to result in proteasomal degradation of B α , the possibility that it may control cell localisation was investigated. In theory, the PP2A_{B α} complex may be shuttled to another cell compartment following insulin stimulation to remove phosphatase activity when not required, and this may be controlled by



ubiquitination of the B α regulatory subunit. In support of this theory, E4ORF4 protein has been shown to increase HA-S6K1 phosphorylation by sequestering B α and shuttling to the nucleus (O'Shea et al., 2005(a)). Untransfected cells were treated with insulin and rapamycin as indicated following overnight serum-starvation. Cells were then fractionated into cytoplasmic, nuclear and membrane compartments and analysed by western blot.

Analysis with B α antibody revealed a predominantly cytoplasmic protein with a trace found within the membrane compartment (Figure 5.2B). No change was seen in the levels of B α within the cytoplasm or membrane fraction under each of the conditions. This indicates that cell localisation is not used as a method of regulation of the B α subunit upon insulin stimulation, or that ubiquitination of the B α subunit results in any change in localisation of the protein. In addition, no B α was apparent within the nuclear fraction. As the shift in apparent molecular weight observed in Figure 5.2A was not apparent on analysis of total B α levels in any of the cell compartments fractionated, ubiquitinated B α may only form a small pool of B α that is bound to PP2Ac and is not apparent when viewing total protein levels. Ubiquitinated B α therefore appears to bind PP2Ac specifically, and may only form a small pool of total protein. In summary, ubiquitination of B α when bound to PP2Ac does not appear to influence localisation of the subunit. Therefore ubiquitination of B α within the PP2A_{B α} complex may play an as of yet unidentified role in the regulation of activity.

5.2.3 Depletion of B α increases S6K1 phosphorylation and activity of HIF1

V5-E4ORF4 increases HA-S6K1 phosphorylation in a manner dependent on the binding of the B α regulatory subunit of PP2Ac (Figure 5.1B). It therefore appears that B α may regulate phosphatase activity within the mTORC1 pathway. The effect of B α knockdown on mTORC1 signalling using shRNA was investigated. A panel of shRNA vectors was tested to find the most effective clone for knockdown of B α . Firstly, the time between transfection of cells with shRNA and observation of B α knockdown was optimised. Cells were seeded in triplicate on day one, transfected on day 2 and lysed on either day 3, 4 or 5. This showed that effective knockdown of B α occurred 72h post transfection within cells lysed on day 5 (data not shown). To minimise the time between cell seeding and lysis, the reverse transfection procedure

was attempted. Cells were seeded and transfected in one step on day 1, with cell lysis occurring on day 4. This resulted in improved cell condition prior to lysis. Control cells were transfected with non-coding shRNA. Levels of B α were analysed by western blot. Figure 5.3 shows that following reverse transfection and cell lysis on day 4, clone 5 was the most effective at knocking down B α levels, which resolves at a molecular weight of 55kDa. This clone was then used in all future shRNA experimentation.

V5-E4ORF4 expression results in an increase in mTORC1 substrate phosphorylation that is dependent on binding to the B α regulatory subunit of PP2Ac (Figure 5.1B). B α may therefore regulate mTORC1 substrate dephosphorylation via PP2Ac. To investigate the effect of B α knockdown on the mTORC1 pathway, following shRNA transfection on day 1 (targeted or non-coding), cells were transfected on day 3 with HA-S6K1 and serum-starved overnight. Prior to lysis on day 4, cells were treated with insulin and rapamycin as indicated and subsequently analysed by western blot. Signal intensity under each condition was averaged from three experiments and used for densitometry analysis. Maximal phosphorylation taken as 100% was used to calculate relative signal intensity. Error is indicative of standard deviation. A student's T test was used to calculate p values comparing data between experimental and control cells. HA-S6K1 phosphorylation at T389 showed a small but reproducible increase under serum starved ($p < 0.05$) and insulin stimulatory ($p < 0.05$) conditions following knockdown of B α in comparison to control cells transfected with non-coding shRNA (Figure 5.4A). There was no difference in HA-S6K1 phosphorylation following treatment with rapamycin or under serum starved conditions in either cell sample. This indicates that B α may be acting as a regulatory subunit for PP2Ac towards S6K1 as knockdown results in a slight increase in phosphorylation concurrent with a reduction in phosphatase activity. This result is complicated however as no increase was seen under serum-starved conditions or following treatment of cells with rapamycin. Under these conditions it is predicted that a phosphatase would be active.

As regulatory subunits of PP2Ac may be involved in substrate recognition, it would be expected that a reduction in the level of such a subunit within the cell would result in reduced ability of PP2Ac to bind a substrate and therefore result in an increase in



Figure 5.3: Optimisation of B α knockdown. A panel of five obtained shRNA clones directed to B α were transfected into HEK293 cells and levels of B α protein analysed by western blot in comparison to control cells transfected with non-coding shRNA. The results showed that clone 5 was the most effective at lowering levels of B α protein and was used in subsequent experimentation. Results are representative of three independent experiments.

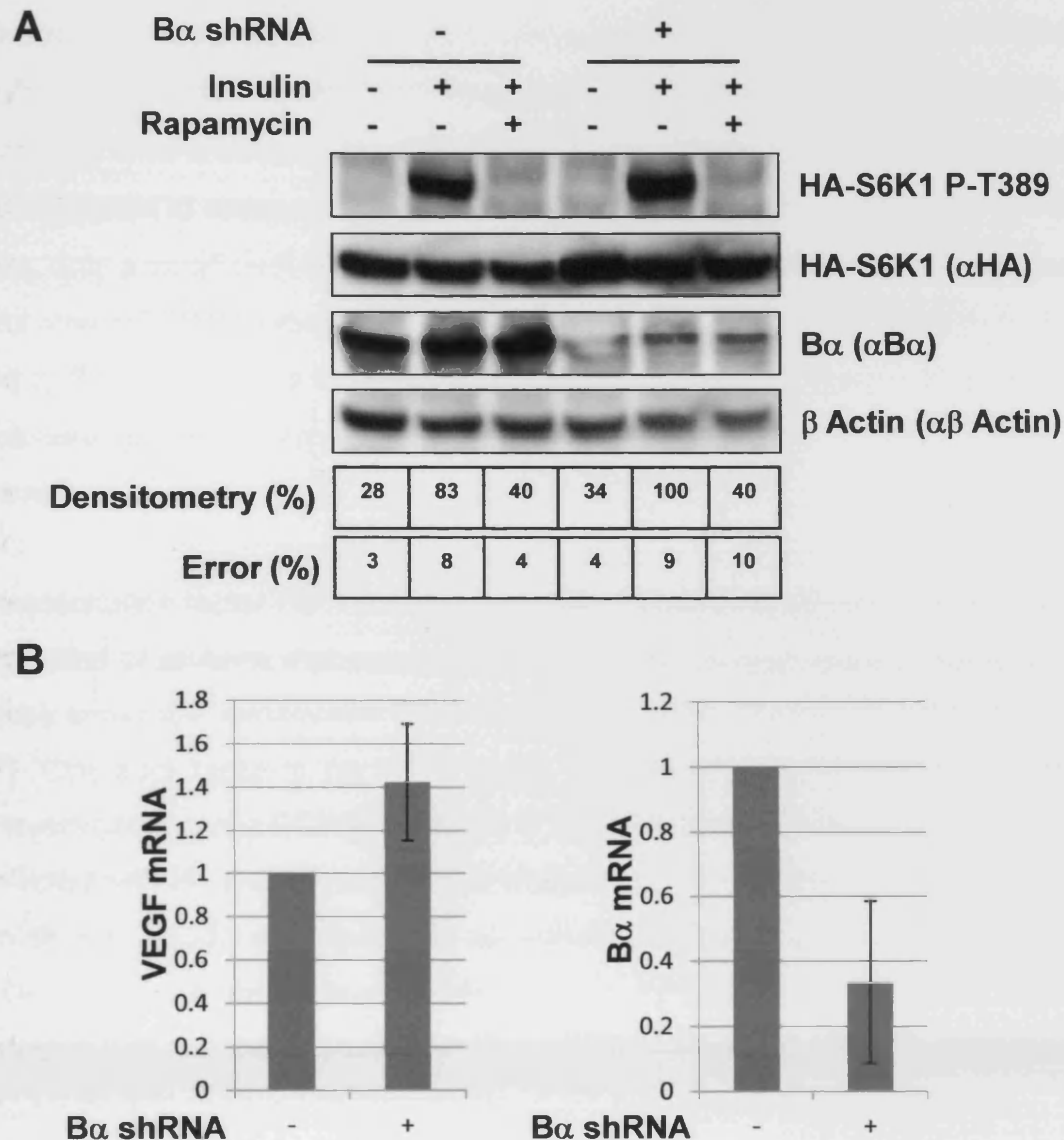


Figure 5.4: Knockdown of B α increases mTORC1 activity. **A.** HEK293 cells transfected with B α shRNA were co-transfected with HA-S6K1. Control cells were transfected with non-coding shRNA. Prior to lysis, cells were cultured in serum free medium and treated with insulin and rapamycin as indicated. HA-S6K1 phosphorylation was measured by SDS-PAGE followed by western blot and densitometry analysis. Absolute signal intensity was averaged between three experiments and converted to % using the highest intensity as 100%. Error is indicative of standard deviation. Following test for equal variances, p values were calculated using a student's T test. The data show that on knockdown of B α a small but significant increase in HA-S6K1 phosphorylation is observed following serum starvation ($p < 0.05$) and insulin stimulation ($p < 0.05$). No significant difference was observed following treatment with rapamycin in cells deficient in B α compared to control cells. **B.** On transient knockdown of B α Q-PCR of VEGF mRNA was used as a measure of HIF1 activity. Levels of B α mRNA were also analysed to ensure efficient knockdown. The data show that knockdown of B α increased expression of VEGF mRNA, and by extension activity of HIF1 ($p < 0.05$) in comparison to control cells transfected with non-coding shRNA. Error bars are indicative of standard deviation. Results are representative of three independent experiments.

substrate phosphorylation under conditions when the phosphatase is activated. As B α knockdown does not alter HA-S6K1 phosphorylation under predicted PP2Ac activating conditions, the role of PP2Ac_{B α} within the mTORC1 pathway may be more complicated than previously thought. Alternatively, the extent of B α depletion may not be sufficient to reduce phosphatase regulation. If B α levels within the cell are in excess, only a small pool of the protein may be required to maintain phosphatase control over mTORC1 signalling. In defence of this notion, only a small pool of B α , bound to PP2Ac, appears to be regulated by ubiquitination (Figure 5.2) as the ubiquitinated protein is not detected on analysis of total lysate or following cell fractionation.

The transcription factor HIF1 is activated under hypoxic conditions and results in upregulation of proteins that are required in order to combat hypoxic stress and increase anaerobic metabolism (Semenza et al., 1994, Chen et al., 2001, Kim et al., 2001). One such target is the VEGF gene. The effect of B α knockdown on HIF1 was investigated using RTPCR analysis of VEGF mRNA. Cells were reverse transfected with B α shRNA on day one and were serum-starved and treated overnight with insulin and rapamycin as indicated on day 3, under hypoxic conditions (1% O₂). On day 4 mRNA levels were analysed by Q-PCR. Control cells were transfected with non-coding shRNA. The results in Figure 5.4B show increased (42%, +/- 10.5%) in VEGF mRNA levels under serum starved conditions on knockdown of B α in comparison to control cells. Error bars are indicative of standard deviation. As it is predicted that PP2Ac would be active under these conditions, it appears that PP2Ac_{B α} negatively regulates VEGF expression under hypoxic conditions. Whilst this may be due to activation of mTORC1 and therefore HIF1 induced by loss of the B α regulatory subunit, it is important to note that VEGF is also regulated by other transcription factors and signalling pathways. Therefore the effect of B α knockdown resulting in enhanced VEGF expression may be due to an effect on a parallel pathway that also regulates the growth factor.

Q-PCR was then used to quantify the extent of B α knockdown. Following transfection of HEK293 cells with B α shRNA vector or non-coding shRNA, Q-PCR procedure and analysis was undertaken as detailed in Materials and Methods.

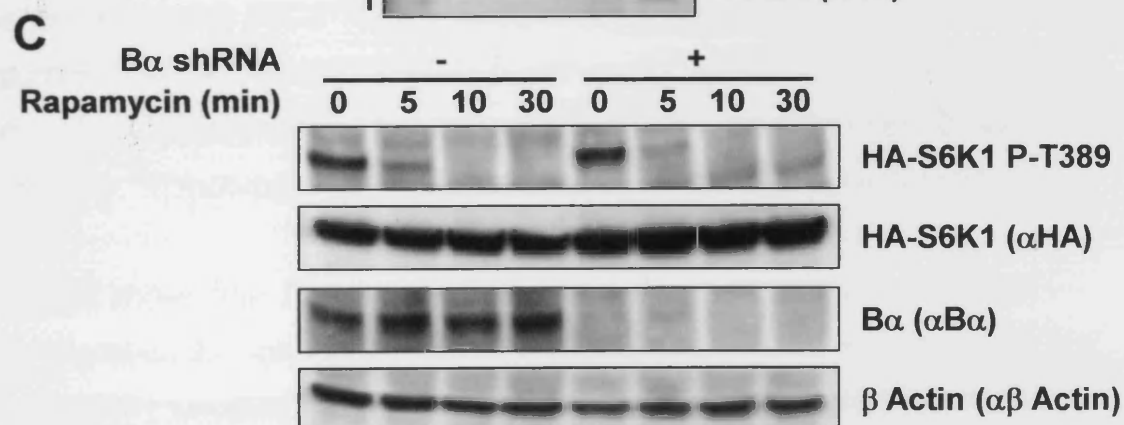
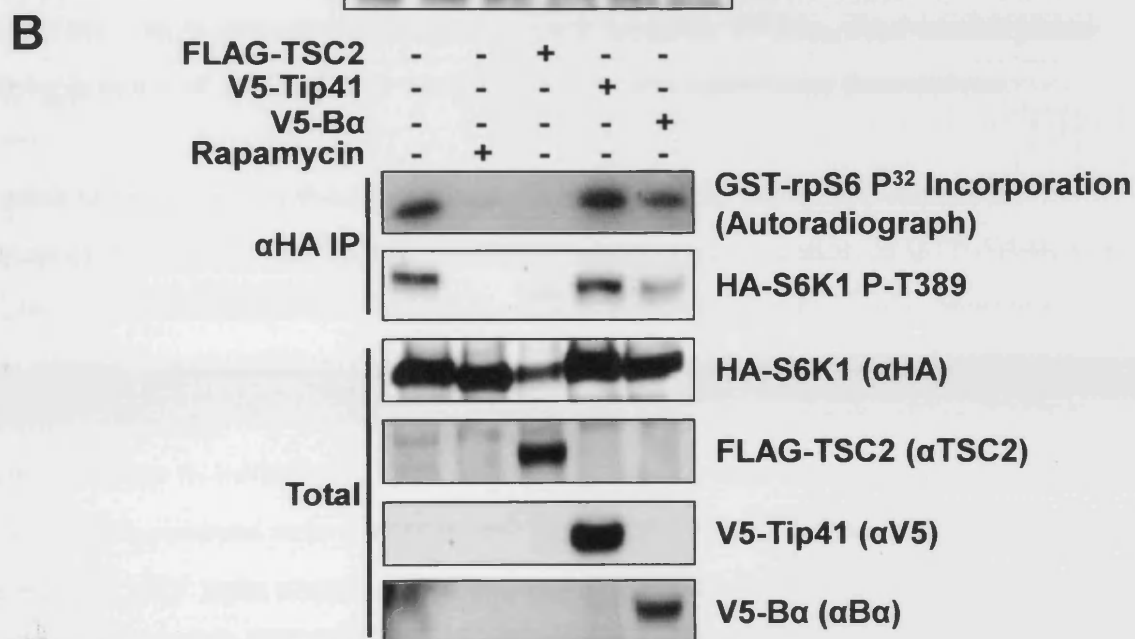
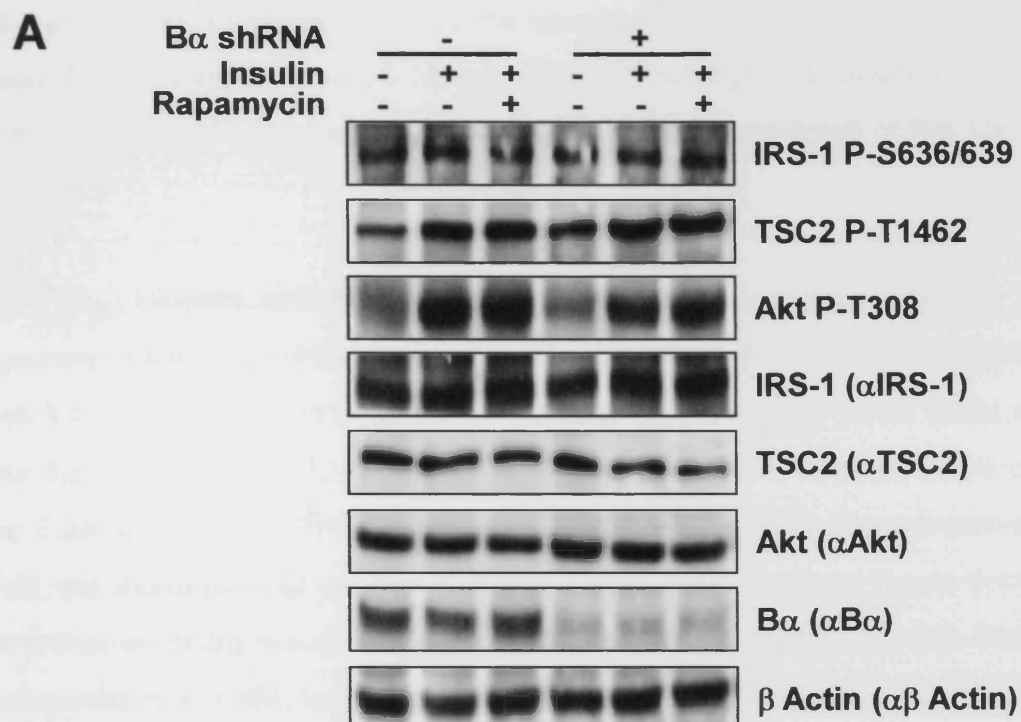
Figure 5.4B shows that B α mRNA levels following knockdown were at 33% (+/- 22%) of that seen in control cells and confirms the result from the western blot seen in Figure 5.3. Thus B α shRNA reduced B α mRNA levels leading to a subsequent reduction in B α protein levels as expected ($p < 0.05$). Error bars are indicative of standard deviation.

5.2.4 PP2A_{B α} does not regulate phosphorylation within the PI3K/Akt/TSC2 pathway upstream of mTORC1

Although B α knockdown appeared to increase phosphorylation of mTORC1 substrates, whether it was acting as a PP2A α regulatory subunit upstream or downstream of mTORC1 was yet to be determined. To examine this, HEK293 cells where B α levels were reduced using shRNA were analysed by western blot for phosphorylation of targets upstream of the mTORC1 complex. Akt positively regulates mTORC1 activity in response to upstream activation of the insulin signalling pathway.

Phosphorylation of Akt at T308 was analysed on knockdown of B α in comparison to control cells transfected with non-coding shRNA (Figure 5.5A). This showed no change in phosphorylation of Akt in response to B α knockdown and indicates that the increase in mTORC1 substrate phosphorylation observed on B α knockdown was not caused by an increase in phosphorylation at or above the level of Akt. This shows that PP2A_{B α} does not dephosphorylate Akt directly or influence its phosphorylation in order to inhibit the mTORC1 pathway.

The TSC1/2 complex lies downstream of Akt in the insulin signalling pathway and inhibits mTORC1. Phosphorylation of TSC2 results in inhibition of the complex thereby increasing activity of mTORC1. B α knockdown appears to have no effect on TSC2 phosphorylation at T1462 (Cai et al., 2005). This indicates that the increase of mTORC1 substrate phosphorylation seen on B α knockdown was not caused by inhibition of the TSC1/2 complex caused due to a reduction in PP2A α activity. This again shows that PP2A_{B α} causes dephosphorylation of mTORC1 substrates by dephosphorylating a target downstream of TSC2 within the mTORC1 pathway. These data indicate that the increase in activity of HIF1 and S6K1 observed in Figure



5.4 in response to B α knockdown are the result of upregulation of the mTORC1 pathway downstream of the TSC1/2 complex. Thus PP2A_{B α} appears to dephosphorylate mTORC1 substrates at some point downstream of the TSC1/2 complex.

5.2.5 PP2A_{B α} inhibits mTORC1 downstream of the TSC1/2 complex

B α appears to act as a PP2Ac regulatory subunit within the mTORC1 pathway (Figure 5.4) but the point of action is still unclear as the results so far could indicate a role for the complex both up- or downstream of mTORC1. Although the results in Figure 5.5A indicate that PP2A_{B α} decreases mTORC1 activity downstream of TSC1/2, the exact point of intervention is unclear. The results of Figure 5.4A show that knockdown of B α results in a small but reproducible increase in HA-S6K1 phosphorylation at T389. In addition, B α knockdown increases the activity of HIF1 (Figure 5.4B). Both effects would be apparent whether PP2A_{B α} dephosphorylates targets upstream of mTORC1 or acts directly on the substrates themselves.

Activation of upstream pathways that positively regulate mTORC1 results in inhibition of the TSC1/2 complex. This then allows accumulation of GTP-Rheb and the subsequent activation of mTORC1. Cells deficient in *TSC2*, the gene encoding TSC2, display hyperactive mTORC1 signalling that is unresponsive to upstream regulatory pathways. To investigate whether B α acts downstream of the TSC1/2 complex in order to inhibit mTORC1 signalling, V5-B α was expressed in *TSC2*^{-/-} MEF cells. If PP2A_{B α} indeed acts downstream of the TSC1/2 complex, over-expression of B α in *TSC2*^{-/-} MEF cells should reduce phosphorylation of mTORC1 substrates. V5-B α was expressed in *TSC2*^{-/-} MEFs along with HA-S6K1. Following lysis, HA-S6K1 was purified from cell lysates using α HA immunoprecipitation and an S6K1 assay carried out as detailed in the 'Materials and Methods'. HA-S6K1 activity was visualised by ³²P incorporation of the substrate GST-rpS6 using autoradiography.

Figure 5.5B shows that *TSC2*^{-/-} MEF cells have a high basal level of HA-S6K1 activity displayed as high levels of GST-rpS6 phosphorylation. As rapamycin is a direct mTORC1 complex inhibitor, treatment with the drug reduced HA-S6K1 activity in comparison to untreated cells. Cells over-expressing FLAG-TSC2 also showed

reduced HA-S6K1 activity in comparison to control cells. Expression of V5-B α resulted in a marked reduction in HA-S6K1 activity as shown by a reduction in GST-rpS6 phosphorylation in comparison to *TSC2*^{-/-} MEF cells expressing HA-S6K1 only. These data show that B α over-expression reduces HA-S6K1 activity in cells devoid of mTORC1 inhibition by TSC2. Excess purified HA-S6K1 was retained for analysis by western blot. Phosphorylation of HA-S6K1 at T389 mirrored results seen in the S6K1 assay. High basal levels of HA-S6K1 phosphorylation were observed in the *TSC2*^{-/-} cells expressing HA-S6K1 only. This was reduced by treatment with rapamycin and expression of FLAG-TSC2. V5-B α expression reduced HA-S6K1 phosphorylation at T389 in comparison to control cells expressing HA-S6K1 only. Collectively, this data strengthens the idea that PP2Ac-B α controls mTORC1 substrate phosphorylation downstream of the TSC1-TSC2 complex.

As rapid dephosphorylation of mTORC1 substrates occurs following treatment with rapamycin, the existence of a phosphatase that is activated following mTORC1 inhibition is likely (Peterson et al., 1999). In order to investigate whether PP2A_{B α} may be the phosphatase complex responsible for dephosphorylating substrates in response to mTORC1 inhibition with rapamycin, a rapamycin time course experiment was used. If PP2A_{B α} dephosphorylates mTORC1 substrates in response to rapamycin inhibition, knockdown of the B α subunit may reduce rapamycin efficacy. To test this, cells were transfected with B α shRNA on day 1, or non-coding shRNA in the case of control cells, followed by HA-S6K1 on day 3. Cells were serum-starved overnight. Rapamycin was added to cells at 5 min, 10 min and 30 min prior to lysis, following stimulation with insulin. Phosphorylation of HA-S6K1 at T389 was analysed by western blot and compared to control cells expressing HA-S6K1 only.

Figure 5.5C shows that in control cells, HA-S6K1 was reduced after 5 min and completely ablated 10 min post treatment with rapamycin. Following B α knockdown, although an initial increase in HA-S6K1 phosphorylation at T389 was observed, the reduction in phosphorylation post rapamycin treatment was identical, with phosphorylation completely ablated 10 min after treatment with rapamycin. These data show that B α knockdown has no effect on the rapamycin-induced dephosphorylation of HA-S6K1. PP2Ac-B α therefore does not appear to be the phosphatase complex involved in the rapid dephosphorylation of mTORC1

substrates that takes place following treatment with rapamycin. Alternatively, as observed in the results of Figures 5.3 and 5.4B, the extent of B α knockdown may not be sufficient to completely remove PP2Ac regulation of mTORC1 substrates.

5.2.6 PP6c is a PP2A family member with links to mTORC1 signalling

TOR signalling in yeast is controlled by the phosphatase Sit4 in addition to Pph21/22 (Jiang & Broach, 1999). Following inhibition of TOR, Sit4 is activated by removal of Tap42 mediated inhibition resulting in dephosphorylation of downstream substrates (Schmidt et al., 1998, Duvel et al., 2003). As the human homologue of Sit4 is PP6c, a potential role in mTORC1 signalling is possible (Bastians & Ponstingl, 1996, Stefansson & Brautigan, 2007). In order to investigate a possible role for PP6C within mTORC1 signalling, knockdown of the PP6c protein using shRNA was used in order to evaluate the effect on phosphorylation within the mTORC1 signalling pathway. A panel of PP6C shRNA vectors was tested to find the most effective clone for knockdown of PP6C in comparison to control cells transfected with non-coding shRNA. Firstly, the amount of time between transfection of cells with shRNA and actual knockdown taking place was optimised. Cells were seeded in triplicate on day one, transfected on day 2 and lysed on either day 3, 4 or 5. This showed that effective knockdown of PP6c occurred 72h post transfection within cells lysed on day 5 (data not shown). To minimise the time between cell seeding and lysis, the reverse transfection procedure was attempted. Cells were seeded and transfected in one step on day 1, with cell lysis occurring on day 4. This resulted in improved cell condition prior to lysis. Levels of PP6c were analysed by western blot, where the protein resolves at a molecular weight of 33kDa. Figure 5.6 shows that following reverse transfection and cell lysis on day 4, clone 1 was the most effective at knocking down PP6c levels. This clone was then used in all future shRNA experimentation.

5.2.7 Depletion of PP6c inhibits mTORC1 signalling

As Sit4 dephosphorylates TOR substrates in yeast, the potential role of PP6c in mTORC1 signalling was explored. If PP6c is involved in the dephosphorylation of mTORC1 substrates, a predicted increase in mTORC1 substrate phosphorylation would occur following knockdown due to reduced phosphatase mediated

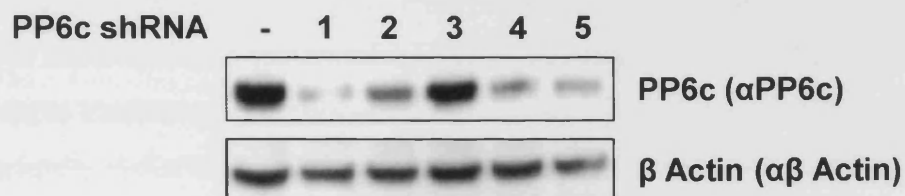


Figure 5.6: Optimisation of PP6c knockdown. A panel of five obtained shRNA clones directed to PP6c were transfected into HEK293 cells and levels of PP6c protein analysed by western blot in comparison to control cells transfected with non-coding shRNA. The results showed that clone 1 was the most effective at lowering levels of PP6c protein and was used in subsequent experimentation. Results are representative of three independent experiments.

dephosphorylation. To investigate the effect of PP6c knockdown on the mTORC1 pathway, following shRNA transfection on day 1, cells were transfected on day 3 with HA-S6K1 and serum-starved overnight. Prior to lysis on day 4, cells were treated with insulin and rapamycin as indicated and subsequently analysed by western blot. Control cells were transfected with non-coding shRNA. HA-S6K1 phosphorylation at T389 was markedly reduced on PP6c knockdown in comparison to control cells expressing HA-S6K1 only (Figure 5.7A). This reduction was only apparent following insulin stimulation as no HA-S6K1 phosphorylation was detected in control cells (or following PP6c knockdown) under serum-starved conditions or following treatment with rapamycin. This result was unexpected as a rise in phosphorylation of proteins would be predicted on knockdown of a phosphatase caused by a reduction in dephosphorylation events.

The activity of the transcription factor HIF1 was also analysed upon knockdown of PP6. HIF1 is a less well characterised mTORC1 substrate and it is not yet clear exactly how its regulation within the pathway is controlled. However HIF1 activity is increased by insulin treatment and reduced following rapamycin inhibition so appears to be a downstream target within the pathway. The effect of PP6c knockdown on activity of the transcription factor was investigated. Following shRNA transfection on day 1, cells were transfected with the HIF1 luciferase reporter element on day 3 followed by overnight serum-starvation under hypoxic (1% O₂) conditions. Following cell lysis the luciferase assay was carried out as described in the 'Materials and Methods'. Knockdown of PP6 resulted in a reduction of HIF1 activity to 57% in comparison to control cells ($p < 0.05$) (Figure 5.7B). Error bars are indicative of standard deviation. This is concurrent with the result in Figure 5.7A where PP6c knockdown resulted in a reduction in HA-S6K1 phosphorylation. Again this is an unexpected result as knockdown of a phosphatase would cause a predicted rise in activity of mTORC1 substrates resulting from reduced dephosphorylation.

These data therefore give rise to two possibilities. Firstly, that PP6c knockdown is resulting in an initial rise in mTORC1 signalling, which in turn activates the negative feedback loop as was found to be the case in Figure 4.9 while investigating the role of Tip41 within the mTORC1 pathway. Alternatively the result may indicate that PP6c is not acting as a phosphatase within the mTORC1 signalling pathway.

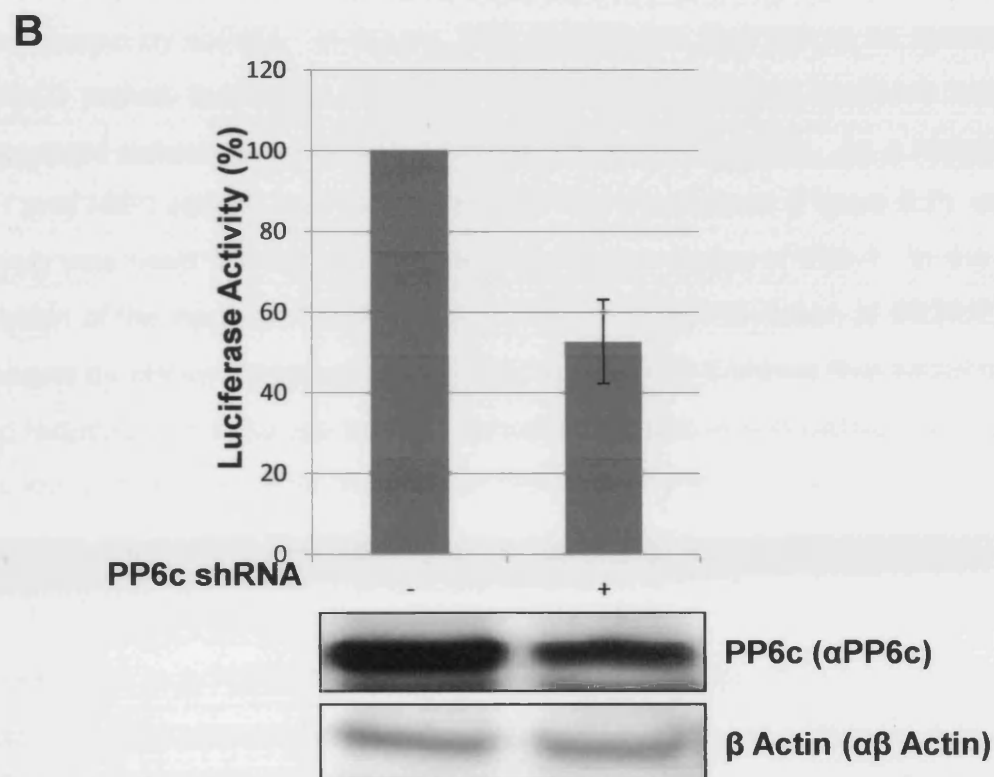
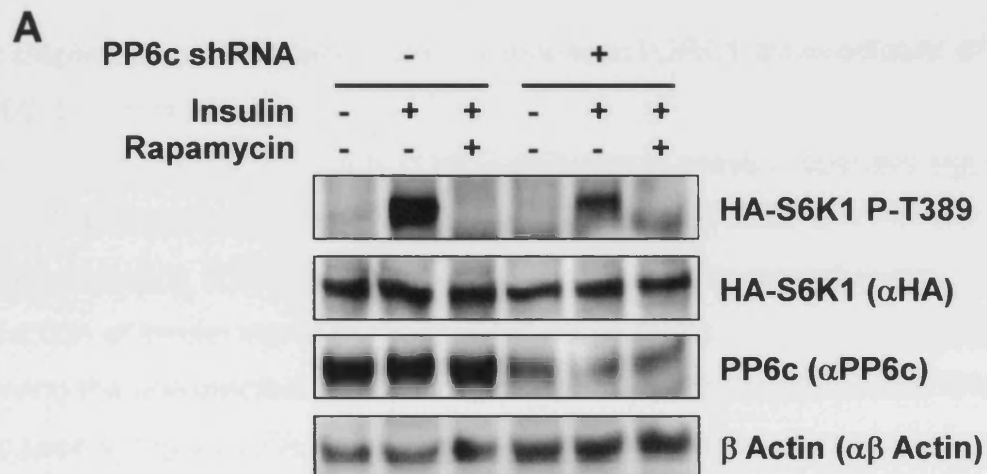


Figure 5.7: Knockdown of PP6c reduces activity of mTORC1 substrates. A. HA-S6K1 was co-expressed in cells transfected with PP6c shRNA vector and used as a measure of mTORC1 activity. Cells were cultured overnight in serum free media and treated with insulin or rapamycin immediately prior to lysis as indicated. The results showed that insulin-induced phosphorylation of HA-S6K1 was reduced on knockdown of PP6c in comparison to control cells transfected with non-coding shRNA. **B.** Activity of HIF1 on knockdown of PP6c was also analysed using the HIF luciferase reporter construct as a measure of HIF1 transcriptional activity. Cells transfected with PP6c shRNA vector on day one were transfected with the HIF1 luciferase reporter element on day three. Cells were cultured overnight in serum free media on day three in 1% O₂ and lysed on day four. The results showed that knockdown of PP6c reduced activity of HIF1 in comparison to cells transfected with non-coding shRNA ($p < 0.05$). Results are representative of three independent experiments.

5.2.8 Depletion of PP6c specifically inhibits mTORC1 downstream of the TSC1/2 complex

Feedback loops act within the mTORC1 pathway to prevent aberrant signalling.

Following activation of the pathway, phosphorylation of IRS1 at a number of serine residues including S636/639 results in its degradation or relocalisation. This causes a reduction of insulin signalling following through to the mTORC1 complex.

Following the unexpected reduction in HA-S6K1 phosphorylation after knockdown of PP6c seen in Figure 5.7A, samples from this experiment were used to investigate the possibility that the feedback loop had been initiated in cells where PP6c levels were reduced by shRNA. In theory, PP6 knockdown may cause an increase in mTORC1 activity leading to activation of the IRS-1 mediated feedback loop and thus an apparent reduction in mTORC1 substrate phosphorylation. As a reduction in HA-S6K1 and HIF1 activity was observed on PP6c knockdown (Figure 5.7), western blot analysis was used to evaluate the phosphorylation status of IRS-1. In the event of activation of the negative feedback loop, IRS-1 phosphorylation at S636/639 is increased by phosphorylation by mTORC1. Figure 5.8 shows that knockdown of PP6c resulted in no change in IRS-1 phosphorylation in comparison with control cells. PP6c knockdown therefore appears to reduce phosphorylation of mTORC1 substrates via another mechanism.

The reduction in mTORC1 substrate phosphorylation resulting from PP6c knockdown may also be explained by increased activity of pathways that are inhibitory to mTORC1 and feed into upstream targets such as Akt. To investigate whether PP6c knockdown reduces phosphorylation of targets upstream of the mTORC1 complex, the phosphorylation of Akt and TSC2 were also investigated. Akt phosphorylation at T308 was unchanged on PP6c knockdown in comparison to control cells, as was TSC2 phosphorylation at T1462. This indicates that the reduction of mTORC1 substrate phosphorylation resulting from PP6c knockdown is not due to increased activity of upstream inhibitory pathways.

PP6c is also involved in NF- κ B signalling. To activate this pathway, the cytokine IL-1 β binds to its cognate receptor IL-1R resulting in activation of IKK β . This requires

TAK1 along with a number of TAB (TAK1 activating) proteins that control activation of the complex and therefore IKK β . Active IKK β phosphorylates the inhibitory protein I κ B, resulting in its degradation by the 26S proteasome, and thus activates NF- κ B. PP6c dephosphorylates and therefore inhibits TAK1 leading to suppression of IKK β phosphorylation and signalling to NF- κ B (Kajino et al., 2006). Knockdown of PP6c may therefore activate signalling to IKK β . As activated IKK β inhibits insulin signalling in response to TNF α via phosphorylation of IRS-1 at S312 (Gao et al., 2002) and therefore inhibits mTORC1 activity, the inhibition of mTORC1 seen on PP6c knockdown may be due to increased signalling via IKK β .

To examine this possibility, the phosphorylation of IKK β at S176/180 was investigated by western blot. Analysis of IKK β phosphorylation showed that PP6c knockdown had no effect at S176/180 in comparison to control cells (Figure 5.8). This indicates that the reduction in mTORC1 substrate phosphorylation resulting from PP6c knockdown is not caused by activation of NF- κ B signalling, which negatively feeds into mTORC1 thereby reducing substrate phosphorylation. In conclusion, these data indicate that the reduction in mTORC1 substrate phosphorylation caused by PP6c knockdown is due to a specific effect downstream of the TSC1/2 complex. As the effect is inhibitory, PP6c is unlikely to play a direct role within the mTORC1 signalling pathway as knockdown of a phosphatase should result in increased signalling. Instead, knockdown of PP6c may be inducing an unrelated consequence resulting in inhibition of the mTORC1 complex.

5.3 Discussion

These data indicate that adenoviral protein E4ORF4 binds the B α regulatory subunit of PP2A in order to activate phosphorylation of mTORC1 substrates (Figure 5.1). Expression of E4ORF4, but not mutants Δ 359 and L51/54A that are deficient in B α binding, increased S6K1 phosphorylation in the absence of serum (Figure 5.1B). This implicates PP2A_{B α} as a phosphatase complex acting towards mTORC1 substrates. E4ORF4 inhibits PP2A_{B α} by modulating activity towards specific substrates rather than inhibiting catalytic activity *per se* (Li et al., 2009). As E4ORF4 modulates PP2A_{B α} substrate-specific catalytic activity, inhibition of S6K1 phosphorylation by E4ORF4 further implies that PP2A_{B α} specifically targets

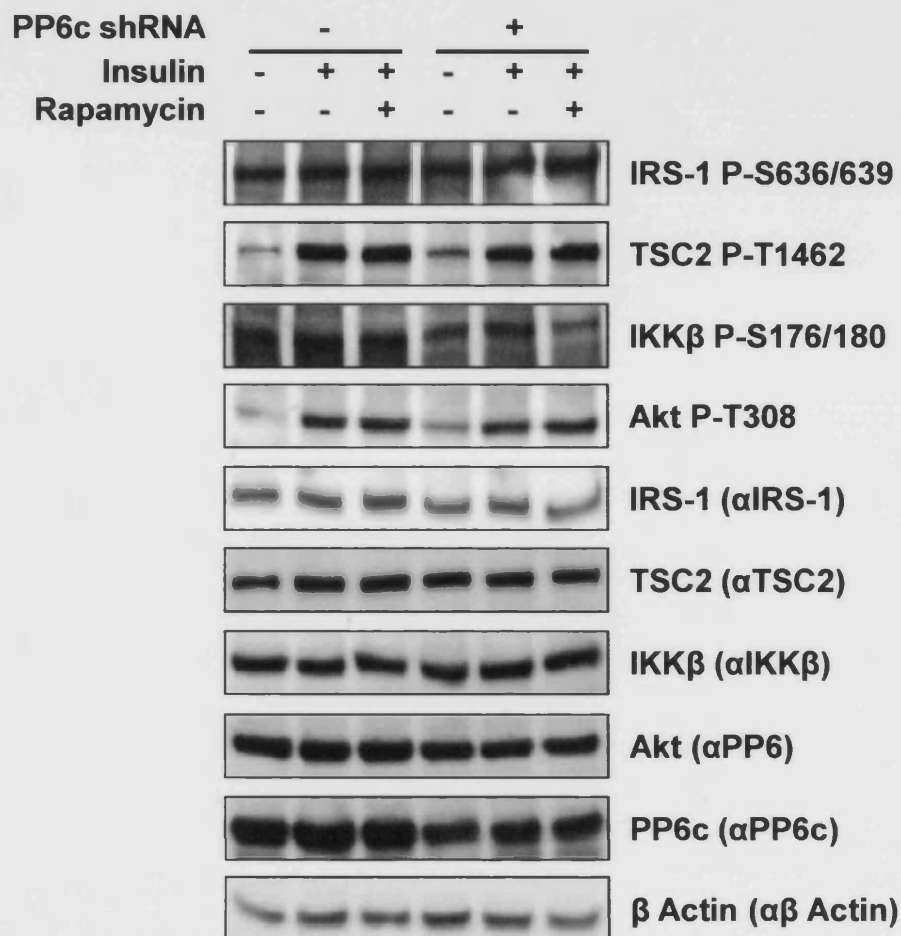


Figure 5.8: Knockdown of PP6c does not alter insulin signalling upstream of mTORC1. Hyperactivity of mTORC1 induces a negative feedback loop via IRS-1 downregulation. To determine whether PP6c knockdown was promoting negative feedback leading to an apparent reduction in mTORC1 signalling, phosphorylation of upstream effectors was analysed in HEK293 cells transfected with PP6c shRNA vector in comparison to control cells transfected with non-coding shRNA. The results showed that IRS-1 phosphorylation at S636/639 was unaffected by PP6c knockdown in comparison to control cells, indicating that negative feedback was not induced. In addition, Akt (T308) and TSC2 phosphorylation (T1462) was unchanged in comparison to control cells. Therefore the effect of PP6c knockdown on mTORC1 activity occurs downstream of the TSC1/2 complex. As PP6c is known to be involved in signalling to NF- κ B, the possibility that PP6c was inhibiting mTORC1 via this pathway was investigated via phosphorylation of IKK β . The results showed that phosphorylation of IKK β (S176/180) was unchanged on knockdown of PP6c in comparison to control cells. Results are representative of three independent experiments.

mTORC1 signalling, rather than kinase signalling in general. As rapamycin effectively inhibited the E4ORF4 induced increase in S6K1 phosphorylation, PP2A_{Bα} appears to act upstream of mTORC1, rather than directly towards S6K1. Further evidence for this comes as 4EBP1 phosphorylation has been found to increase on expression of E4ORF4 (Li et al., 2009). E4ORF4 therefore inhibits phosphatase activity of PP2A_{Bα} in a substrate-specific manner upstream of mTORC1.

In order to further characterise the role of PP2A_{Bα} in mTORC1 signalling, activity of mTORC1 substrates was analysed on knockdown of the Bα subunit. This resulted in a small but significant increase in phosphorylation of S6K1, and enhanced HIF1 activity induced by hypoxia (Figure 5.4). Thus PP2A_{Bα} appears to negatively regulate phosphorylation of mTORC1 targets. As both S6K1 and HIF1 are affected, PP2A_{Bα} appears to act upstream of mTORC1 in agreement with Figure 5.5. In addition, the increase in S6K1 phosphorylation is sensitive to rapamycin, again implying that PP2A_{Bα} lies upstream of mTORC1 (Figure 5.4A).

In using VEGF as a marker of HIF1 activity it is important to consider that other transcription factors, and by extension signalling pathways, also regulate the growth factor (Arany et al, 2008). Therefore although knockdown of Bα increased expression of VEGF, this theoretically could be due to alteration in signalling to another pathway that regulates expression of the growth factor. Therefore whilst PP2A_{Bα} may inhibit mTORC1-mediated HIF1 activity, it may also act on a separate pathway that also enhances VEGF expression under hypoxia.

The slight increase in S6K1 phosphorylation and HIF1 activity, as opposed to the marked increase caused by expression of E4ORF4, may be explained by two mechanisms. Firstly, as PP2A_{Bα} acts upstream of mTORC1, another phosphatase may regulate mTORC1 substrate phosphorylation directly downstream of mTORC1. PP2A inhibition with calyculin A increased phosphorylation of S6K1 under amino acid starved conditions and following treatment with rapamycin (Peterson et al., 1999, Bielinski & Mumby, 2007). In addition, PP2A_B has been implicated as a direct phosphatase towards S6K1 (Hahn et al., 2010). Thus phosphatases may act at various points within the mTORC1 pathway each targeting their specific substrates.

This may prevent aberrant signalling on mutation of a single subunit and may give the cell greater control over proliferation. As a central regulator of a number of processes including transcription, translation, proliferation and the cell cycle, extensive regulation of mTORC1 is therefore required. As PP2A_{B α} acts upstream of mTORC1, the effect of B α knockdown may be overcome by upregulation of downstream phosphatase activity acting directly on mTORC1 substrates.

A second explanation is that other isoforms of the B regulatory subunit also target PP2A towards a target upstream of mTORC1. There are four isoforms of the B regulatory subunit and while each is encoded by a single gene, a high degree of sequence similarity exists (Mayer et al., 1991, Zolnierowicz et al., 1994). Both B α and B δ are ubiquitously expressed and share 81% protein sequence homology. Therefore B α and B δ may share some functional redundancy, and indeed both subunits regulate PP2A activity towards Calcium/calmodulin-dependent protein kinase IV (Reece et al., 2009). As E4ORF4 binds all isoforms of the B subunit, the increase in mTORC1 signalling seen on expression of E4ORF4 may also be mediated by binding to B δ (Branton & Roopchand, 2001). Therefore in order to reduce PP2A_B activity upstream of mTORC1, knockdown of both B α and B δ may have to be performed. In support of this theory, over-expression of B α resulted in a distinct decrease in S6K1 phosphorylation (Figure 5.5B). Whereas knockdown of B α alone may have not altered S6K1 phosphorylation markedly due to functional redundancy with B δ , over-expression would not encounter these difficulties. Therefore as over-expression of B α clearly reduces (Figure 5.5B), and knockdown of B α only slightly enhances (Figure 5.4A), S6K1 phosphorylation it appears that phosphorylation of mTORC1 substrates is negatively regulated by PP2A_{B α} and possibly PP2A_{B δ} .

The substrate of PP2A_{B α} upstream of mTORC1 is unclear. Previous studies have shown that PP2A-B α complex acts in parallel to PI3K/Akt/TSC2 as over-expression of E4ORF4 had no effect on Rheb-GTP loading (O'Shea et al., 2005(a)). Results in Figure 5.5A agree with this as knockdown of B α had no effect on phosphorylation of Akt or TSC2. Intriguingly, results in Figure 5.5B indicate that PP2A_{B α} can overcome

mTORC1 hyperactivation induced by loss of inhibitory TSC2 activity. It follows, therefore, that the effect of PP2A_{Bα} upstream of mTORC1 can overcome the effect of GTP-Rheb accumulation. It is possible that PP2A_{Bα} may interfere with a mechanism of mTORC1 activation in parallel to that induced by Rheb. Two possibilities for this exist.

Firstly, PP2A_{Bα} may regulate amino acid-induced activation of mTORC1 which is separate to PI3K signalling and relies heavily on cellular localisation of mTORC1 rather than activation or inhibition of mTORC1 catalytic activity. Rheb localises to membranes via a farnesylated residue (Tee et al., 2003(b)). Activation of mTORC1 by hVps34, RalA and the Rag GTPase proteins on amino acid stimulation results in translocation of mTORC1 from the cytosol to the membrane (Gulati et al., 2008, Maehama et al., 2008, Sancak et al., 2008). This allows interaction with Rheb and thus activation of mTORC1. PP2A_{Bα} may therefore alter the cellular localisation of mTORC1 rather than catalytic activity in order to inhibit signalling to substrates. A second possibility is that PP2A_{Bα} acts on an unknown intermediary between Rheb and mTORC1. Possible targets include PLD1, which binds GTP-bound Rheb specifically and activates phosphorylation of S6K1 (Sun et al., 2008). PP2A_{Bα} may negatively regulate this or an alternative unidentified Rheb-mTORC1 intermediate thus mediating inhibition of mTORC1 activity.

Regulation of PP2A activity is often mediated via the regulatory B subunit. Analysis of purified GST-PP2Ac complex showed that B_α was ubiquitinated in response to insulin treatment (Figure 5.2A). This ubiquitination is prevented by treatment with rapamycin. As phosphatase activity towards mTORC1 substrates would be predicted to drop following mTORC1 activation this implies that phosphatase activity of PP2A-B_α is likely inhibited by ubiquitination following insulin stimulation. Classically, polyubiquitination targets proteins for proteasomal degradation (reviewed in Bhat & Greer, 2011) but polyubiquitin can also provide a binding platform allowing protein-protein interactions between ubiquitinated substrates and proteins containing a UBD. For example, upstream of NF-κB, polyubiquitination of TAK1 provides a binding site for TAB proteins required for autophosphorylation and activation (Shibuya et al., 1996, Sakurai et al., 2000).

As total B α levels were unchanged following insulin stimulation, it is possible that ubiquitination does not result in proteasomal degradation of the protein.

Ubiquitination of B α may therefore provide a binding site for negative regulatory proteins. As PP2A_{B α} is inhibited by methylation (by PME1) and phosphorylation of PP2Ac, ubiquitin could mediate interaction with proteins involved in these events. A second possibility is that ubiquitination of B α prevents substrate interaction with PP2A_{B α} . As B α mediates interaction with PP2Ac substrates, ubiquitin may provide steric interference thus inhibiting dephosphorylation. A third, more likely, explanation is that the short timescale of insulin treatment did not allow time for degradation of ubiquitinated B α . Analysis of B α levels following longer-term insulin treatment may reveal a decrease in protein levels.

As ubiquitination of B α is prevented by rapamycin, this indicates that it is mediated downstream of mTORC1. The CUL4-DDB1 E3 ligase has been shown as essential for mTORC1 phosphorylation of S6K1 and 4EBP1. Raptor and mLST8 bind CUL4-DDB1 directly, and loss of CUL4 or DDB1 blocks phosphorylation of both mTORC1 substrates. Thus CUL4-DDB1 binds directly to mTORC1 and is required for phosphorylation of downstream substrates (Ghosh et al., 2008). The function of this E3 ligase activity associated with mTORC1 is unknown but results in Figure 5.2A indicate that it may ubiquitinate B α thereby removing inhibitory phosphatase activity allowing activation of mTORC1. If this model is correct, how this is prevented by rapamycin requires further investigation. Rapamycin weakens the interaction between mTOR and Raptor (reviewed in Foster & Toschi, 2009) and CUL4-DDB1 binds Raptor (Ghosh et al., 2008). It is possible that the intact mTOR-Raptor complex may be required for CUL4 E3 ligase activity, resulting in ubiquitination of B α . In summary, although phosphatase activity of PP2A-B α acts upstream of mTORC1, it is regulated downstream of mTORC1 by mTORC1 itself, possibly via the CUL4-DDB1 E3 ligase.

PP6c is a functional homologue of budding yeast Sit4 (Bastians & Ponstingl, 1996, Stefansson & Brautigan, 2007). As Sit4 regulates TORC1 activity, PP6c may be involved in mTORC1 activity in human cells. To assay the role of PP6c in mTORC1

signalling, S6K1 phosphorylation and HIF1 activity were analysed on knockdown of the phosphatase. In contrast to the expected outcome, PP6c knockdown reduced phosphorylation of S6K1 and activity of HIF1 (Figure 5.7). Analysis of Akt and TSC2 phosphorylation showed no change on PP6c knockdown, which may implicate PP6c as having a positive regulatory role within mTORC1 signalling. This also shows that apparent decrease in mTORC1 signalling is not due to induction of a negative feedback loop.

PP6c could activate mTORC1 by removing inhibitory phosphorylation sites within TSC2. Only TSC2 phosphorylation at T1462 was analysed (Figure 5.8). TSC2 is also activated via phosphorylation by AMPK at T1227 and S1345, leading to inhibition of mTORC1 (Inoki et al., 2003). PP6c may dephosphorylate these sites leading to inhibition of TSC1/2 and activation of mTORC1. This is particularly relevant given that AMPK may be active in the cells analysed owing to overnight serum-starvation. This would therefore explain why PP6c knockdown results in reduced S6K1 phosphorylation and HIF1 activity.

As a control, the effect of PP6c knockdown on IKK β phosphorylation was analysed (Figure 5.8). PP6c attenuates IKK β activation upstream of NF- κ B by dephosphorylating the IKK β activating protein TAK1 (Kajino et al., 2006). Signalling modules upstream of NF- κ B have been shown to have numerous links with mTORC1. For example, IKK β phosphorylates TSC1 at S487 and S511 resulting in inactivation and therefore activation of mTORC1 (Lee et al., 2007). IKK β can also inactivate mTORC1 signalling by triggering the degradation of IRS-1 (Gao et al., 2002). Therefore IKK can activate and inhibit mTORC1. Thus in order to confirm that any effects on mTORC1 activity caused by knockdown of PP6c were not caused by NF- κ B activation, phosphorylation of IKK β was analysed. Figure 5.8 showed that IKK β phosphorylation was unaffected by PP6c knockdown. Therefore the effect of PP6c knockdown on mTORC1 activity appears to be specific to mTORC1.

In summary, both PP2A_{B α} and PP6c are involved in regulation of mTORC1. Whereas PP2A_{B α} inhibits signalling to mTORC1, PP6c is unexpectedly an activating phosphatase towards the complex. Although PP2A_{B α} appears to act upstream of

mTORC1, possibly by altering the ability of amino acids to activate the complex, mTORC1 directly regulates activity of PP2A_{Bα} by ubiquitination and possibility proteasomal degradation. Therefore PP2A_{Bα} is the subject of an mTORC1 feedback mechanism. The method of PP6c-mediated mTORC1 activation remains elusive.

CHAPTER 6 – GENERAL DISCUSSION

6.1 Phosphatases and mTORC1 – selection of targets

The yeast model of TORC1 signalling places phosphatase activity as central in the regulation of the pathway. In particular, the regulatory subunit Tip41 is critical in allowing dephosphorylation of TORC1 substrates by Pph21/22 and Sit4 in the absence of activating signals. This project set out to test the hypothesis that the function of Tip41 may be conserved from yeast to mammalian systems, with Tip41 as a potential phosphatase regulatory subunit in relation to mTORC1 signalling. In support of this theory, human Tip41 shares extensive sequence similarity with its yeast counterpart, including conservation of a TOS motif that in other mTORC1 substrates is essential for interaction with Raptor. Also, Tip41 has been shown to interact with the principle mammalian phosphatases PP2Ac, PP4 and PP6c (McConnell et al, 2007). Despite this, little further investigation into the role of Tip41 in mTORC1 signalling had been performed. As the role of a PP2Ac-Tip41 complex in mammalian systems is unknown, this project investigated the regulation of the complex by cell localisation, phosphorylation of Tip41, alteration of intra-complex interactions or changes to substrate binding. This project also investigated the impact of PP2A_{Tip41} on mTORC1 substrate phosphorylation.

An additional PP2A subunit with links in mTORC1 regulation is the B α regulatory subunit. This was identified following studies using the adenoviral protein E4ORF4, which enhances mTORC1 signalling by inhibition of PP2A_{B α} (O'Shea et al, 2005 (a)). In contrast to the PP2A complex containing Tip41, PP2A_{B α} is a classical trimeric enzyme, with B α as the subunit involved in substrate recognition. This project set out to identify whether PP2A_{B α} was involved in attenuation of mTORC1 signalling, by dephosphorylating substrates within the pathway. The principle means of investigation used shRNA knockdown to ascertain impacts on the loss of PP2A_{B α} on mTORC1 activity.

Finally, PP6c is the human homologue of yeast Sit4, which is responsible for dephosphorylation of TORC1 substrates. Although PP6c has been shown as a functional homologue of Sit4, no investigation has been performed into a possible

role of PP6c in mTORC1 signalling. This project tested the hypothesis that PP6c is indeed involved in dephosphorylation of mTORC1 substrates, using shRNA directed to PP6c.

6.2 Principal findings

6.2.1 A PP2A_{Tip41} complex regulates mTORC1 activity

Yeast Tip41 interacts indirectly with Pph21/22 via Tap42 (Jacinto et al., 2001). Previously, mammalian Tip41 was shown to inhibit PP2Ac activity *in vitro*, although whether Tip41 bound PP2Ac directly or via Alpha4 remained unclear (McConnell et al., 2007, Smetana & Zanchin, 2007). In addition, although PP2A_{Tip41} interaction was shown as insensitive to rapamycin, the role of Tip41 within mTORC1 signalling had not been investigated (McConnell et al., 2007). This is despite Tip41 in budding yeast playing a key role in TORC1 signalling. The data presented in this thesis show that Tip41 binds directly to PP2Ac *in vivo*, as the PP2Ac(E42A) mutant deficient in Alpha4 binding purified equal levels of Tip41 in comparison to that of wild-type PP2Ac (Figure 3.3). Furthermore, the data shown in this thesis reveals for the first time that Tip41 can interact with endogenous PP2Ac and Alpha4 (Figure 3.2A).

As Tip41 purification did not show interaction with other PP2Ac subunits such as regulatory B or structural A subunits, Tip41 is proposed as a *bona fide* regulatory subunit of PP2A (Figure 3.2A). Over-expression of Tip41 resulted in inhibition of S6K1 and 4EBP1 phosphorylation and activity of HIF1 (Figures 4.1A, 4.2A and 4.4), all of which are regulated downstream of mTORC1. Tip41 therefore appears to act as a positive regulatory subunit of PP2Ac towards mTORC1 substrates. Previous data also implicate Tip41 as a negative regulator of PP2Ac (McConnell et al., 2007). Although this conflicts with data showing Tip41 as a positive regulatory PP2Ac subunit in relation to mTORC1 signalling, an equivalent paradigm exists for Alpha4 in the literature. Whilst Alpha4 activated PP2Ac activity *in vitro*, Alpha4 has been shown to inhibit PP2Ac dephosphorylation of S6K1 when expressed in cells (Murata et al., 1997, Yamashita et al., 2005). Therefore, the effects of both Alpha4 and Tip41 on PP2Ac activity must be regarded as substrate specific.

Modulation of Tip41 levels rendered cells sensitive to the induction of feedback loops. Over-expression of Tip41 induced feedback via upregulation of Akt (Figure 4.3),

whereas Tip41 depletion resulted in increased phosphorylation of IRS-1 (Figure 4.9) and thus inhibition of mTORC1 signalling. Analysis of upstream regulators of mTORC1 allowed identification of Tip41 as a positive PP2Ac regulatory subunit (Figure 4.3 and 4.9). Over-expression of Alpha4 may also induce negative feedback towards mTORC1, raising the possibility that abundance of either Tip41 or Alpha4 may enhance sensitivity of cells to feedback loops towards mTORC1. Over-expression of Alpha4 has been shown to both increase and decrease phosphorylation of mTORC1 substrates, as is the case with Tip41 (Nanahoshi et al., 1998, Nien et al., 2007, Grech et al., 2008). As PP2A_{Alpha4} association is reduced following mTORC1 inhibition, this indicates that Alpha4 is a PP2Ac negative regulatory subunit (Murata et al., 1997, Yamashita et al., 2005). Where over-expression of Alpha4 has reduced phosphorylation of 4EBP1 (Nien et al., 2007), the negative feedback loop may be induced via phosphorylation of IRS-1 leading to decreased association with PI3K and inhibition of signalling downstream. This may account for the opposing published findings of Alpha4, as has been the case with findings regarding Tip41 in this project. Therefore, over-expression of Tip41 or Alpha4 may render the mTORC1 pathway sensitive to induction of feedback loops. The reason for this is unclear but may involve other regulatory mechanisms within the pathway. Negative feedback loops attenuate mTORC1 signalling to prevent aberrant signalling. Loss of phosphatase regulation may induce upregulation of mTORC1 compensatory mechanisms, such as the situation with chronic rapamycin treatment.

6.2.2 Tip41 acts in parallel to PI3K/Akt mediated activation of mTORC1

Over-expression of Tip41 resulted in inhibition of mTORC1 activity, indicating that Tip41 acts as a positive regulatory PP2Ac subunit towards mTORC1, either directly or indirectly. As three substrates of mTORC1 were affected by over-expression or knockdown of Tip41 (4EBP1, S6K1 and HIF), it is likely that PP2A_{Tip41} either acts upstream of mTORC1 or modulates direct dephosphorylation of these substrates (or both) (Figures 4.1A, 4.2A and 4.4). Analysis of upstream kinases showed that over-expression of Tip41 had no effect on phosphorylation of Akt (S473 or T308) or TSC2 (T1462), narrowing down the list of potential substrates within the cell signal cascade above mTORC1 (Figures 3.10 and 4.3). To try and identify the PP2A_{Tip41} target upstream of mTORC1, Tip41 was expressed in *TSC2*^{-/-} MEFs (Figure 4.12A). In this

instance Tip41 over-expression could not overcome the constitutive activation of mTORC1 achieved by loss of TSC2.

Of interest, Tip41 expression lead to the depletion of Raptor, which was rescued by treatment with the proteasomal inhibitor MG132 (Figure 3.7). This indicates that PP2A^{Tip41} may inhibit mTORC1 by promoting the proteasomal degradation of Raptor. Phosphorylation of Raptor at S863 by mTORC1 is induced by Rheb binding and is required for phosphorylation of downstream substrates (Wang et al., 2009). In addition to mTOR-mediated phosphorylation events of Raptor, AMPK inhibits mTORC1 by phosphorylation of Raptor at T1227 and S1345. AMPK-dependent phosphorylation of Raptor promotes dissociation of Raptor from mTORC1 to the cytosol, where it is sequestered by 14-3-3 (Gwinn et al., 2008). As binding to 14-3-3 to Raptor protects from interaction with the 26S proteasome, free Raptor may be subject to degradation (Li et al., 2002, Shumway et al., 2003, Cai et al., 2006). As Raptor is subject to both activating and inhibitory phosphorylation events, PP2A^{Tip41} may be involved in regulation of Raptor phosphorylation leading to its destabilisation (Gwinn et al., 2008). During completion of this thesis, Dr A. Tee's lab acquired a panel of phospho-Raptor antibodies. It would have been of interest to see whether Tip41 over-expression could result in Raptor dephosphorylation, interaction with 14-3-3 and its destabilisation. Rapamycin is known to cause reduction in Raptor phosphorylation that may be accountable for its destabilisation within mTORC1. Thus Tip41 may inhibit mTORC1 by regulating phosphorylation of Raptor, thereby leading to accumulation of free Raptor and resultant degradation of the protein (Figure 3.7).

On consideration, these data create a clear paradigm - if Tip41 inhibits mTORC1 by promoting Raptor degradation, how did Tip41 over-expression not overcome constitutive Rheb loading caused by loss of TSC2? Although the exact mechanism of Rheb-induced mTORC1 activation is not known, we do know that Rheb induces substrate binding (Avruch et al., 2009, Sato et al., 2009). Therefore it may be more accurate to describe Rheb as a modulator of substrate binding rather than an activator of mTORC1 *per se*. As Raptor is the mTORC1 subunit involved in substrate binding, it follows that Rheb may modify Raptor in some way, in a GTP-dependent manner. If Rheb is constitutively GTP-bound, as in the TSC2^{-/-} MEFs, it

may therefore be permanently bound to membrane-bound mTORC1 and continuously promote Raptor-substrate binding. In these instances, Rheb may prevent PP2A_{Tip41}, or an unidentified intermediate, gaining access to Raptor and prevent PP2A_{Tip41} mediated dephosphorylation. In line with this evidence, it has been shown that Rheb over-expression can induce Raptor phosphorylation. Thus it is likely that PP2A_{Tip41} may indirectly oppose Rheb-induced mTORC1 activation.

6.2.3 Alpha4 may regulate activity of PP2A_{Tip41}

Tip41 purification did not show interaction with the regulatory B and structural A subunit indicating that PP2A_{Tip41} is a distinct phosphatase complex to the classical trimeric complex (Figure 3.2). In contrast, purified Tip41 also co-purified Alpha4 (Figure 3.2A). As Tip41 was shown to directly bind PP2Ac (Figures 3.3A and 3.3B), this indicates that Alpha4 and Tip41 have individual binding sites within PP2Ac and may indeed form a distinct trimeric complex. As Alpha4 has been shown as an inhibitor of PP2Ac (Nanahoshi et al., 1998, Grech et al., 2008), and Tip41 appears to be an activating subunit of PP2Ac, this could be a unique PP2Ac-containing trimeric complex composed of an activating and inhibitory subunit. Whilst both regulatory subunits in the classical PP2A heterotrimer are required for catalytic activity, this presents Tip41-PP2Ac-Alpha4 as a potential novel PP2A trimeric enzyme. Tip41 therefore may have an analogous role to the role in budding yeast. For instance, rather than binding Alpha4 to remove inhibitory activity towards PP2Ac, Tip41 activates PP2Ac directly.

Analysis of the PP2A_{Alpha4} complex shows reduced association following mTORC1 inhibition (Murata et al., 1997, Yamashita et al., 2005). This provides further evidence for the interesting possibility that Tip41 and Alpha4 provide individual and opposing roles towards PP2Ac. Alpha4 may interact with PP2Ac resulting in inhibition, whereas these data show that Tip41 is constitutively bound to PP2Ac (Figure 3.3C). Activity of PP2A_{Tip41} may therefore depend on the binding of Alpha4, which may interact with PP2A_{Tip41} during activation of mTORC1. Further research to investigate this possibility and characterise the way that Tip41 prevents Alpha4-mediated inhibition of PP2Ac is required.

As Alpha4 has many roles in the cell, it is very likely that Tip41 also has additional roles than just to modulate mTORC1 signalling. In addition to a role in mTORC1 signalling, PP2A_{Alpha4} is also involved in dephosphorylation of Midline1 to allow migration along MTs (Aranda-Orgilles et al., 2008). This is required for MT regulation and mutations within the Alpha4 binding region of Midline1 lead to the disease OS (Trockenbacher et al., 2001). In addition PP2A_{Alpha4} dephosphorylates CaMKII in the CNS which is required for memory and learning (Yamashita et al., 2006).

6.2.4 PP2A_{Tip41}-substrate binding may be regulated by phosphorylation of Tip41

Tip41 has been identified as a phosphoprotein in budding yeast that was sensitive to treatment with rapamycin (Jacinto et al., 2001). Although interaction with PP2Ac has been investigated briefly, regulation of PP2A_{Tip41} has not been investigated extensively. As PP2A_{Tip41} interaction was unaffected by insulin or rapamycin (Figure 3.3C), regulation of the activity of this complex is not likely achieved by intra-complex interactions. PP2Ac was shown to interact with S6K1 in response to rapamycin treatment (Figure 3.4), Although the subunit involved in substrate recognition was not identified, regulation of PP2Ac with S6K1 may take place via modification of substrates binding. As PP2Ac substrate binding is mediated by regulatory subunits, it follows that Tip41 regulates PP2Ac substrate binding in relation to mTORC1 signalling. Regulation of Tip41, and therefore PP2A_{Tip41} substrate binding, may occur via phosphorylation of Tip41. As Tip41 phosphorylation decreases in response to insulin, Tip41 appears to be regulated by a kinase that is inhibited in response to growth factors (Figure 3.11). If Tip41 acts as an activating PP2A subunit to inhibit mTORC1 signalling, dephosphorylation following insulin stimulation may induce inactivation of Tip41.

The classical Akt kinase substrate GSK3 inhibits eIF2B via phosphorylation in unstimulated cells. Upon insulin stimulation, GSK3 is inactivated upon phosphorylation by Akt leading to accumulation of hypophosphorylated eIF2B that then promotes translation initiation (Wang et al., 2001, Wang et al., 2002, Mariappan et al., 2008). Tip41 phosphorylation may be regulated in a similar manner. If indeed PP2A_{Tip41} is also regulated by binding of Alpha4, this may provide a dual

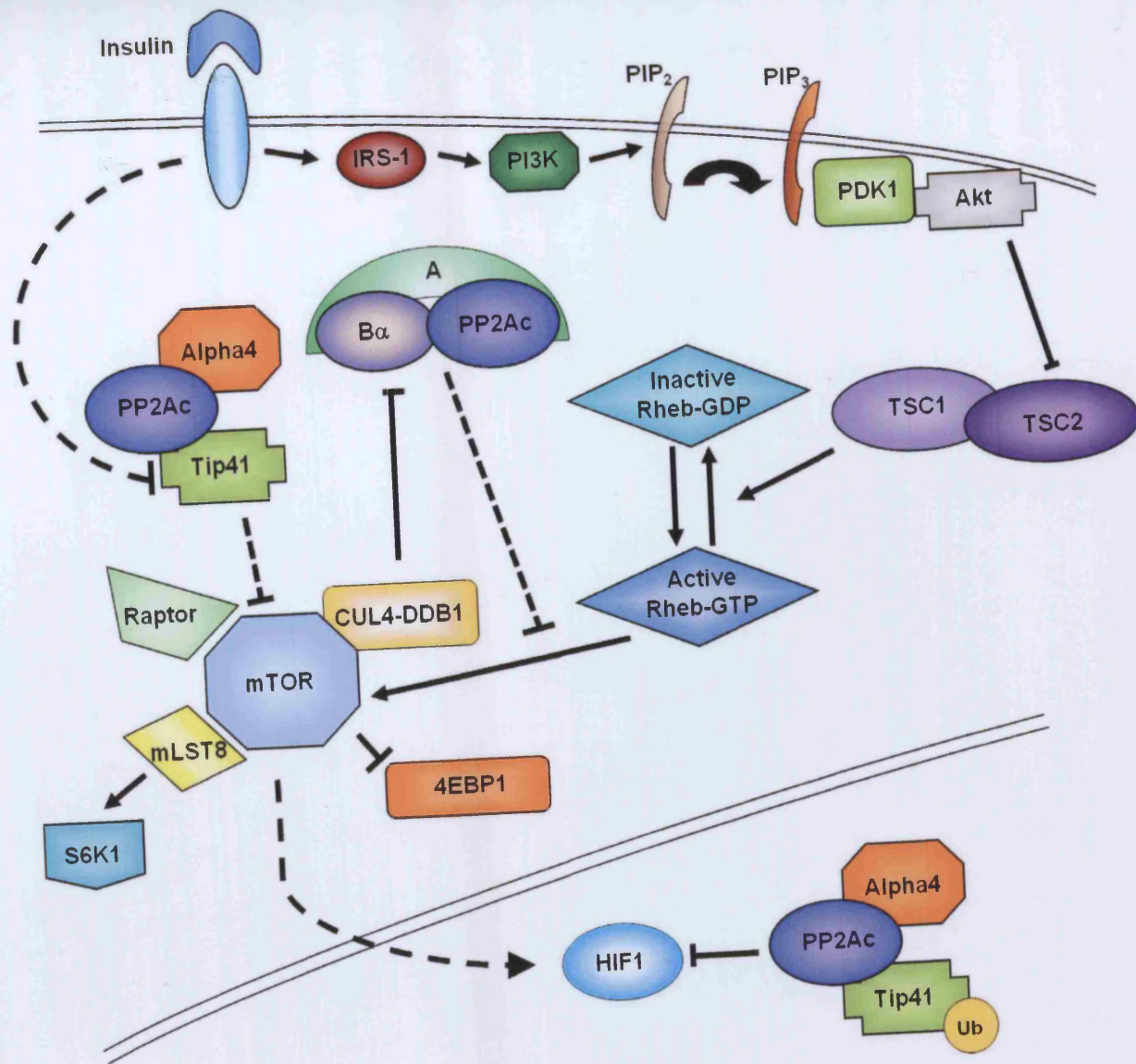
mechanism for regulation of the complex. As the interaction between PP2A and Alpha4 is modulated upon insulin treatment while the interaction between PP2A and Tip41 is constitutive, it is possible that binding of Alpha4 negatively regulates the PP2A_{Tip41} complex in addition to Tip41 phosphorylation in response to insulin. Alternatively, phosphorylation of Tip41 may promote binding of Alpha4 to PP2Ac, or indeed Alpha4 binding may promote phosphorylation of Tip41. In summary, it appears that upon insulin stimulation, Tip41 phosphorylation is impaired that leads to inhibition of the PP2A_{Tip41} complex and mTORC1 activation.

6.2.5 PP2A_{Tip41} may oppose Rheb-mediated activation of mTORC1

Analysis of Tip41 regulation and function raises the interesting possibility that Tip41 in human cells is a direct regulatory subunit of PP2Ac. If this is the case, Tip41 may mediate PP2Ac substrate binding. As an inhibitor of mTORC1 signalling, one of the PP2A substrates that interacts with Tip41 clearly lies within the pathway. PP2A activity is regulated by substrate binding, as rapamycin upregulates S6K1 interaction with the phosphatase. In the case of PP2A_{Tip41}, substrate binding could be induced by hyperphosphorylation of Tip41 in nutrient deprived cells. This may lead to dephosphorylation of Raptor resulting in release from the mTORC1 complex (Figure 6.1). Free Raptor may then be subject to degradation by the 26S proteasome. As Tip41 over-expression was not able to overcome Rheb-induced mTORC1 hyperactivation, GTP-Rheb may permanently interact with membrane-localised mTORC1 thus blocking interaction with Tip41. It is therefore possible that PP2A_{Tip41} opposes the Rheb-induced increase in Raptor-substrate binding by regulating phosphorylation of Raptor leading to release and proteasomal degradation. Rheb counteracts this by binding mTORC1 and enhancing mTOR substrate interaction with Raptor. In summary, in unstimulated cells it appears that Tip41 is phosphorylated leading to activation of PP2A_{Tip41} and degradation of Raptor. Stimulation with growth factors leads to dephosphorylation of Tip41 and inhibition of PP2A_{Tip41} activity with Raptor. In addition, the accumulation of GTP-Rheb activates mTORC1 by increasing Raptor-substrate binding.

6.2.6 A specific nuclear isoform of Tip41 may regulate activity of HIF1

Figure 6.1: Tip41 and B α are PP2Ac regulatory subunits involved in inhibition of mTORC1 signalling. Tip41 appears to form part of a trimeric complex with PP2Ac and Alpha4, which directly opposes Rheb-mediated activation of mTORC1 and promotes Raptor degradation. Accumulation of hypophosphorylated Tip41 occurs on activation of insulin signalling, and may provide a mechanism of PP2A_{Tip41} inhibition. PP2A_{B α} may alter membrane localisation of mTORC1 thus preventing interaction with membrane-bound Rheb. Ubiquitination of B α , possibly via CUL4-DDB1, may be involved in an mTORC1 feedback loop resulting in inhibition of PP2A_{B α} . A specific nuclear PP2Ac complex with ubiquitinated Tip41 may regulate activity of HIF1.



The cellular distribution of Tip41 has previously not been investigated. Cellular fractionation showed that both nuclear and cytoplasmic forms of Tip41 exist, but that the nuclear form of Tip41 is subject to a large, approximately 8kDa, post-translational modification (Figure 4.11). This nuclear form of Tip41 is present equally on insulin and rapamycin treatment hence is not regulated directly by mTORC1, although of interest would be the proportion of nuclear Tip41 on serum starvation as phosphorylation of Tip41 is responsive to insulin but not rapamycin. As knockdown of Tip41 results in a massive increase in HIF1 activity (Figure 4.10), nuclear Tip41 could specifically be involved (directly or indirectly) in inhibition of mTORC1 responsive transcription factors in the nucleus (Figure 6.1). In support of this, a nuclear PP2A α 4 complex has previously been observed in complex with mTORC1, which is involved in regulation of STAT1 (Fielhaber et al., 2009).

The molecular shift caused by post-translational modification of Tip41 is indicative of ubiquitination. Both mono- and polyubiquitination occur within the cell, which both have diverse consequences for the target protein. Tip41 appears to be monoubiquitinated, which could alter its cellular localisation. The transcription factor FOXO4, for example, is ubiquitinated in response to oxidative stress resulting in nuclear translocation and activation of transcriptional activity (van der Horst et al., 2006, Brenkman et al., 2008). Therefore, Tip41 may similarly either be ubiquitinated leading to nuclear shuttling. An alternative is that Tip41 forms part of a complex that translocates to the nucleus where it is ubiquitinated. In summary, a nuclear ubiquitinated form of Tip41 exists which may be involved in regulation of transcription factors downstream of mTORC1. Therefore two pools of Tip41 may be present within the cell with distinct biological functions.

6.2.7 PP2A $\beta\alpha$ attenuates mTORC1 signalling

PP2A $\beta\alpha$ is a classical PP2A heterotrimeric complex consisting of PP2Ac along with a structural A subunit and regulatory B α subunit. Previously PP2A $\beta\alpha$ has been shown to inhibit phosphorylation of S6K1, as indicated by studies with the adenoviral protein E4ORF4. Although PP2A $\beta\alpha$ causes dephosphorylation of S6K1 and 4EBP1, E4ORF4 mediated activation of S6K1 is rapamycin sensitive. Furthermore, E4ORF4 was shown not to affect PI3K/Akt activation (O'Shea et al., 2005(a)). It was proposed

that PP2A_{Bα} acts in a pathway parallel to insulin/growth factor activation of mTORC1. Previous data regarding the role of PP2A_{Bα} in mTORC1 signalling had been mainly indirect via studies using over-expressed E4ORF4 protein. Thus the role of PP2A_{Bα} specifically within mTORC1 warranted further investigation.

Knockdown of Bα resulted in a small but significant ($p < 0.05$) increase in S6K1 phosphorylation and HIF1 activation (Figure 5.4). Again as both mTORC1 substrates were affected, this implicated PP2A_{Bα} as acting upstream of mTORC1. The small effect of Bα knockdown in comparison to the action of E4ORF4 may be due to functional redundancy of Bα. Bα and Bδ are part of the same family and are ubiquitously expressed. Although encoded by separate genes, Bα and Bδ share 81% amino acid sequence homology (Strack et al., 1999). It is therefore conceivable that the proteins share some functional redundancy, and indeed both subunits mediate PP2A_C regulation of Calcium/calmodulin-dependent protein kinase IV (Reece et al., 2009). In support of this, E4ORF4 binds all isoforms of B regulatory subunit. As only Bα and Bδ are ubiquitously expressed, the activation of mTORC1 may be due to inhibition of PP2A_D in complex with Bα or Bδ. In contrast to knockdown of Bα, over-expression of Bα resulted in inhibition of S6K1 phosphorylation (Figure 5.5B). This further supports the theory of functional redundancy as whereas knockdown of Bα may result in compensatory activity of Bδ, no such system could exist for an over-expression.

As well as redundancy between Bα and Bδ, other unknown and alternative phosphatase complexes work downstream of mTORC1 (which would also involve PP2A_{Tip41}) may be upregulated as a consequence of Bα knockdown. Such compensation between phosphatase complexes may mask any effect of Bα knockdown towards the phosphorylation of 4EBP1 and S6K1. In support of this, other PP2A complexes involved in specific substrate dephosphorylation downstream of mTORC1 have previously been identified. For instance, PP2A_{B'} specifically dephosphorylates S6K1 but not 4EBP1 (Hahn et al., 2010). Therefore phosphatase complexes appear to act at multiple points in the pathway dependent on the regulatory subunits.

6.2.8 PP2A_{Bα} acts downstream of the TSC1/2 complex

As over-expression of B α inhibits S6K1 phosphorylation in cells lacking TSC2 (Figure 5.5B), this shows that PP2A_{B α} inhibits mTORC1 signalling downstream of the TSC1/2 complex, although the exact point of action is unclear. Knockdown of B α had no effect on phosphorylation of Akt or TSC2 (Figure 5.5A) providing evidence that the protein acts in a parallel pathway to insulin and growth factors to inhibit phosphorylation of downstream mTORC1 targets. Amino acids stimulate mTORC1 activity by altering cellular localisation of the complex. Cytosolic mTORC1 is translocated to membrane structures in order to interact with active Rheb, which is membrane-bound, and facilitates phosphorylation of downstream substrates (Hara et al., 1998, Gulati et al., 2008, Maehama et al., 2008, Sancak et al., 2008). This translocation event of mTORC1 is required as a priming activation of mTORC1 and is dependent on amino acid sufficiency, as insulin fails to activate mTORC1 in the absence of amino acids (Byfield et al., 2005, Smith et al., 2005). PP2A_{B α} may therefore prevent mTORC1 translocation in the absence of amino acids (Figure 6.1). Over-expression of B α may prevent translocation of mTORC1 in *TSC2*^{-/-} MEFs, which may inhibit activation of mTORC1 regardless of the GTP-loading of Rheb (Figure 5.5B).

A number of different pathways for mTORC1 translocation in response to amino acids have been identified. Human Vps34, RalA and the Rag GTPases have all been shown to mediate mTORC1 membrane localisation (Gulati et al., 2008, Maehama et al., 2008, Sancak et al., 2008). PP2A_{B α} may therefore inhibit any of these proteins in order to inhibit mTORC1 signalling. Also in parallel to PI3K-mediated mTORC1 activation is the response to PA. The lipid second messenger is synthesised by PLD1, which is activated by Rheb. It is thought that PA-binding to mTORC1 may enable membrane localisation. Therefore PP2A_{B α} may interfere with Rheb-induced PLD1 activation therefore preventing membrane localisation in this manner (Fang et al., 2002, Sun et al., 2008). MAPK also activates mTORC1 downstream of the TSC1/2 complex. RSK phosphorylates Raptor at a number of sites resulting in enhanced mTORC1 activity by an unknown mechanism (Carriere et al., 2008). This provides an additional point of action for PP2A_{B α} to inhibit mTORC1 that could involve modulation of Raptor phosphorylation.

6.2.9 PP2A_{B α} may be downregulated by an mTORC1 feedback mechanism involving the ubiquitin ligase CUL4-DDB1

Purification of GST-PP2Ac identified B α polyubiquitination in response to insulin treatment that was abrogated by rapamycin (Figure 5.2A). This novel finding provides evidence that PP2A_{B α} may be subject to mTORC1-mediated polyubiquitination. In addition, this data implies that ubiquitination of the B α subunit may regulate PP2Ac phosphatase activity in an mTORC1 dependent manner. As the CUL4-DDB1 E3 ligase is required for mTORC1 activity, this indicates that CUL4-DDB1 may ubiquitinate B α in response to insulin stimulation thus removing inhibition from the phosphatase (Figure 6.1). Polyubiquitination classically targets proteins for degradation by the 26S proteasome. Thus the requirement of CUL4-DDB1 for mTORC1 activity may be to remove the negative regulatory phosphatase activity of PP2A_{B α} , by targeting B α for degradation (or inhibition via ubiquitination). B α total protein levels were unaffected upon its ubiquitination, suggesting that ubiquitinated B α is still relatively stable. As the time of treatment with insulin was only 30 min, this may not be long enough for proteasomal degradation to occur. It would therefore be of interest to investigate the role of extended insulin stimulation on B α levels within the cell to determine whether ubiquitination results in its protein degradation. Given that rapid dephosphorylation of mTORC1 substrates occur upon treatment with rapamycin, it is unlikely that protein degradation of B α would be the main regulatory mechanism.

Alternatively, polyubiquitination of B α may not target the protein for degradation, as polyubiquitination has other known consequences within the cell. These alternative roles centre around the ability of ubiquitin to act as a binding platform for proteins containing a ubiquitin binding domain or ubiquitin interacting motif. For example, signalling to NF- κ B requires polyubiquitination of TAK1 in order to allow binding of TAB proteins required to activate autophosphorylation of the protein (Shibuya et al., 1996, Sakurai et al., 2000, Takesu et al., 2000, Kanayama et al., 2004, Kishida et al., 2005). Also, ubiquitination of Akt is essential for activation and may similarly provide a binding site for cofactors (Yang et al., 2009, Yang et al., 2010). If the role of

polyubiquitination of B α is not to cause degradation of the protein, then it may provide a binding site for unknown inhibitory proteins.

6.2.10 PP6c enhances mTORC1 signalling

PP6c is the functional homologue of budding yeast Sit4 (Bastians & Ponstigl, 1996). As Sit4 dephosphorylates TORC1 substrates in yeast, a role of PP6c in mTORC1 signalling was investigated (Wang et al., 2003). On knockdown of PP6c, phosphorylation of S6K1 (Figure 5.7A) and activity of HIF1 (Figure 5.7B) were reduced, indicating inhibition of mTORC1. This is in contrast to the expected result, as knockdown of a phosphatase would be expected to increase phosphorylation of substrates. This may indicate that PP6 is involved in dephosphorylation of an mTORC1 inhibitory protein such as DEPTOR or PRAS40. Post-translational modification of DEPTOR is required for activation of mTORC1 signalling (Peterson et al., 2009). Similarly, PRAS40 is phosphorylated by Akt and mTOR relieving inhibition on mTORC1 (Oshiro et al., 2007, Sancak et al., 2007, Wang et al., 2008). PP6c may dephosphorylate either of these inhibitors to allow mTORC1 activation. A more intriguing explanation stems from a finding that Tip41 also binds PP6c, in addition to PP2Ac (Smetana & Zanchin, 2007). Knockdown of PP6c may relieve Tip41 from PP6c binding and allow free Tip41 to bind PP2Ac thus leading to increased inhibitory PP2A_{Tip41} acting towards mTORC1. Higher levels of PP2A_{Tip41} complexes in cells as a consequence of loss of PP6c expression would explain the observation why the activity of S6K1 and HIF1 activity were reduced upon PP6c knockdown. As PP6c knockdown had no effect on phosphorylation of Akt or TSC2 (Figure 5.8), the role of PP6c appears to mirror that of Tip41, providing further evidence for this theory.

6.3 Significance in Relation to Human Disease

6.3.1 Cancer

The *raison d'être* of basic science is to increase our understanding of the molecular processes underlying human disease. Considering that upregulation of mTORC1 is strongly associated with a number of human diseases, understanding the inhibition of the pathway is a crucial element that has received comparatively little attention. This project has identified two phosphatase complexes that negatively regulate

mTORC1. Both Tip41 and B α are PP2Ac regulatory subunits that inhibit signalling to characterised substrates of mTORC1 including 4EBP1, S6K1 and HIF1 α (Figures 4.1A, 4.2A, 4.4 and 5.5A). As hyperactivation of mTORC1 is a hallmark of a number of cancers, inactivation of either of these complexes could therefore promote carcinogenesis. Indeed a direct link between B α and carcinogenesis was uncovered by miRNA analysis of lung and hepatic cell carcinomas. Increased levels of miRNA31 and miRNA222 are found in lung and hepatic cell carcinoma (Liu et al., 2010, Wong et al., 2010). As these promote degradation of B α mRNA, downregulation of PP2A_{B α} is directly associated with these cancers. In addition, high levels of miRNA31 and miRNA222 are associated with increased tumorigenicity and reduced life expectancy (Liu et al., 2010, Wong et al., 2010). Theoretically this could be due to activation of mTORC1, indicating that deregulation of mTORC1 may be associated with a more aggressive phenotype in relation to lung and hepatic cell carcinoma. S6K1 activity is strongly associated with breast and colon cancer (Barlund et al., 2000, Slattery et al., 2010). Therefore inactivating mutations of Tip41 or B α could promote tumour growth associated with hyperactivation of S6K1.

Stabilisation of HIF1 is associated with metabolic advantage of solid tumours by inducing transcription of genes involved in glycolysis and cell survival, thus allowing tumours to progress in the absence of oxygen. Specifically, tumours with loss of PTEN or upregulation of Akt are sensitive to transformation by increased transcription of HIF1-regulated genes via activation of mTORC1 (reviewed in Denko, 2008). For example, HIF1 activates transcription of a number of glycolytic enzymes including GLUT1 and GLUT3, which increases cellular glucose uptake (Chen et al., 2001). In addition, HIF1 increases transcription of enzymes involved in glycolysis (Semenza et al., 1994). Therefore, promotion of glycolysis by HIF1 is a two-winged approach, by both increasing glucose uptake and increasing glycolytic enzymes required to process with glucose once in the cell in order to generate energy. By promoting glycolysis over oxidative phosphorylation, tumours bypass the requirement for oxygen allowing growth in the absence of vascularisation. HIF also encourages vascularisation, which then feeds the tumour with additional oxygen and nutrients. If a specific nuclear isoform of Tip41 exists that specifically regulates HIF1 (Figure 4.11), Tip41 down-regulation could theoretically promote tumorigenesis

through this mechanism. Therefore Tip41 could be critical in the regulation of HIF1 directly in the nucleus, and thus preventing expression of glycolytic genes. Loss of nuclear Tip41 activity, by loss of post translational modification that targets Tip41 to the nucleus or by mutation, for instance, could prevent regulation of HIF1. This could induce transformation of a cell by loss of requirement for vascularisation.

6.3.2 TS

The inherited hamartoma syndrome TS is characterised by the development of benign tumours in the brain, kidneys, heart, lungs and skin. Tumours within the brain lead to seizures and developmental delay, with many TS patients also being diagnosed with autism (reviewed in Inoki et al., 2005, Tee & Blenis, 2005, Rosner et al., 2008). Either *TSC1* or *TSC2* are mutated in patients with TS resulting in hyperactivation of signalling to mTORC1. For this reason rapamycin and related analogues are undergoing clinical trial for treatment of the disease (Davies et al., 2008, McCormack et al., 2008). As mTORC1 hyperactivation underlies many of the phenotypic characteristics of TS, understanding inhibitory networks working within the pathway will provide much needed information about the cellular events that cause the disease.

Both Tip41 and B α are PP2Ac regulatory subunits that impact on mTORC1 and over-expression of either subunit in *TSC2*^{-/-} MEFs provided crucial information about how the respective PP2A complexes act within the signalling pathway (Figures 4.12A and 5.5B). Over-expression of Tip41 did not reduce S6K1 phosphorylation in *TSC2*^{-/-} MEFs, indicating that Tip41 acts upstream of mTORC1 to inhibit signalling (Figure 4.12A). This also implies that cells within TS patients are resistant to the inhibitory action of PP2A_{Tip41}. On the other hand, PP2A_{B α} still reduces S6K1 phosphorylation in the absence of TSC2 (Figure 5.5B). Therefore, deregulation of mTORC1 by loss of *TSC2* is still sensitive to the inhibitory action of PP2A_{B α} . Therefore it appears that loss of inhibitory PP2A_{Tip41} activity also contributes to the progression of TS, in addition to hyperactivation of mTORC1. So it appears that two mechanisms of mTORC1 activation are at work in TS. Firstly, direct activation of mTORC1 by loss of inhibitory TSC1/2 activity, and secondly through loss of inhibitory activity of PP2A_{Tip41}. Intriguingly, mTORC1 activation in *TSC2*^{-/-} MEFs is still

sensitive to PP2A_{Bα} regulation. As Bα may be negatively regulated by ubiquitination mediated by the CUL4-DDB1 E3 ligase associated with mTORC1, this may provide a therapeutic opportunity for treatment of TS. If ubiquitination of Bα could be prevented, inactivation of mTORC1 by PP2A_{Bα} could be overcome leading to activation of phosphatase regulation and inhibition of mTORC1 activity.

6.3.3 Type II diabetes

Activation of a negative feedback loop downstream of mTORC1 underlies the development of type II diabetes, which is characterised by the failure of hyperinsulinaemia to rescue the uptake of glucose in adipose and skeletal muscle tissue. Chronic activation of mTORC1 leads to phosphorylation of IRS-1 at S636 by mTOR and S6K1 leading to reduced association with PI3K (Bouzakri et al., 2003, Veileux et al., 2010). This renders Akt insensitive to signalling from PI3K, and by extension, insulin. Indeed mice lacking *S6K1* are resistant to the development of type II diabetes, underlining the importance of mTORC1 feedback in the development of the disease (Um et al., 2004, Tremblay et al., 2007). Direct evidence for the role of mTORC1 feedback was gained by analysis of human skeletal muscle biopsies. Patients with type II diabetes showed reduced association between PI3K and IRS-1 that was concurrent with increased phosphorylation of IRS-1 at S636 (Bouzakri et al., 2003). In addition, chronic insulin-induced mTORC1 activity leads to reduced membrane translocation of the glucose transporter GLUT4 (Taha et al., 1999, Gaster et al., 2001, Garcia-Souza et al., 2008). This is thought to induce glucose-mediated tissue damage associated with type II diabetes. Therefore dissociation of PI3K and IRS-1 is central to the pathological phenotype of type II diabetes due to the inability of the cell to respond to insulin. Knockdown of Tip41 using shRNA resulted in activation of the negative feedback loop by increasing phosphorylation of IRS-1 at S636/639 (Figure 4.9B). Therefore activation of mTORC1 by loss of regulatory PP2A-Tip41 activity results in a phenotype that mimics type II diabetes. This indicates that PP2A_{Tip41} inactivation is required for the development of the disease. Therefore loss of PP2A_{Tip41} activation and/or function may be an underlying signalling event in type II diabetes.

6.3.4 AD

The pathology within the AD brain consists of a number of extracellular senile plaques made up of A β along with hyperphosphorylated Tau leading to formation of neurofibrillary tangles. The disease has been associated with a degree of insulin resistance and hence the term 'type III diabetes' has been used to describe AD (Castri et al., 2003, Gupta et al., 2011). In normal brain tissue, the level of Tau phosphorylation decreases with age. In the AD brain, Tau is hyperphosphorylated, which is thought to result in aggregation and formation of neurofibrillary tangles. Phosphorylation of Tau is controlled by GSK3 and PP2A_{B α} , which phosphorylate and dephosphorylate the protein, respectively (Martin et al., 2009, Qian et al., 2010). Dysfunction of PP2A_{B α} and mTORC1 have individually been associated with development of AD (Evans & Hemmings, 2000, Nunbakhdi-Craig et al., 2007, Xu et al., 2008, Deters et al., 2009, Qian et al., 2010). Upregulation of insulin signalling, with a concomitant increase in mTORC1 activity, is associated with AD in both model systems and patient tissue (An et al., 2003, Ferrando-Miguel et al., 2005, Griffin et al., 2005, Damjanac et al., 2007, Meske et al., 2008). Hyperphosphorylation of Tau is directly linked with hyperactivation of mTORC1, as rapamycin inhibits Tau phosphorylation (An et al., 2003, Ferrando-Miguel et al., 2005).

Combining this information leads to the interesting conclusion that PP2A_{B α} may be directly inhibited by mTORC1, and upregulation of mTORC1 activity in the AD brain may lead to inhibition of PP2A_{B α} and therefore hyperphosphorylation of Tau. The data in this thesis present the possibility that PP2A_{B α} inhibits mTORC1 and is negatively regulated by the complex by ubiquitination (Figure 5.2A) via the DDB1-CUL4 E3 ligase that is associated with mTORC1 (Ghosh et al., 2008). Therefore it may be of interest to investigate the level of B α ubiquitination in the AD brain, and could present a therapeutic opportunity for treatment of the disease. Theoretically, inhibition of ubiquitination of B α may prevent neurofibrillary tangle formation by suppression of Tau phosphorylation.

6.4 Future directions

Following the results of this project, it is clear that PP2A_{T τ 41} is involved in attenuation of mTORC1 signalling. What is not clear, however, is the exact point of action. Results from Figures 4.3A and 4.12A indicate that PP2A_{T τ 41} acts upstream of the

TSC1/2 complex, whereas Figures 3.10 and 4.3A show that Akt phosphorylation is unaffected by modulation of Tip41 levels. Further insight into this comes from Figure 3.7, which shows that Tip41 causes proteasomal degradation of Raptor. From this arises the interesting possibility that Tip41 modulates Rheb-mediated activation of mTORC1. The method of mTORC1 activation by Rheb has not been identified, nor has the GEF responsible for the GTP-loading of the protein. It is therefore possible that identifying the substrate of PP2A_{Tip41} could concurrently identify either of these factors.

Also of particular interest would be to further investigate the role of a specific nuclear isoform of Tip41 involved in the regulation of HIF1. As HIF1 has particular implications in tumorigenesis, identifying regulatory mechanisms of the protein may provide further insight into cancer progression at a cellular level. In addition, the fact that a specific nuclear isoform of Tip41 exists raises the possibility that PP2A_{Tip41} is involved in regulation of other transcription factors. For example, mTORC1 is also involved in the regulation of STAT3 and YY1. It would therefore be of interest to investigate whether PP2A_{Tip41} is involved in attenuation of the activity of these additional factors, in addition to HIF1.

Regarding findings on the B α regulatory subunit of PP2A, the possibility of PP2A_{B α} regulation via mTORC1-associated ubiquitin ligase activity to be particularly interesting (Figure 5.2A). Clearly this is theoretical, but a small number of simple experiments may shed light onto whether CUL4-DDB1 indeed negatively regulates PP2A_{B α} through ubiquitination. If this is the case, this would provide another mechanism of mTORC1 activation, whereby mTORC1 itself regulates phosphatase activity upstream of the complex.

Finally, the interesting possibility that knockdown of PP6c leads to enhanced PP2A_{Tip41} activity would provide further clarity as to the role of PP2A_{Tip41} in mTORC1 signalling. Figure 5.7 shows that phosphorylation of S6K1 is attenuated on knockdown of PP6c, which is contrary to the expected result taking into consideration that loss of phosphatase activity would be expected to enhance signalling. As Tip41 has been shown to bind PP6c, in addition to PP2Ac (McConnell et al, 2007), loss of PP6c could therefore promote PP2A_{Tip41} complex formation

leading to inhibition of mTORC1 and substrates. Uncovering whether this theory is correct would not only provide further evidence for the inhibitory role of Tip41 towards mTORC1, but would also clarify that PP6c is not acting to somehow directly enhance signalling to the complex.

6.5 Summary

Analysis of phosphatase complexes in the mTORC1 signalling pathway has provided a degree of clarity regarding regulation of the pathway that may be bypassed in a number of pathologies. Further investigation into phosphatase regulation will allow greater understanding of the molecular processes underlying diseases such as TS and AD, which could provide points of therapeutic intervention. In addition, inhibition of HIF1 is a topic where further investigation could lead to the understanding of molecular principles underlying cancer progression that affects countless individuals. Type II diabetes is attributed to obesity and presents the biggest challenge in health to the western world. Therefore, understanding the molecular causes of the disease is crucial to providing effective treatment. As loss of PP2A-Tip41 activity mimics the molecular features of type II diabetes, loss of Tip41 regulation may play a key role in insulin resistance. These discussions show that phosphatase regulation of the mTORC1 pathway may prove central to understanding the mechanism behind a wide variety of disease, from neurological to metabolic, amongst others. Whilst the data in this thesis provides further insights into the role of PP2A in the mTORC1 pathway, further research is required to fully elucidate the regulatory mechanisms and the exact point of action. Therefore, whilst specific PP2A complexes acting in the mTORC1 pathway have been identified, fully appreciating the opposition of kinase activity by phosphatases is required and will no doubt lead to important steps on the road to our understanding of the molecular causes of disease.

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