The Regulation of Mammalian Target of Rapamycin

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Abstract

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mTOR is a highly conserved Serine/Threonine protein kinase which couples to a variety of stimulatory signals to control both anabolic and catabolic cellular processes. It exists in two biochemically and functionally distinct multi-component complexes termed as mTORC1 and mTORC2. Increasing evidence indicates that both mTOR complexes play crucial roles in the regulation of pancreatic β-cell function and mass. For example, hormones like GLP-1 that augment intracellular cAMP ($[cAMP]_i$) are able to activate mTORC1 in β-cells, and it has been shown that GLP-1 treatment can lead to an enhancement of β-cell proliferation. However, in other cell types such as hepatocytes, neurons and many cancer cell lines, increases in $[cAMP]_i$ lead to the inhibition of mTORC1 which coincides with an impairment of cell replication. Therefore, the aims of this thesis were to reveal how cAMP regulates mTOR and to elucidate the molecular mechanism upon which mTOR complexes control pancreatic β-cell mass. My data provides evidence that the PKB-mTORC1 pathway by GLP-1 positively regulates β-cell proliferation, and that prolonged rapamycin treatment results in an induction of β-cell death which is caused by the inhibition of mTORC2 rather than mTORC1. These results further elucidated the mechanism by which mTOR complexes control pancreatic β-cell mass and therefore contribute to the design of future treatment against diabetes mellitus.

In addition, I have also shown that cAMP, in contrast to its effects in β-cells, inhibits both mTORC1 and 2 in mouse embryonic fibroblasts (MEFs) and human embryonic kidney (HEK293) cells by promoting complex dissociation and blocking mTOR kinase activity. Increases in $[cAMP]_i$ also inhibit protein synthesis and prevent cell cycle progression in MEFs. In conclusion, this work has further unravelled how mTOR complexes control pancreatic β-cell mass, and provided a rationale of using cAMP-inducing agents to treat cancer.

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I would like to dedicate this work to my grandparents.

Abbreviations

$[cAMP]_i$	intracellular cAMP levels
4EBP	eIF4E-binding protein
ABTS	2,2'-azino-bis(3-ethylbenzothiazoline-6-sulphonic acid)
AC	adenylate cyclase
ADB	assay dilution buffer
AdDN-AMPK	adenovirus expressing dominant-negative AMPK
ADP	adenosine diphosphate
AGC	protein kinase A, G and C
AICAR	5-Aminoimidazole-4-carboxamide ribotide
AID	*C*-terminal auto-inhibitory domain
$Akt1^{-/-}$	PKBα knock-out
$Akt2^{-/-}$	PKBβ knock-out
AL	activation loop
AMP	adenosine monophosphate
AMPK	5'-AMP-activated protein kinase
aPKC	atypical PKC
APS	ammonium persulfate
Arf1	ADP-ribosylation factor 1
ART	Dr. Andrew R Tee
ATG	autophagy-related protein

ATGL	adipose triglyceride lipase
ATM	ataxia-telangiectasia mutated
ATP	adenosine triphosphate
BAD	Bcl-2-associated death promoter
Bcl-2	B-cell lymphoma 2
BNIP3	Bcl2/adenovirus E1B 19-kD protein-interacting protein 3
βRicKO	β-cell specific knock-out of RICTOR
BSA	bovine serum albumin
βTrCP	β-transducin repeat-containing protein
$\beta TSC2^{-/-}$	β-cell specific TSC2 knock out
cAMP	cyclic AMP
CB	Cambridge Biosciences
CBP	nuclear cap-binding protein
CCTβ	chaperonin containing TCP-1
cdc2	cell division control protein 2
CDK1	cyclin dependent kinase 1
CDK4	cyclin D dependent kinase 4
CHAPS	3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate
CHUK	conserved helix-loop-helix ubiquitous kinase
CK1α	casein kinase 1α
cPKC	conventional PKC
CPM	counts per minute
CREB	cAMP response element modulator

ddH_2O	double distilled water
DEP	dishevelled, egl-10, pleckstrin
DEPC	diethylpyrocarbonate
DEPTOR	DEP domain-containing mTOR interacting protein
DMEM	Dulbecco's modified Eagle's medium
DNA-PK	DNA-dependent protein kinase
DPM	disintegrations per minute
DPP-IV	dipeptidyl peptidase IV
$dPRAS40^{-/-}$	*Drosophila* PRAS40 knockout
Dvl	dishevelled
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEF	eukaryotic elongation factors
eEF2K	eEF2 kinase
EGF	epidermal growth factor
EGFR	EGF receptor
EGTA	ethylene glycol tetraacetic acid
eIF	eukaryotic initiation factor
EJC	exon junction complex
EPAC	exchange protein directly activated by cAMP
ERK	extracellular signal-regulated kinase
ERK1/2	extracellular signal-regulated kinase 1 and 2
ESC	embryonic stem cell

FAK	focal adhesion kinase
FAT	FRAP, ATM, TRAP
FATC	*C*-terminal FAT
FBS	foetal bovine serum
FIP200	FAK family kinase-interacting protein of 200 kDa
FKBP12	FK506-binding protein of 12kDa
FKBP38	FK506 binding protein 38
FLAG-Rheb	FLAG tagged Rheb
FMRP	fragile X mental retardation protein
FOXO1	forkhead box protein O1
FRB	FKBP12-rapamycin binding
FSC-H	Forward scatter-height
GAP	GTPase-activating protein
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
GEF	guanine nucleotide exchange factor
GFP	green fluorescent protein
GLK	germinal centre-like kinase
GLP-1	glucagon like peptide 1
GLP-1R	GLP-1 receptor
GLUT1	glucose transporter 1
GNEF	guanine nucleotide exchange factors
GPCRs	G-protein coupled receptors
Grb10	growth factor bound-receptor protein 10

GRp58	58 KDa glucose-regulated protein
GSIS	glucose-stimulated insulin secretion
GSK3	glycogen synthase kinase 3
GsPCR	Gs-protein coupled receptor
GβL	G_{β}-like protein
HA	hemagglutinin
HA-S6K1	HA tagged S6K1
HBS	HEPES-Buffered Saline
HEAT	Huntington, EF3, a subunit of PP2A, TOR1
HEK293	human embryonic kidney 293
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIF1α	hypoxia-inducible factor 1α
HM	hydrophobic motif
HSL	hormone-sensitive lipase
Hsp90	heat shock protein 90
hVps34	homologue of vacuolar protein sorting 34
IAPP	islet amyloid polypeptide
IBMX	3-isobutyl-1-methylxanthine
ICK	intestinal cell kinase
IGF	insulin-like growth factor
IGF1R	IGF1 receptor
IKKα	inhibitor of NF-κB kinase subunit α
IMP2	IGF2 mRNA-binding protein 2

IMS	industrial methylated spirit
INS1E	rat insulinoma cell line 1E
InsR	insulin receptor
IP	immunoprecipitation
IPMK	inositol polyphosphate multikinase
IRS	insulin receptor substrate
JAK	Janus kinase
JNK	c-Jun N-terminal kinase
KRB	Krebs-Ringer bicarbonate
LB	Luria-Bertani
LCD	domain conserved with *Drosophila* lobe protein
LKB1	liver kinase B1
LRS	Leucyl-tRNA synthetase
m^7GTP	7-methylguanosine triphosphate
Mad1	mitotic arrest deficient 1
Maf1	MFP1 associated factor 1
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3
MAPK	mitogen-activated protein kinase
MAR	matrix attachment region
MDM2	murine double minute 2
MEF	mouse embryonic fibroblast
MEK	MAPK/ERK kinase
MEM	Eagle's minimum essential media

MFP1	MAR binding filament–like protein 1
MIN6	mouse insulinoma cell line 6
mLST8	mammalian ortholog of lethal with sec thirteen
MP1	MEK partner 1
mSIN1	mammalian stress activated protein kinase interacting protein 1
mTOR	mechanistic (mammalian) target of rapamycin
myc	myelocytomatosis
myc-mTOR	myc-tagged mTOR
NAD^+	nicotinamide adenine dinucleotide
NADH	NAD plus hydrogen
NCBP1	nuclear cap binding protein subunit 1
NcoRI	nuclear receptor co-repressor 1
NEB	New England Biolabs
NF	neurofibromatosis
NF-κB	nuclear factor κ-light-chain-enhancer of activated B cells
NFL	negative feedback loop
nPKC	novel PKC
OD_{600}	optical density measured at 600 nm
P/S/N	100 μg/ml streptomycin, 100 units/ml penicillin sulphate, 100 units/ml neomycin
PA	phosphatidic acid
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline

PDCD4	programmed cell death protein 4
PDE	phosphodiesterase
PDK1	phosphoinositide dependent protein kinase 1
Pdx1	pancreatic duodenal homeobox 1
PDZ	postsynaptic density 95, discs large, zonula occludens-1
PGC1α	peroxisome proliferator-activated receptor 1α
PH	pleckstrin homology
PHAS	phosphorylated heat- and acid-stable protein
PI3K	phosphatidylinositol 3-kinase
PIKKs	PI3K related kinases
PIM-1	proviral integration site for Moloney murine leukemia virus-1
PIP2	phosphatidylinositol (4,5)-biphosphate
PIP3	phosphatidylinositol (3,4,5)-triphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PLD	phospholipase D
PML	promyelocytic leukaemia
POLDIP3	polymerase (DNA-directed), delta interacting protein 3
PP2A	protein phosphatase 2A
$PP2A_{T61\varepsilon}$	PP2A oligomer containing the PR61ε-targeting subunit
PPARα	peroxisome proliferator activated receptor α
PRAK	p38-regulated/activated kinase

PRAS40	Pro-rich Akt substrate of 40 kDa
PROTOR	protein observed with RICTOR
PTEN	Phosphatase and tensin homolog
Rab	Ras-related in brain
Rac1	Ras-related C3 botulinum toxin substrate 1
RAIP	letters of amino acids 13-16 of 4EBP1/2
RalA	Ras-like protein A
RAPTOR	regulatory-associated protein of mTOR
Ras	rat sarcoma
Rb	retinoblastoma protein
Rbx1	RING-box protein 1
rDNA	ribosomal DNA
REDD1	regulated in development and DNA damage responses 1
Rheb	Ras homolog enriched in brain
RIα	regulatory subunit Iα of PKA
RICTOR	rapamycin-insensitive companion of TOR
RIP-KdAkt1	mice expressing kinase dead PKBα
RNC	RAPTOR *N*-terminal conserved
ROS	reactive oxygen species
RP	ribosomal protein
RPB5	retinol binding protein 5
RPMI	Roswell Park Memorial Institute
rpS6	ribosomal protein S6

$rpS6^{P-/-}$	rpS6 knock-in
rRNA	ribosomal RNA
RSK	p90 ribosomal protein S6 kinase
S6K1	p70 rpS6 kinase 1
$S6K1^{-/-}$	S6K1 knock out
$S6K2^{-/-}$	S6K2 knock out
$S6KCA^{RIP}$	over-expression of a constitutively active form of S6K1 in mouse β-cells
SCB	Santa Cruz Biotechnology
SCF	Skp1/Cul1/F box
$SCF^{\beta TrCP}$	SCF protein adaptor βTrCP
SDS	sodium dodecyl sulphate
SGK1	serum- and glucocorticoid-induced protein kinase 1
shRNA	small hairpin RNA
siRNA	small interfering RNA
SirT1	silent information regulator two protein 1
SKAR	S6K1 Aly/REF-like target
SLC1A5	solute carrier family 1 member 5
SLC7A5	solute carrier family 7 member 5
SOC	super optimal broth with catabolite repression
SREBP	sterol regulatory element-binding protein
STAT3	signal transducers and activators of transcription 3
STK11	serine/threonine kinase 11
T1DM	type 1 diabetes mellitus

T2DM	type 2 diabetes mellitus
TANK	TRAF family member-associated NF-κB activator
TASCC	TOR-autophagy spatial coupling compartment
TBK1	TANK binding kinase 1
TCA	trichloroacetic acid
TCF7L2	transcription factor 7-like 2
TCP	T-complex protein
TCTP	translationally controlled tumour protein
TE	Tris-EDTA
Tel2	telomere maintenance 2
TEMED	N, N, N', N'-tetramethylethylenediamine
TFEB	transcription factor EB
TFIIIC	transcription factor 3C
TM	turn motif
TNFα	tumour necreosis factor α
TOR	target of rapamycin
TOS	TOR signalling
TRAF	tumour necrosis factor receptor-associated factor
Tris	tris(hydroxymethyl)aminomethane
$TSC2^{-/-}$	TSC2 knock-out
Tti1	Tel2 interacting protein 1
ULK1	UNC-51-like kinase
UNC	uncoordinated

URI	unconventional prefoldin RPB5 interactor
Vam6	vacoular membrane protein 6
v-ATPase	vacuolar H^+-ATPase
vps39	vacuolar protein sorting 39
WB	western blotting
WD	terminating in a tryptophan-aspartic acid (W-D) dipeptide
Wnt	wingless, integration
YY1	ying-yang 1

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Chapter 1. General Introduction

1.1 An introduction to mTOR

1.1.1 The complexity of mTOR complexes

Mammalian target of the immunosuppressant drug rapamycin (mTOR, also known as FRAP, RAFT or RAPT) is a highly conserved Ser/Thr protein kinase which couples nutrient and growth factor signals to control cell growth, proliferation, viability and function (Heitman, Movva and Hall. 1991, Sabatini et al. 1994, Brown et al. 1994). mTOR is present in two biochemically and functionally distinct multi-component complexes termed mTORC1 and mTORC2 (Fig. 1.1). Rapamycin is an allosteric inhibitor of mTOR, it associates with FKBP12 (for FK506-binding protein of 12kDa), which in turn binds to the FRB (for FKBP12-rapamycin binding) domain of mTOR and inhibits its activity (Chen et al. 1995). Upon rapamycin treatment mTORC1 is rapidly dissociated and therefore inhibited (Oshiro et al. 2004, Yip et al. 2010), whereas mTORC2 is resistant to short-term rapamycin treatment, albeit prolonged presence of rapamycin does inhibit mTORC2 in a few cell types, due to the binding of rapamycin-FKBP12 to newly synthesized and unbound mTOR pool that prevents mTORC2 assembly (Sarbassov et al. 2006). Both mTOR complexes are comprised of mTOR, mLST8 [for mammalian ortholog of lethal with sec thirteen, also known as GβL (G_β-like protein)] (Kim et al. 2003), DEPTOR [for DEP (dishevelled, egl-10, pleckstrin) domain-containing mTOR interacting protein] (Peterson et al. 2009), Tti1 [Tel2 (telomere

maintenance 2) interacting protein 1], Tel2 (Kaizuka et al. 2010, Takai et al. 2010), GRp58 (for 58 KDa glucose-regulated protein, also referred to as ERp57) (Ramirez-Rangel et al. 2011) and Rac1 [Ras (rat sarcoma)-related C3 botulinum toxin substrate 1] (Saci, Cantley and Carpenter. 2011). mTORC1 specific components include RAPTOR (regulatory-associated protein of mTOR) (Kim et al. 2002, Hara et al. 2002) and PRAS40 (for Pro-rich Akt substrate of 40 kDa) (Sancak et al. 2007, Wang et al. 2007, Fonseca et al. 2007, Oshiro et al. 2007, Thedieck et al. 2007). mTORC2 specific components consist of RICTOR (rapamycin-insensitive companion of TOR) (Sarbassov et al. 2004), mSIN1 (mammalian stress activated protein kinase interacting protein 1) (Jacinto et al. 2006, Frias et al. 2006, Yang et al. 2006) and PROTOR (protein observed with RICTOR) (Pearce et al. 2007).

1.1.2 Phosphorylation and function of mTORC components

The control of mTOR complex activity relies on a sophisticated cellular signalling network largely dependent upon protein phosphorylations. To date, six mTOR phosphorylation sites (Ser2448, Ser2481, Thr2446, Ser1261, Ser2159 and Thr2164, in order of discovery, numbering of the amino acid residues mentioned in this thesis are for human proteins only, unless specified) have been identified so far. Ser2448 was the first reported mTOR phosphorylation site yet, to date, its function remains unclear. It was believed that mTOR Ser2448 phosphorylation was mediated by PKB (protein kinase B, also referred to as Akt) (Nave et al. 1999, Sekulic et al. 2000, Reynolds, Bodine and Lawrence. 2002, Bolster et al. 2002), however, more recent works have revealed that S6K1 [p70 ribosomal protein S6 (rpS6) kinase 1] is the mTOR Ser2448 kinase (Holz and

Blenis. 2005, Chiang and Abraham. 2005). Ser2481 is the mTOR autophosphorylation site (Peterson et al. 2000), which serves as a biomarker of mTOR intrinsic kinase activity (Soliman et al. 2010). mTORC1 but not mTORC2-associated mTOR Ser2481 phosphorylation is reduced upon acute rapamycin treatment or nutrient withdraw (Soliman et al. 2010). Opposite to the phosphorylation pattern of Ser2448, Thr2446 is phosphorylated in response to nutrient deprivation (Cheng et al. 2004). Both PKB and AMPK [5'-AMP (adenosine monophosphate)-activated protein kinase] are able to phosphorylate mTOR Thr2446 *in vitro* (Cheng et al. 2004), yet this observation remained to be proved *in vivo*. Ser1261, Ser2159 and Thr2164 are newly identified mTOR phosphorylation sites of which their respective kinases are currently unknown (Acosta-Jaquez et al. 2009, Ekim et al. 2011). Nevertheless, the phosphorylation of these sites is a prerequisite for mTOR autophosphorylation on Ser2481, and unlike the early identified sites (Ser2448, Ser2481 and Thr2446), Ser1261, Ser2159 and Thr2164 phosphorylations are important to mTOR function, especially the regulation of cell growth and proliferation (Acosta-Jaquez et al. 2009, Ekim et al. 2011).

mLST8 is essential for the integrity and activation of mTORC2 but its deletion has no effect on mTORC1 (Guertin et al. 2006). In contrast, GRp58 is required for the assembly and stimulation of mTORC1, but it is dispensable for mTORC2 (Ramirez-Rangel et al. 2011). The newly identified mTORC subunit Rac1 is a positive regulator of both mTOR complexes, and its deletion in MEFs and lymphocytes can cause a reduction in cell size (Saci, Cantley and Carpenter. 2011).

DEPTOR is a negative regulator of both mTORC1 and 2 (Peterson et al. 2009). It is expressed at low levels in most cancer cells, which coincides with enhanced activities of mTORC1 and 2 and improved resistance to nutrient deprivation-induced apoptosis, yet striking exceptions occur in many multiple myelomas where DEPTOR is often over-expressed. Although the activation of mTORC1 is turned off in those cells, mTORC2 is surprisingly "re-activated", this is because PI3K (phosphatidylinositol 3-kinase) stimulates mTORC2 activity (Gan et al. 2011), and DEPTOR over-expression alleviates PI3K from the negative feedback inhibition of mTORC1, and therefore survival rates of these tumour cells are also improved (Peterson et al. 2009). Twenty-one *in vivo* phosphorylation sites within DEPTOR have been identified (Peterson et al. 2009, Gao et al. 2011). The phosphorylation of at least six of these sites (Ser286, Ser287, Ser291, Ser293, Thr295 and Ser299) can be directly phosphorylated by either mTOR (on Ser286, Ser293, Thr295 and Ser299), CK1α (casein kinase 1α) (on Ser286 and Ser287) (Gao et al. 2011), S6K1 or RSK (p90 ribosomal protein S6 kinase) (Ser286, Ser287, Ser291) (Zhao, Xiong and Sun. 2011) and are essential for the binding of $SCF^{\beta TrCP}$ [Skp1/Cul1/F box (SCF) protein adaptor β-transducin repeat-containing protein (βTrCP)] E3 ubiquitin ligase to DEPTOR (Gao et al. 2011, Zhao, Xiong and Sun. 2011, Duan et al. 2011). As a positive feedback mechanism, the phosphorylation of these sites in response to extracellular stimuli results in the ubiquitination and degradation of DEPTOR, which releases mTOR from DEPTOR inhibition (Gao et al. 2011, Zhao, Xiong and Sun. 2011, Duan et al. 2011).

Unlike its role in the maintenance of telomere length in budding yeast (Lustig and Petes. 1986), Tel2 has no telomere function in mammalian cells, but it is essential for embryonic development and cell growth, as it positively regulates the maturation and stability of PIKKs (PI3K related kinases) family members including mTOR (Takai et al. 2007), dependent upon the action of chaperone Hsp90 (heat shock protein 90) (Takai et al. 2010). Importantly, it has been demonstrated that Tel2 and its associated protein Tti1 bind to both mTORC1 and 2, and they are crucial factors for mTOR complex assembly (Kaizuka et al. 2010).

Raptor is essential to the integrity of mTORC1, and it is responsible for the recruitment of mTOR downstream targets, such as S6Ks and 4EBPs [eukaryotic initiation factor (eIF) 4E-binding proteins, also referred to as phosphorylated heat- and acid-stable protein (PHAS)], to mTOR, via interacting with their TOS (for TOR signalling) motif (Schalm and Blenis. 2002, Schalm et al. 2003, Nojima et al. 2003). Raptor can be phosphorylated at multiple sites by many protein kinases (see table 1-1) and others which are phosphorylated by unknown kinase(s) (Wang et al. 2009, Foster et al. 2010, Langlais, Yi and Mandarino. 2011, Carriere et al. 2011). Phosphorylations of RAPTOR by ERK, RSK, p38β, mTOR, JNK (c-Jun N-terminal kinase) and ICK (intestinal cell kinase) are likely to be stimulatory to the activation of mTORC1 (Wang et al. 2009, Foster et al. 2010, Langlais, Yi and Mandarino. 2011, Carriere et al. 2011, Carriere et al. 2008, Wu et al. 2011a, Kwak et al. 2012, Wu et al. 2012), whereas the phosphorylation by AMPK, which induce the binding of 14-3-3 to RAPTOR (Gwinn et al. 2008), and ULK1 (Dunlop et al. 2011), lead to the inhibition of mTORC1 during energy stress and autophagy induction.

Table 1-1 Phosphorylation sites of RAPTOR and the protein kinases responsible for their phosphorylations. Abbreviations: AMPK: 5'-AMP (adenosine monophosphate)-activated protein kinase; ERK: extracellular signal-regulated kinase; ICK: intestinal cell kinase; JNK: c-Jun N-terminal kinase; MAPK: mitogen-activated protein kinase; mTOR: mammalian target of rapamycin; RSK: p90 ribosomal protein S6 kinase; ULK1: UNC (uncoordinated)-51-like kinase.

Phosphorylation	***Protein kinase(s)***	***References***
Ser8	ERK	(Carriere et al. 2011)
Ser696	ERK, JNK	(Langlais, Yi and Mandarino. 2011, Carriere et al. 2011, Kwak et al. 2012)
Thr706	JNK	(Kwak et al. 2012)
Ser719	RSK	(Carriere et al. 2008)
Ser722	RSK, AMPK	(Carriere et al. 2008, Gwinn et al. 2008)
Ser771	p38β MAPK	(Wu et al. 2011a)
Ser792	AMPK, ULK1	(Gwinn et al. 2008, Dunlop et al. 2011)
Ser855	ULK1	(Dunlop et al. 2011)
Ser859	mTOR, ULK1	(Wang et al. 2009, Dunlop et al. 2011)
Ser863	mTOR, ULK1, ERK, p38β MAPK and JNK	(Wang et al. 2009, Foster et al. 2010, Langlais, Yi and Mandarino. 2011, Carriere et al. 2011, Wu et al. 2011a, Kwak et al. 2012, Dunlop et al. 2011)
Ser877	ULK1	(Dunlop et al. 2011)
Thr908	ICK	(Wu et al. 2012)

PRAS40 was first identified as an inhibitory binding partner of mTORC1 (Sancak et al. 2007, Wang et al. 2007, Fonseca et al. 2007, Oshiro et al. 2007, Vander Haar et al. 2007),

upon its phosphorylation on Thr246 by mTORC1 upstream regulator PKB (Sancak et al. 2007, Wang et al. 2007, Fonseca et al. 2007, Oshiro et al. 2007, Vander Haar et al. 2007), followed by subsequent phosphorylation (Nascimento et al. 2010) on Ser183 (Oshiro et al. 2007), Ser212 and Ser221 (Wang, Harris and Lawrence. 2008) by mTORC1 itself, PRAS40 is released from the complex and results in mTORC1 activation (Sancak et al. 2007, Wang et al. 2007, Fonseca et al. 2007, Oshiro et al. 2007, Vander Haar et al. 2007, Nascimento et al. 2010). Recently it has been shown that the protein kinase PIM-1 (proviral integration site for Moloney murine leukemia virus-1), an oncoprotein kinase implicated in the progression of haematopoietic malignancies and prostate carcinomas (Nawijn, Alendar and Berns. 2011), and protein kinase A (PKA) (Blancquaert et al. 2010), are also able to phosphorylate PRAS40 on Ser246 (Zhang et al. 2009). PRAS40 knockout in *Drosophila* (dPRAS40$^{-/-}$) has no effect on the growth of the flies, but they have bigger ovaries and improved fertility compared to their wild-type littermates (Pallares-Cartes, Cakan-Akdogan and Teleman. 2012). Interestingly, only dTORC1 in ovaries is constitutively activated in dPRAS40$^{-/-}$ flies whereas dTORC1 in other tissues is unaffected, which implies that the contribution of PRAS40 to TORC1 activity is tissue-specific (Pallares-Cartes, Cakan-Akdogan and Teleman. 2012).

RICTOR and mSIN1 are essential for the maintenance of mTORC2 complex integrity and the regulation of mTORC2 downstream targets (Sarbassov et al. 2004, Sarbassov et al. 2005, Hresko and Mueckler. 2005), whereas PROTOR is dispensable for the assembly of other subunits into mTORC2 (Pearce et al. 2007), although it is required for the activation of specific substrates such as SGK1 (serum- and glucocorticoid-induced

protein kinase 1) (Pearce et al. 2011) and tristetraprolin (Holmes et al. 2012). mSIN1, a selective adaptor for the recruitment of mTORC2 downstream targets (Cameron et al. 2011), has been found recently to be phosphorylated (sites yet to be identified) by mTOR (Chen and Sarbassov. 2011), mTOR kinase activity is essential for mSIN1 phosphorylation and mTORC2 integrity (Chen and Sarbassov. 2011).

Four independent groups (Dibble, Asara and Manning. 2009, Julien et al. 2010, Treins et al. 2010, Boulbes et al. 2010) have identified Thr1135 as a RICTOR phosphorylation site which is modulated by growth factor and nutrient availability. Interestingly, mTORC1 downstream target S6K1 (Chen et al. 1995, Chung et al. 1992) is the RICTOR Thr1135 kinase (Dibble, Asara and Manning. 2009, Julien et al. 2010, Treins et al. 2010), providing a potential link between the two mTOR complexes. However, although Thr1135 phosphorylation have been found to promote 14-3-3 binding to RICTOR (Dibble, Asara and Manning. 2009, Treins et al. 2010), the significance of this phosphorylation event is far from understood and it may play roles in mTORC2-independent RICTOR functions (Boulbes et al. 2010). In a study carried out by Gao *et al*. it was shown that RICTOR Thr1135 phosphorylation has a negative effect on its association with cullin-1 and Rbx1 (RING-box protein 1) to form a functional E3 ubiquitin ligase, which promotes the ubiquitination and degradation of SGK1 (Gao et al. 2010). Interestingly, studies in *Caenorhabditis elegans* (Soukas et al. 2009, Jones et al. 2009) and mammalian cells (Chen and Sarbassov. 2011) have revealed that a single residue [G1120 (C. elegans)/G934 (mammals)] in RICTOR is essential for its association with SIN1/mSIN1 and mTORC2 function.

A schematic representation of mTOR components and their respective phosphorylation sites is provided in Fig. 1.1.

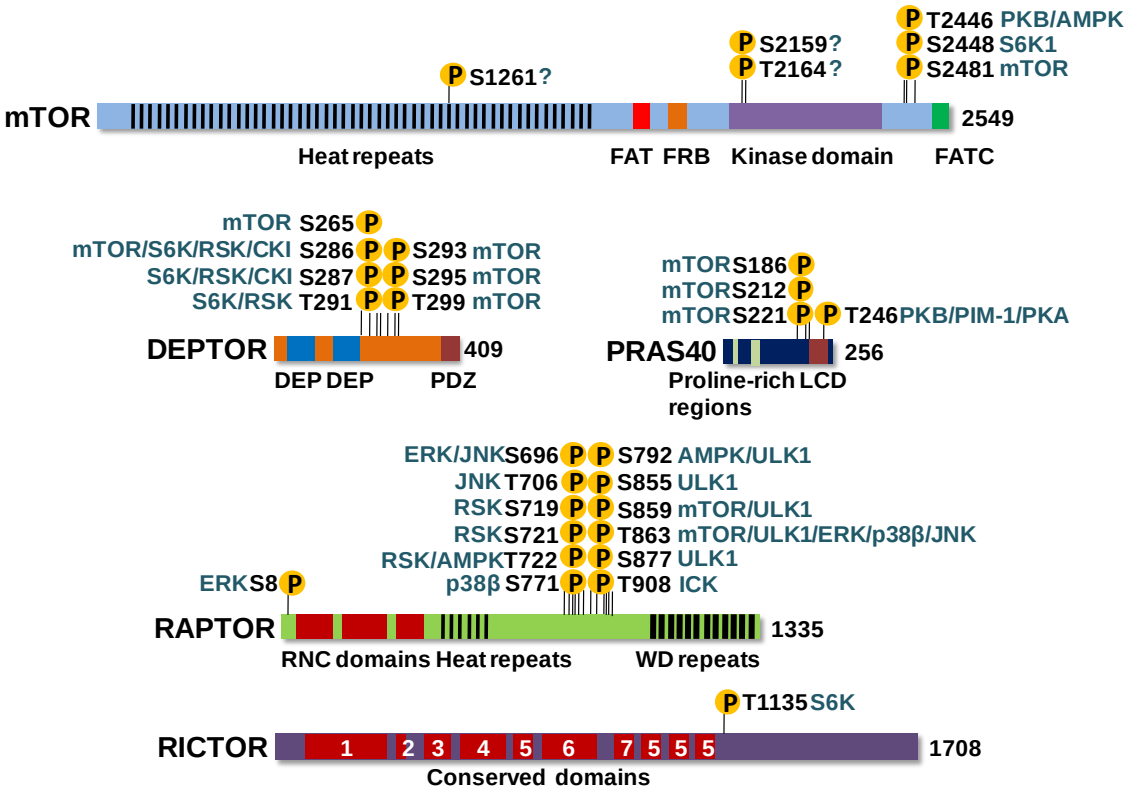


Figure 1.1 Protein structural domains and phosphorylation of selected mTOR complex components. mTOR is comprised of 2549 amino acids which can be divided into several structural domains, including HEAT (for Huntington, EF3, a subunit of PP2A, TOR1) repeats, FAT (for FRAP, ATM, TRAP), FRB, kinase domain and FATC (for *C*-terminal FAT) domain. The HEAT repeats, located close to the *N*-terminus, are required for mTOR multimerization (Takahara et al. 2006). FRB domain, as its name implied, is the binding site of mTOR to FKBP12 and rapamycin (Chen et al. 1995). FAT, kinase domain and FATC domain are conserved within the PIKKs, and they are essential for maintaining the activity of PIKKs (Lempiainen and Halazonetis. 2009). DEPTOR contains two DEP domain and one PDZ (for postsynaptic

density 95, discs large, zonula occludens-1) domain (Peterson et al. 2009), both PDZ domain (DEPTOR) and FAT domain (mTOR) are essential for mTOR-DEPTOR binding (Peterson et al. 2009). PRAS40 contains two proline-rich domains at the *N*-terminus and a LCD (for domain conserved with *Drosophila* lobe protein) domain at the *C*-terminus (Sancak et al. 2007). Structural domains for Raptor include: RNC (for RAPTOR *N*-terminal conserved) domains, HEAT repeats, and followed by WD [for terminating in a tryptophan-aspartic acid (W-D) dipeptide] repeats. RNC is crucial for RAPTOR association with TOS (for TOR signalling) motif-containing proteins such as 4EBP1 (Dunlop et al. 2009). Point mutations within RNC and WD domains can also disturb mTOR binding (Dunlop et al. 2009). Rictor has seven conserved domains among living species, with domain five repeated four times (Sarbassov et al. 2004). For all proteins mentioned in this figure, only human homologs are shown. Phosphorylation sites and their corresponding kinase(s) (both *in vitro* and *in vivo*) are also presented. See text for details.

1.2 Upstream regulators of mTOR

1.2.1 Regulation of mTORC1 by growth factors

mTORC1 is activated upon association with the small G-protein Rheb (Ras homolog enriched in brain), when the latter is in its GTP bound state (Zhang et al. 2003, Saucedo et al. 2003, Garami et al. 2003). Another small G protein Rhes (Ras homolog enriched in striatum) has also recently been shown to stimulate mTORC1 in its GTP-bound state (Subramaniam et al. 2011). Nevertheless, unlike Rheb, Rhes also activates mTORC2 (Subramaniam et al. 2011). Rheb is regulated through the activity of its GTPase-activating protein (GAP) TSC1/2 (tuberous sclerosis complex 1 and 2, also known as hamartin and tuberin, respectively) (Zhang et al. 2003, Saucedo et al. 2003, Garami et al. 2003) (Fig. 1.2a). Importantly, Growth factors, hormones, nutrients, energy, inflammation and cell cycle signals activate signalling pathways that lead to changes in

the phosphorylation status of TSC1/2 which results in its inactivation (Fig. 1.2b). This promotes the formation of Rheb-GTP and hence the activation of mTORC1. Although it has been suggested that Rheb is able to release mTOR from the inhibitory binding of FKBP38 (FK506 binding protein 38) (Bai et al. 2007), and TCTP (translationally controlled tumour protein) have been proposed to act as guanine nucleotide exchange factors (GNEF) for Rheb (Hsu et al. 2007), other studies have questioned this (Wang et al. 2008, Uhlenbrock et al. 2009). PLD (phospholipase D), an enzyme which catalyses the hydrolysis of phosphatidylcholine to phosphatidic acid (PA), is an important regulator of mTOR activity upon mitogenic stimuli (Fang et al. 2001), and there is evidence that PLD1 acts downstream of Rheb to stimulate mTORC1 (Sun et al. 2008), while recent studies have also suggested a role of PLD in the activation of mTORC1 by nutrient sensing (see section 1.2.2). How PLD/PA leads to mTORC1 activation is poorly understood, albeit it is suggested that they may stimulate mTORC1 by promoting mTORC1 complex assembly (Toschi et al. 2009) and activating MAPK pathway (Winter et al. 2010).

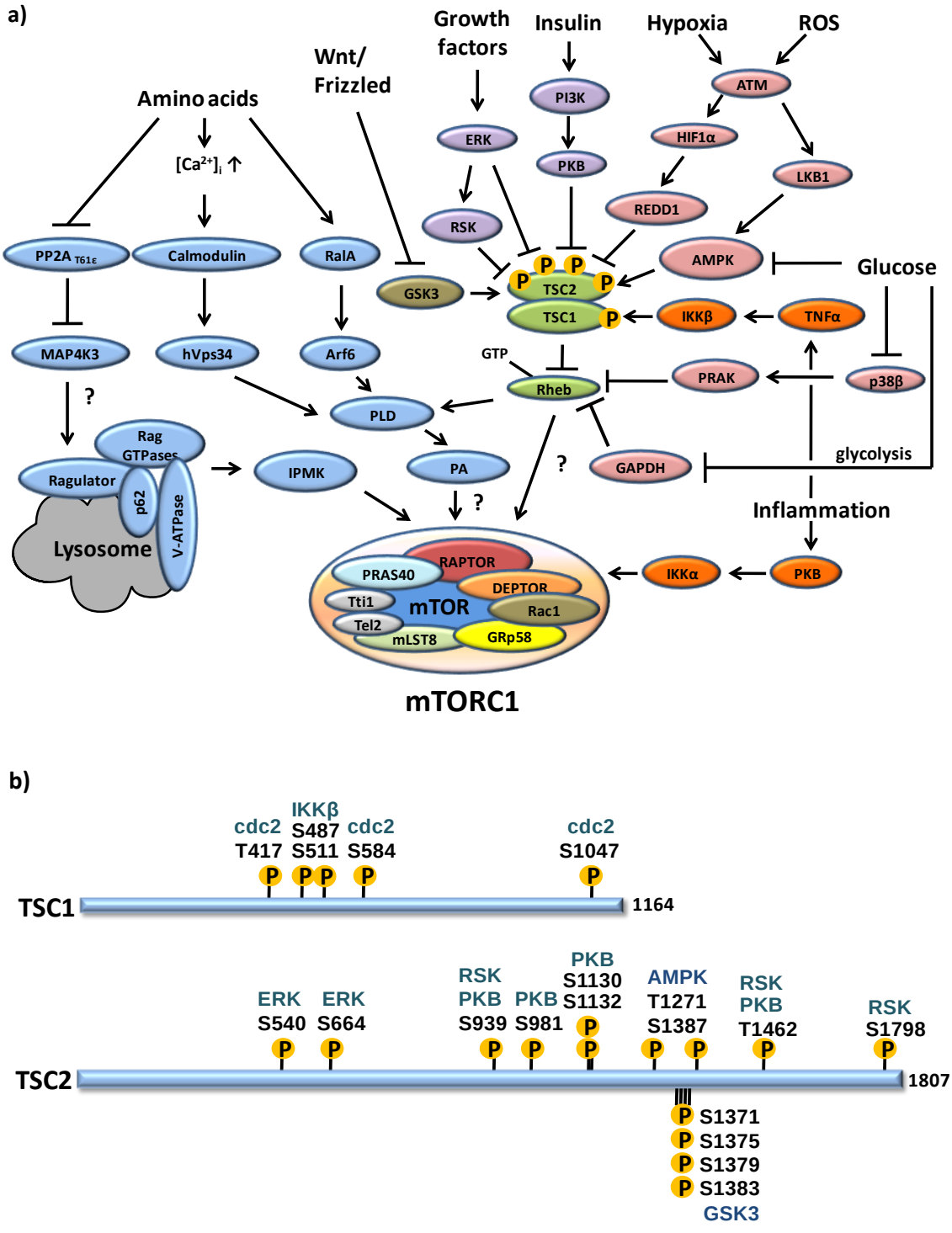


Figure 1.2 Schematic representation of signalling pathways that regulate mTORC1. **a)** mTORC1 components include: mTOR, RAPTOR (Kim et al. 2002, Hara et al. 2002), PRAS40 (Sancak et al. 2007,

Wang et al. 2007, Fonseca et al. 2007, Oshiro et al. 2007, Thedieck et al. 2007), mLST8 (Kim et al. 2003), DEPTOR (Peterson et al. 2009), Tti1, Tel2 (Kaizuka et al. 2010, Takai et al. 2010), GRp58 (Ramirez-Rangel et al. 2011) and Rac1 (Saci, Cantley and Carpenter. 2011). mTORC1 senses a wide range of upstream signals such as: glucose levels through the regulation of p38β MAPK/PRAK (Zheng et al. 2011), AMPK (Inoki, Zhu and Guan. 2003) or GAPDH (Lee et al. 2009); oxygen levels through LKB1/AMPK (Wolff et al. 2011) and ATM/HIF1α/REDD1(Wolff et al. 2011, Brugarolas et al. 2004, Sofer et al. 2005, Cam et al. 2010, Jin et al. 2011); ROS levels through ATM-LKB1 (Alexander et al. 2010); inflammation signals through PKB/IKKα (Dan, Adli and Baldwin. 2007) or TNFα/IKKβ/TSC1 (Lee et al. 2007); amino acids availability, which modulates the activity of mTORC1 through $PP2A_{T61\varepsilon}$/MAP4K3-Rag GTPases (Yan et al. 2010, Sancak et al. 2008, Kim et al. 2008) pathway, Ca^{2+}/Calmodulin-hVps34-PLD-PA pathway (Nobukuni et al. 2005, Gulati et al. 2008, Yoon et al. 2011) and RalA-Arf6-PLD-PA pathway (Xu et al. 2011). Recently IPMK (Kim et al. 2011), p62 (Duran et al. 2011) and v-ATPase (Zoncu et al. 2011) have all been shown to be implicated in the binding of Rag GTPases to RAPTOR and the activation of mTORC1 by amino acids; growth factors, which activate MAPK and stimulate mTORC1 via ERK and RSK (Carriere et al. 2011, Carriere et al. 2008, Ma et al. 2005, Roux et al. 2004, Rolfe et al. 2005, Fonseca et al. 2011); Insulin via activation of PI3K and PKB (Gingras et al. 1998, Scott et al. 1998) and Wnt/Frizzled pathway via inhibition of GSK3 (Inoki et al. 2006). These pathways impinge on TSC1/2, a GTPase activating protein of the small G protein Rheb, and GTP bound Rheb in turn activates mTORC1. For simplicity, not all known pathways mentioned in the text are shown. Protein colours of mTORC1 regulators: pink: stress signals; purple: growth factors and hormones; light blue: amino acids; tan: Wnt/Frizzled; orange: inflammation. **b)** Sites of phosphorylation on TSC1/2 and their respective kinases, adapted from (Huang and Manning. 2008).

Insulin, for example, activates mTORC1 through the PI3K (phosphatidylinositol 3-phosphate kinase)-protein kinase B (PKB, also known as Akt) pathway. PI3K catalyses the conversion of PIP2 [phosphatidylinositol (4,5)-biphosphate] to PIP3 [phosphatidylinositol (3,4,5)-triphosphate] (Irvine et al. 1986), and the latter recruits

PDK1 (phosphoinositide dependent protein kinase 1) and PKB to the plasma membrane through the binding to their PH (pleckstrin homology) domain, where PDK1 phosphorylates PKB on Thr308 (Alessi et al. 1997), PKB in turn phosphorylates TSC2 on Ser939, Ser981, Ser1130, Ser1132 and Thr1462, which leads to the inactivation of TSC1/2 (Potter, Pedraza and Xu. 2002, Manning et al. 2002, Dan et al. 2002, Inoki et al. 2002) (Fig. 1.2b). Also to mention is that full activation of PKB also requires the phosphorylation on Ser473, which is driven by mTORC2 (Sarbassov et al. 2005). Tumour suppressor PTEN (Phosphatase and tensin homolog) counteracts the action of PI3K to dephosphorylate PIP3 and thereby inhibits the activation of PDK1/PKB/TSC2/mTORC1 pathway. Interestingly, as a negative feedback, over-stimulation of mTORC1 and 2 leads to an increase in PTEN expression and subsequent PKB inhibition (Das et al. 2012).

Growth factors can also activate mTORC1 via stimulating ERK1/2 (extracellular signal-regulated kinase 1 and 2) and/or RSK-dependent phosphorylation of TSC2 at Ser540 and Ser664 (Ma et al. 2005), or Ser939, Thr1462 and Ser1798 (Roux et al. 2004) respectively, which lead to the suppression of TSC1/2. In addition, both ERK and RSK are able to phosphorylate RAPTOR on sites that have been reported to promote the activation of mTORC1 (Carriere et al. 2011, Carriere et al. 2008) (Fig 1.1 and 1.2).

1.2.2 Regulation of mTORC1 by amino acids

Growth factor or hormonal activation of mTORC1 is dependent upon the nutrient status of the cell. The presence of amino acids is an obligate requirement for the activation of

mTORC1, regardless of stimuli. This amino acid "sensing" by mTORC1 occurs independently of TSC1/2 (Smith et al. 2005), but it is dependent on a group of Ras-related small GTPases comprised of four proteins (Rag A, B, C and D) that form obligate heterodimers (Sancak et al. 2008, Kim et al. 2008), which are associated with a newly identified trimeric protein complex termed as "ragulator" (comprised of p14, p18 and MP1 [MEK (MAPK/ERK kinase) partner 1]) (Sancak et al. 2010). RagA/B^{GTP} and RagC/D^{GDP} are the most active forms and are essential for the recruitment of mTORC1 to endosomal and lysosomal compartments, where it encounters and is activated by Rheb-GTP (Sancak et al. 2008, Kim et al. 2008). Importantly, Rag GTPases can be stimulated by amino acids (Sancak et al. 2008, Kim et al. 2008). Leucyl-tRNA synthetase (LRS) has been identified as the GAP of RagD (Han et al. 2012). Vam6 [vacoular membrane protein 6, also referred to as Vps39 (vacuolar protein sorting 39)] has been identified as a GEF (guanine nucleotide exchange factor) of yeast RagA/B homolog Gtr1 (Binda et al. 2009), yet it is still unknown whether Vam6 plays the same role in mammalian cells. Crystal structural analysis of RagA and C has revealed that the N-terminal GTPase domains and dimerisation are essential for RAPTOR binding and subsequent mTORC1 activation (Gong et al. 2011). A recent study has identified p62 (also referred to as sequestosome 1) as a binding partner of Rag GTPases (Duran et al. 2011). Together with RAPTOR, p62 stabilizes the dimerization of Rags, it is also required for the association of mTORC1 and Rag GTPases and the translocation of mTORC1 to the lysosome (Duran et al. 2011) (Fig. 1.2a). Several members of the Rab (Ras-related in brain) family of GTPases and Arf1 [ADP (adenosine diphosphate)-ribosylation factor 1] are able to counteract the positive

effect of amino acids or active Rag GTPases on mTORC1 activation (Li et al. 2010), yet the molecular mechanism behind this remained to be established.

SLC1A5 (solute carrier family 1 member 5), which regulates L-glutamine uptake, and SLC7A5 (solute carrier family 7 member 5), a bidirectional transporter which mediates the efflux of L-glutamine and influx of L-leucine, are essential for the activation of mTORC1 (Nicklin et al. 2009). Amino acids uptake also leads to increases in intracellular Ca^{2+} levels, Ca^{2+}/Calmodulin forms a protein complex with hVps34 (homologue of vacuolar protein sorting 34, which is also the class III PI3K), it has been demonstrated that the binding of Ca^{2+}/Calmodulin-hVps34 signalosome to mTOR is required for amino acids signalling to mTORC1 (Gulati et al. 2008). Recently it has been demonstrated that PLD1 and its metabolite PA are required for hVps34 signals to mTORC1 in response to amino acids, and this pathway is in parallel to the activation of Rag GTPases (Yoon et al. 2011, Xu et al. 2011). Nutrient input is also able to enhance the activity of PLD1 via RalA (Ras-like protein A), a member of the Ras family of GTPases which stimulates PLD through increasing the recruitment of the PLD activator Arf6 to PLD (Xu et al. 2011). Furthermore, amino acids suppress the inhibitory effect of $PP2A_{T61\varepsilon}$ [protein phosphatase 2A (PP2A) oligomer containing the PR61ε-targeting subunit] towards MAP4K3 [mitogen-activated protein kinase kinase kinase kinase 3, also referred to as GLK (germinal centre-like kinase)], upon being released from $PP2A_{T61\varepsilon}$, MAP4K3 in turn activates mTORC1 indirectly via RagC/D by a yet unknown mechanism (Yan et al. 2010).

Furthermore, IPMK (inositol polyphosphate multikinase) (Kim et al. 2011) and v-ATPase (vacuolar H^+-ATPase) (Zoncu et al. 2011, Pena-Llopis et al. 2011) have also been reported to impact on amino acids signalling to mTORC1 (Fig. 1.2a). The regulation of amino acids signalling to mTORC1 by IPMK is independent of its catalytic function but relies on its ability in maintaining mTORC1 complex integrity (Kim et al. 2011). In the model proposed by Zoncu *et al*., Amino acids-induced mTORC1 activation begins with the accumulation of amino acids inside the lysosomal lumen, and increases in intralysosomal amino acids provoke ATP hydrolysis and subsequent structural rerrangement of v-ATPase, which strengthen the interaction between v-ATPase and ragulator, facilitating the nucleotide loading of Rag GTPases, and ultimately promote mTORC1 recruitment to the lysosomes which results in its activation (Zoncu et al. 2011). Interestingly, mTORC1 also up-regulates the expression of v-ATPase through the phosphorylation [site(s) remain(s) to be determined], subsequent nuclear localization and inhibition of TFEB (transcription factor EB) (Pena-Llopis et al. 2011, Settembre et al. 2012), a master transcription factor for lysosomal biogenesis (Sardiello et al. 2009). TFEB and v-ATPase are essential for mTORC1-mediated endocytosis (Pena-Llopis et al. 2011).

1.2.3 Regulation of mTORC1 by energy status

mTORC1 also responds to changes in the energy status of the cell (Dennis et al. 2001). For example, glucose can stimulate mTORC1 through the inactivation of AMPK (Inoki, Zhu and Guan. 2003, Kimura et al. 2003) (Fig. 1.2a), which responds to changes in the cellular AMP:ATP ratio (for a historical review, see Hardie, Carling and Carlson. 1998).

AMPK suppresses mTORC1 activity via the phosphorylation of TSC2 on Thr1271 and Ser1387, which stimulates TSC1/2 GAP activity (Inoki, Zhu and Guan. 2003) (Fig. 1.2b). AMPK can also phosphorylate RAPTOR on sites that promote the inhibition of mTORC1 (Gwinn et al. 2008).

Biguanides such as metformin and phenformin are commonly used as antidiabetic drugs because of their inhibitory effect on hepatic gluconeogenesis and lipogenesis, and positive impact on insulin sensitivity and glucose uptake in peripheral tissues (for reviews, see Bailey and Turner. 1996, Buchanan and Xiang. 2010). Although their effect on glucolipogenesis is known to be mediated by the activation of AMPK (Zhou et al. 2001b, Musi et al. 2002, Hawley et al. 2002), it has recently been demonstrated that metformin suppresses mTORC1 in a Rag GTPase dependent yet AMPK independent fashion (Kalender et al. 2010). Metformin is also able to prevent tau phosphorylation dependent upon mTORC1 inhibition and PP2A activation, yet independently of AMPK (Kickstein et al. 2010). These observations has led to the use of biguanides as anti-cancer agents, which have been supported by promising clinical results (Gallagher and Leroith. 2011), and perhaps in the future, a treatment for Alzheimer's disease.

Recent studies have also revealed that energy status is able to regulate mTORC1 independently of AMPK. For example, a decrease in the oxygen levels can augment the expression of REDD1 (regulated in development and DNA damage responses 1) and this has been reported to activate TSC1/2 and inhibit mTORC1 (Brugarolas et al. 2004, Sofer et al. 2005). Cam *et al*. have also recently revealed that under hypoxic conditions, the

DNA damage sensor ATM (ataxia-telangiectasia mutated) (Savitsky et al. 1995) phosphorylates HIF1α (hypoxia-inducible factor 1α) on Ser696 which leads to its activation, subsequent induction of REDD1 and mTORC1 repression (Cam et al. 2010). Energy stress induced by glucose deprivation can also activate p38β MAPK and p38-regulated/activated kinase (PRAK), PRAK in turn phosphorylates Rheb on Ser130, impairs the nucleotide-binding activity of Rheb and suppresses mTORC1 stimulation (Zheng et al. 2011). In contrast, a high concentration of glucose can dissociate Rheb from its inhibitory binding protein, the glycolytic enzyme GAPDH (glyceraldehyde-3-phosphate dehydrogenase), and thereby promote mTORC1 activation (Lee et al. 2009) (Fig. 1.2a). On the other hand, because cellular processes controlled by mTORC1 are highly energy-demanding, mTORC1 inhibition upon energy deprivation is of great importance for the cells to maintain the balance between metabolic consumption and supply, which ultimately contributes to the cell survival rates during nutrient starvation (Choo et al. 2010).

1.2.4 Regulation of mTORC1 by oxidative stress

Reactive oxygen species (ROS) are implicated in the control of a number of cellular processes (D'Autreaux and Toledano. 2007), and elevated levels of ROS have been linked to many diseases such as vascular diseases (Drummond et al. 2011), neurodegenerative diseases (Barnham, Masters and Bush. 2004), diabetes (Tiganis. 2011) and cancer (Trachootham, Alexandre and Huang. 2009). Alexander and colleagues have discovered that an enhancement of ROS levels is able to negatively regulate mTORC1 via the activation of ATM-AMPK-TSC2 pathway (Alexander et al. 2010). Under oxidative stress,

ROS induces the phosphorylation of ATM at Ser1981 which leads to its activation (Alexander et al. 2010). ATM in turn stimulates the phosphorylation of the AMPK kinase LKB1 [liver kinase B1, also referred to as serine/threonine kinase 11 (STK11)] (Woods et al. 2003, Hawley et al. 2003) at Thr366, and thereby promotes the activity of LKB1 and AMPK to inhibit mTORC1 (Alexander et al. 2010). Another study has also shown that modulation of cellular redox potential using cystein oxidants or reducing agents regulates mTORC1 through TSC1/2 and Rheb (Yoshida et al. 2011) (Fig. 1.2a).

1.2.5 Regulation of mTORC1 by inflammation

Inflammation plays an important role in tumour progression [reviewed in (Balkwill. 2009, Chechlinska, Kowalewska and Nowak. 2010, Ben-Neriah and Karin. 2011)] and insulin resistance [reviewed in (Hotamisligil and Erbay. 2008, Eizirik, Colli and Ortis. 2009, Donath and Shoelson. 2011)]. In prostate cancer cells lacking the tumour suppressor PTEN, the inflammation mediated NF-κB (nuclear factor κ-light-chain-enhancer of activated B cells) pathway is activated and elicits mTORC1 stimulation via PKB-dependent binding of IKKα [inhibitor of NF-κB kinase subunit α, also referred to as IKK1 or CHUK (conserved helix-loop-helix ubiquitous kinase)] to mTORC1 (Dan, Adli and Baldwin. 2007), while as a positive feedback, PKB mediated mTORC1 activation also up-regulates the activity of NF-κB dependent upon the interaction between IKK and RAPTOR (Dan et al. 2008). TNFα (tumour necreosis factor α, also known as cachexin or cachectin), a proinflammatory cytokine secreted by tumour-associated macrophages, can also activate the IKK complex and induce IKKβ-mediated TSC1 phosphorylation on Ser487 and Ser511 (Fig. 1.2a) (Lee et al. 2007), the phosphorylation of TSC1 relieves

mTORC1 from the inhibitory effect of TSC1/2 and consequently promotes tumour angiogenesis (Lee et al. 2007). High expression of phosphorylated IKKβ (Ser181) and TSC1 (Ser511) have also been found in high-fat diet/obese mouse models with insulin resistance, characterized by decreased IRS phosphorylations (Ser307, Ser636 and Ser639) (Lee et al. 2008). This observation implies that nutrient overload may link inflammation to chronic mTORC1 activation via IKKβ-TSC1 pathway, which results in the development of insulin resistance. In agreement with this, the deletion of NF-κB1 (p50) from mice leads to a reduction of IKKβ activity, decreased S6K1 expression and improved insulin sensitivity (Gao et al. 2009).

1.2.6 Regulation of mTORC1 by the Wnt pathway

Wnt (for wingless, integration) family of glycoproteins are cysteine-rich signalling molecules that bind to the Frizzled/LRP (low-density-lipoprotein-related protein) family of receptors via Dvl (disheveled) or axin, respectively. Wnt/Frizzled/LRP signalling pathway regulates the expression of the versatile transcription factor β-catenin to control many crucial aspects of the cell, especially during the developmental process (for reviews, see Moon et al. 2004, Staal, Luis and Tiemessen. 2008, Klaus and Birchmeier. 2008). The link between the Wnt pathway and mTOR was first established when Guan's lab showed that Wnt pathway activation inhibits GSK3 (glycogen synthase kinase 3), and therefore prevents the phosphorylation (on Ser1371, Ser1375, Ser1379 and Ser1383, see Fig. 1.2b) and stimulation of TSC2 by GSK3, resulting in the activation of mTORC1 (Inoki et al. 2006). The Wnt/GSK3/mTORC1 pathway plays many roles in a variety of tissues and diseases, for example, it regulates embryonic (Heo, Lee and Han. 2008) and epidermal

(Castilho et al. 2009) stem cell proliferation. In cardiomyocytes, activation of the Wnt/GSK3/mTOR pathway is required for ischemic preconditioning (Vigneron et al. 2011). In neurons, it is essential for the persistent phase of long-term potentiation and synaptic stimulation (Ma et al. 2011). And importantly, increasing evidence suggests that the Wnt/GSK3/mTOR pathway contributes to tumour development (Sun and Jin. 2008, Gazitt et al. 2009, Ahmad et al. 2011, Morton, Myant and Sansom. 2011, Tanwar et al. 2011). Another recent study has also revealed that the non-canonical Wnt7a/Frizzled7 pathway induces myocyte hypertrophy via the PI3K/PKB/mTORC1 pathway (von Maltzahn, Bentzinger and Rudnicki. 2011). As a feedback mechanism, the induction of autophagy, which is negatively regulated by mTORC1 (see section 1.4.2), can attenuate Wnt signalling via promoting the degradation of Dvl2 (Gao et al. 2010).

1.2.7 Regulation of mTOR by tumour suppressors

The mTOR pathway plays a key role in the proliferation, survival and metastasis of tumour cells. The loss of tumour suppressors often results in over-activation of mTOR and therefore effective mTOR inhibition has been the subject of intensive research in the cancer field. As discussed above, loss of function in TSC1/2, LKB1, PTEN and MAPK pathway components, which are characteristic in a variety of cancers, all lead to abnormal over-stimulation of mTOR (for reviews, see Sparks and Guertin. 2010, Zoncu, Efeyan and Sabatini. 2011). Two other examples of tumour suppressors that inhibit mTOR are PML (promyelocytic leukaemia) and NF1/2 (neurofibromatosis-1/2 or neurofibromin 1/2). PML directly associates with mTOR and prevents Rheb binding, and therefore negatively regulates the mTORC1-HIF1α pathway, resulting in the down-regulation of

genes implicated in angiogenesis (Bernardi et al. 2006). NF1 is first known as a GAP for Ras and accordingly Ras is aberrantly stimulated in NF1-deficent cells (Martin et al. 1990). Nonetheless, recent studies have suggested that mTOR is essential for tumorigenesis upon the loss of NF1 (Dasgupta et al. 2005, Johannessen et al. 2005, Yin et al. 2006, Johannessen et al. 2008, Lee da et al. 2010, Banerjee et al. 2010, Banerjee et al. 2011) or NF2 (merlin) (James et al. 2009). NF1 deficiency in fibroblasts causes dual-activation of both Ras-MAPK and PI3K-PKB pathways, which impinge on TSC2 to stimulate mTORC1 (Johannessen et al. 2005). However, astrocyte growth and glioma formation induced by the ablation of NF1 are TSC2/Rheb independent and likely to be mediated by mTORC2 (Lee da et al. 2010, Banerjee et al. 2011), which implies that NF1 may trigger distinct signalling pathways in different cell types to promote tumour progression. NF2 negatively regulates mTORC1 independently of either PI3K/PKB/TSC1/2 or MAPK/TSC1/2 pathways (James et al. 2009), and the exact mechanism remains to be established.

1.2.8 Regulation of mTORC2

mTORC2 has originally been identified as a positive regulator of actin cytoskeletal organization, polarization and cell migration (Sarbassov et al. 2004, Jacinto et al. 2004, Gulati et al. 2009, Liu et al. 2010). The mechanism by which mTORC2 is regulated is not fully understood. Initially it was thought that mTORC2 was not activated by hormones and growth factors. Nonetheless, it has recently been demonstrated that mTORC2 activity towards PKB on S473 can be acutely stimulated by insulin, serum (Gan et al. 2011) and amino acids (Tato et al. 2010) in a PI3K-dependent manner (Fig. 1.3), yet the mechanism

by which PI3K activates mTORC2 is far from understood, and may just be through the PIP3-dependent translocation of PKB to mTORC2 at the plasma membrane. However, mTORC2 stimulation is insensitive to S6K-IRS-PI3K negative feedback loop (NFL) which inhibits mTORC1 (Dalle Pezze et al. 2012), therefore, it was proposed that mTORC2 is activated by PI3K insensitive to NFL (Dalle Pezze et al. 2012). It has also been found that PLD and its metabolite PA are of importance to the complex assembly and activation of both mTORCs (Toschi et al. 2009) (Fig. 1.3), and mTORCs became more sensitive to rapamycin when PA were depleted from cells, implying a competition between PA and rapamycin binding to mTOR (Toschi et al. 2009). In contrast, PA derived from the glycerol-3-phosphate pathway impairs mTORC2 activity and insulin signalling (Zhang et al. 2012), this apparent paradox is most likely due to the structural and functional differences between PA obtained from glycerolipid synthesis and membrane hydrolysis (Zhang et al. 2012). Of note, siRNA (small interfering RNA)-mediated knockdown of proteins implicated in ribosome maturation and formation also abrogates mTORC2 stimulation (Fig. 1.3), indicating that ribosomes may bind to and promote mTORC2 activity (Zinzalla et al. 2011). In endothelial cells, growth factors recruit mTORC2 to lipid rafts containing transmembrane protein syndecan-4 and PKCα (protein kinase C α), syndecan-4 knockout ($S4^{-/-}$) or PKCα knockdown using shRNA (small hairpin RNA) in endothelial cells impairs mTORC2 activity, and this can be rescued upon the addition of myristoylated PKCα (Partovian et al. 2008). Furthermore, Ryh1 GTPase, a human Rab6 ortholog, physically interacts and activates yeast TORC2 (Tatebe et al. 2010), although it remains to be investigated whether Rab6 plays a role in

mTORC2 activation in mammalian cells. And finally, as mentioned in section 1.2.1, Rhes-GTP also positively regulates mTORC2 (Subramaniam et al. 2011).

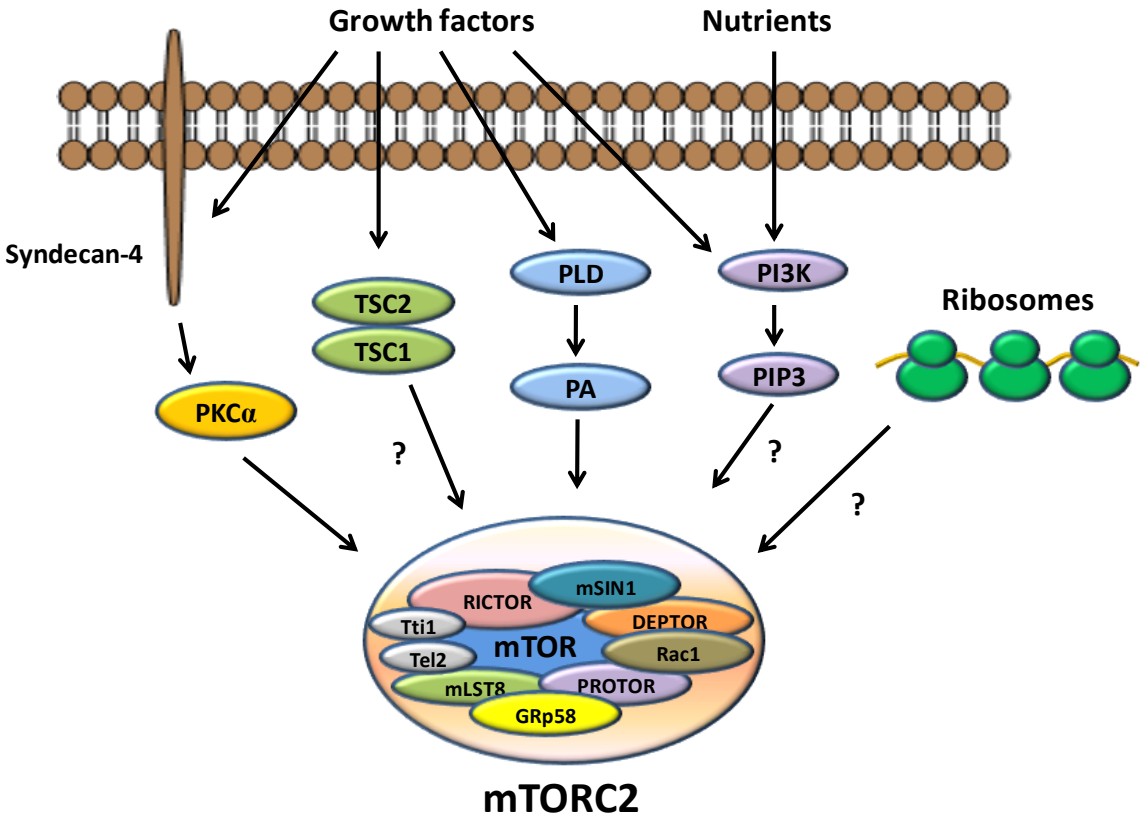


Figure 1.3 Upstream pathways regulating mTORC2. mTORC2 components include: mTOR, RICTOR (Sarbassov et al. 2004), mSIN1 (Jacinto et al. 2006, Frias et al. 2006, Yang et al. 2006), PROTOR (Pearce et al. 2007), mLST8 (Kim et al. 2003), DEPTOR (Peterson et al. 2009), Tti1, Tel2 (Kaizuka et al. 2010, Takai et al. 2010), GRp58 (Ramirez-Rangel et al. 2011) and Rac1 (Saci, Cantley and Carpenter. 2011). It has been recently shown that mTORC2 can be acutely activated by growth factors (Gan et al. 2011) and nutrients (Tato et al. 2010) dependent upon the stimulation of PI3K and the production of PIP3. On the other hand, TSC1/2 (Huang et al. 2008), PLD and its metabolite PA (phosphatidic acid) (Toschi et al. 2009), syndecan-4 and PKCα (Partovian et al. 2008), and proteins implicated in ribosome maturation

(Zinzalla et al. 2011) have all been shown to be implicated in the activation of mTORC2. The exact mechanism of how these pathways exert their effect on mTORC2 awaits further studies.

Interestingly, mTORC1 and 2 can also be differentially regulated in the opposite way by the same upstream signal. For example, TSC1/2 is suppressive to mTORC1 (Inoki et al. 2002, Tee et al. 2002, Gao et al. 2002, Goncharova et al. 2002), yet it associates with mTORC2 and is essential for mTORC2 activation (Huang et al. 2008), although this model has been challenged by a recent study (Dalle Pezze et al. 2012). FoxO1 (forkhead box protein O1) or FoxO3a depleted MEFs have elevated mTORC1 activity whereas mTORC2 is inhibited (Chen et al. 2010), this phenomenon is likely to be caused by the competition between RAPTOR and RICTOR for mTOR binding, and therefore the disruption of one of the mTOR complexes facilitates the formation of the other (Sarbassov et al. 2004).

1.3 Downstream effectors of mTOR

1.3.1 mTORC1 downstream targets

S6K (Chen et al. 1995, Chung et al. 1992) and 4EBPs (Brunn et al. 1997, Gingras et al. 1999) are two of the best characterized mTORC1 downstream targets. Two homologous S6Ks (S6K1 and S6K2) with a high level of homology were discovered in mammalian cells (Gout et al. 1998). Due to alternative splicing and translation, the S6K1 gene encodes four different isoforms: p85S6K1, p70S6K1, p60S6K1 (Kim et al. 2009) and p31S6K1 (Karni et al. 2007, Rosner and Hengstschlager. 2011). The S6K2 gene encodes

two isoforms (p54S6K2 and p56S6K2) which are primarily nuclear (Lee-Fruman et al. 1999). The activity of S6K is regulated by sequential phosphorylation events that occur in a stepwise order (Fig. 1.4, also see Magnuson, Ekim and Fingar. 2012). At least seven phosphorylation sites are of importance to the kinase activity of S6K. Ser371 (amino acid residue numbers are for human p70S6K1), the turn motif phosphorylation site, is the first residue to be phosphorylated, and it has recently been found to be essential for kinase activity (Hauge et al. 2007), and that GSK3β is the kinase responsible for its phosphorylation (Shin et al. 2011). Ser411, Ser418, Thr421 and Ser424, four residues located in the *C*-terminal auto-inhibitory domain (AID) of p70S6K1, are the first to be phosphorylated upon mitogenic stimulation in order to release the kinase domain from the inhibitory binding of AID (Ferrari et al. 1992). It was believed that Ser371 serves as a priming site for the subsequent hydrophobic motif (Thr389) phosphorylation by mTORC1 (Burnett et al. 1998). This provides a docking site for PDK1 to phosphorylate Thr229 (Pullen et al. 1998) located on the activation loop, and leads to the full activation of p70S6K1. However, other earlier studies (Weng et al. 1998, Alessi et al. 1998, Williams et al. 2000) and more recent evidence (Keshwani et al. 2011) suggests that the phosphorylation of Thr229 occurs before Thr389 phosphorylation (Fig. 1.4, also see Magnuson, Ekim and Fingar. 2012). Protein kinases responsible for the four AID phosphorylation sites have not yet been fully established, although cell cycle regulators, such as the cdc2 [cell division control protein 2, also known as cyclin dependent kinase 1 (CDK1)]-cyclin B complex, can phosphorylate these sites *in vitro* (Mukhopadhyay et al. 1992, Papst et al. 1998).

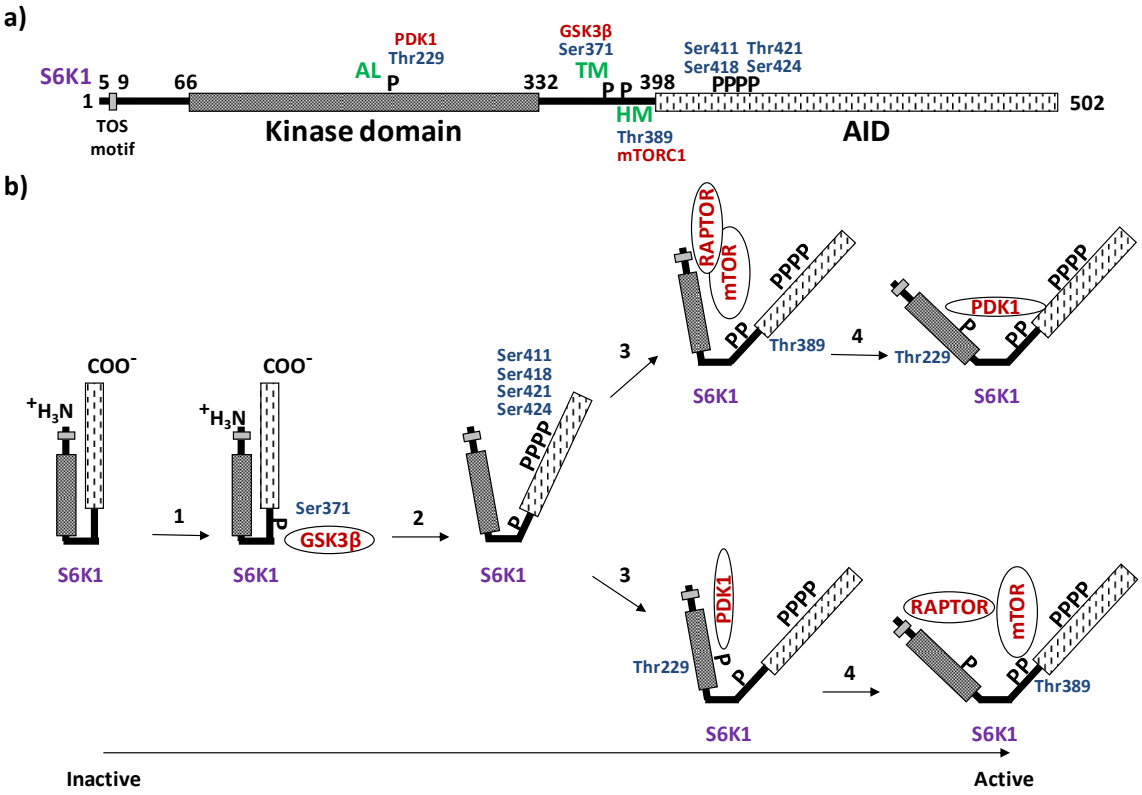


Figure 1.4 Protein structure and the activation of S6K1 by phosphorylation. **a**) S6K1 has a TOS (TOR signalling) motif (Schalm and Blenis. 2002), a kinase domain and a *C*-terminal AID (auto-inhibitory domain). Phosphorylation sites and their respective known protein kinases have been indicated. AL: activation loop; TM: turn motif; HM: hydrophobic motif. Only the protein structure of the p70 isoform is shown here. **b**) Phosphorylation of S6K occurs in an hierarchical fashion (Hannan, Thomas and Pearson. 2003): 1. Phosphorylation of Ser371 (TM) by GSK3β (Shin et al. 2011); 2. Phosphorylation of four serine residues within the AID in response to mitogenic stimuli (Ferrari et al. 1992); 3. Phosphorylation of Thr389 (HM) by mTORC1 (Burnett et al. 1998); 4. Phosphorylation of Thr229 (AL) by PDK1 (Pullen et al. 1998, Alessi et al. 1998). Alternatively, other studies have suggested that the phosphorylation of AL occurs before HM phosphorylation (Weng et al. 1998, Alessi et al. 1998, Williams et al. 2000, Keshwani et al. 2011).

rpS6 is the direct downstream target phosphorylated by both S6K1 and S6K2 (Banerjee et al. 1990, Kozma et al. 1990), predominantly S6K2 (Pende et al. 2004) (Fig. 1.5a). S6K1/2 are able to phosphorylate rpS6 at Ser235, Ser236, Ser240 and Ser244 (Banerjee et al. 1990, Kozma et al. 1990). Three additional *in vivo* rpS6 kinases have since been identified including RSK (Roux et al. 2007) (Ser235/Ser236), PKA (Moore et al. 2009, Valjent et al. 2011) (Ser235/Ser236) and CK1 (Ser247) (Hutchinson et al. 2011). The phosphorylation on Ser235/Ser236 primes for further phosphorylation on Ser240/Ser244, and the latter is required for the phosphorylation of Ser247, while Ser240/Ser244 phosphorylation can also be enhanced by the phosphoryaltion of Ser247 (Hutchinson et al. 2011).

Ruvinsky *et al*. have developed non-phosphorylable rpS6 knock-in ($rpS6^{P-/-}$) mice in which all these five serine residues are substituted by alanine, these mice have a reduction in pancreatic β-cell size and impaired glucose homeostasis (Ruvinsky et al. 2005), $rpS6^{P-/-}$ mice also have smaller myofibers, less muscle strength and deficit in muscle energy storage (Ruvinsky et al. 2009).

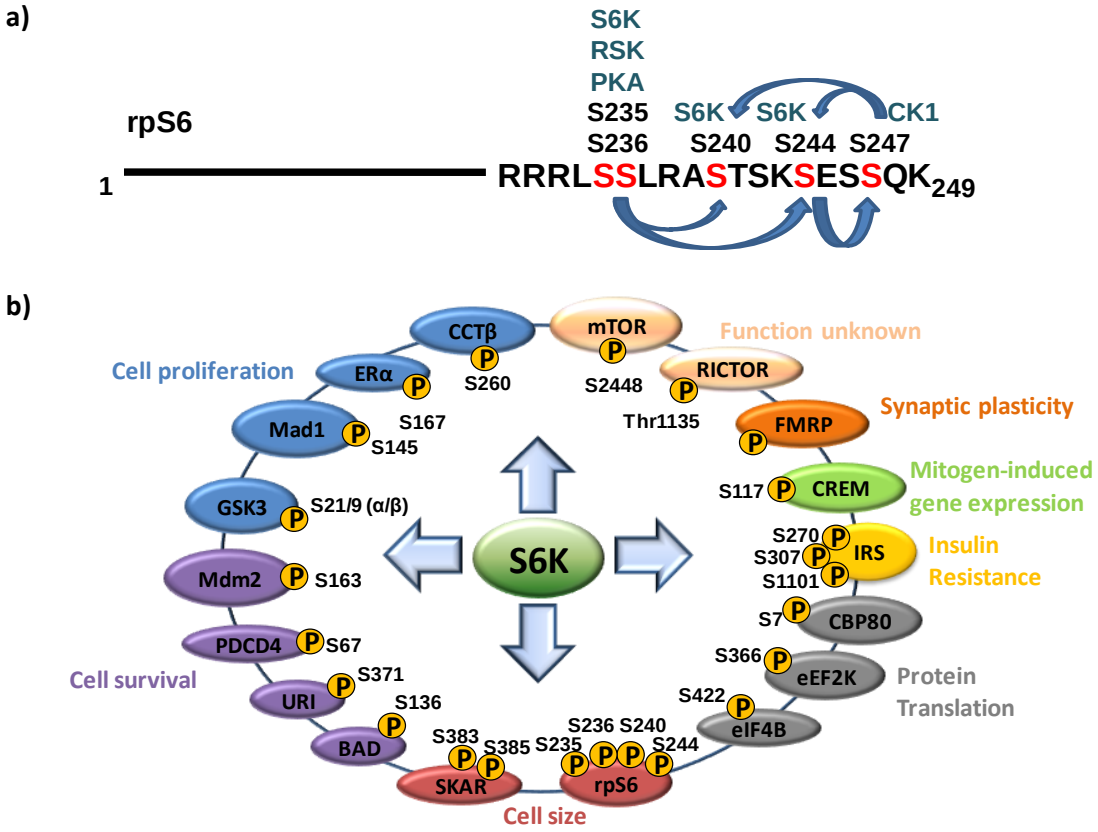


Figure 1.5 Protein substrates downstream of S6K. **a**) Phosphorylation of rpS6. The phosphorylation of Ser235/Ser236 promotes Ser240/Ser244 and Ser247 phosphorylation, while Ser247 phosphorylation also augments the phosphorylation of Ser240/Ser244. Known Ser235/Ser236 kinases: S6K (Banerjee et al. 1990, Kozma et al. 1990), RSK (Roux et al. 2007) and PKA (Moore et al. 2009, Valjent et al. 2011); Ser240/Ser244 kinase: S6K (Banerjee et al. 1990, Kozma et al. 1990); Ser247 kinase: CK1 (Hutchinson et al. 2011). **b**) S6K direct targets and their respective S6K phosphorylation sites and physiological functions: CCTβ (Abe et al. 2009), ERα (Yamnik et al. 2009), Mad1 (Zhu, Blenis and Yuan. 2008), GSK3 (Zhang et al. 2006) (blue: proliferation); Mdm2 (Lai et al. 2010), Pdcd4 (Carayol et al. 2008), URI (Djouder et al. 2007), BAD (Harada et al. 2001) (purple: survival); SKAR (Richardson et al. 2004), rpS6 (Banerjee et al. 1990, Kozma et al. 1990) (red: cell size); eIF4B (Raught et al. 2004), eEF2K (Wang et al. 2001, Browne and Proud. 2004), CBP80 (Wilson, Wu and Cerione. 2000) (grey: protein synthesis); IRS (Harrington et al. 2004, Tremblay et al. 2007, Zhang et al. 2008) (yellow: insulin resistance); CREM (de Groot, Ballou and

Sassone-Corsi. 1994) (green: gene expression); FMRP (Narayanan et al. 2008) (orange: synaptic plasticity). The function of mTOR Ser2448 (Holz and Blenis. 2005, Chiang and Abraham. 2005) and RICTOR Thr1135 (Dibble, Asara and Manning. 2009, Julien et al. 2010, Treins et al. 2010, Boulbes et al. 2010) phosphorylation awaits further study.

At least eighteen S6K direct downstream targets have been discovered so far, and they are regulated by S6Ks through series of phosphorylation events (Fig. 1.5b). These substrates and their respective S6K phosphorylation sites are: FMRP (fragile X mental retardation protein) [site(s) remain(s) to be identified] (Narayanan et al. 2008), CREM [cAMP (cyclic AMP)-response element modulator] (Ser117) (de Groot, Ballou and Sassone-Corsi. 1994), BAD [Bcl-2 (B-cell lymphoma 2)-associated death promoter] (Ser136) (Harada et al. 2001), Mdm2 (murine double minute 2) (Ser163) (Lai et al. 2010), SKAR [S6K1 Aly/REF-like target, also termed as polymerase (DNA-directed), delta interacting protein 3 or POLDIP3] (Ser383 and Ser385) (Richardson et al. 2004), eIF4B (Ser422) (Raught et al. 2004), Pdcd4 (programmed cell death protein 4) (Ser67) (Carayol et al. 2008), eEF2K [eEF2 (eukaryotic elongation factor 2) kinase] (Ser366) (Wang et al. 2001, Browne and Proud. 2004), IRS-1/2 (insulin receptor substrate 1 and 2) [Ser270 (Zhang et al. 2008), Ser307 (Harrington et al. 2004) and Ser1101 (Tremblay et al. 2007) for IRS-1] (Harrington et al. 2004, Tremblay et al. 2007, Tremblay and Marette. 2001, Um et al. 2004, Shah, Wang and Hunter. 2004), CBP80 [for 80 KDa nuclear cap-binding protein, also known as nuclear cap binding protein subunit 1 (NCBP1)] (Ser7) (Wilson, Wu and Cerione. 2000), URI [unconventional prefoldin RPB5 (retinol binding protein 5) interactor] (Ser371) (Djouder et al. 2007), GSK3 (α: Ser21; β: Ser9) (Zhang et al. 2006), ERα (Ser167) (Yamnik et al. 2009), CCTβ [chaperonin containing TCP (T-

complex protein)-1] (Ser260) (Abe et al. 2009), Mad1 (mitotic arrest deficient 1) (Ser145) (Zhu, Blenis and Yuan. 2008), RICTOR (Thr1135) (Dibble, Asara and Manning. 2009, Julien et al. 2010, Treins et al. 2010, Boulbes et al. 2010) and mTOR itself (Ser2448) (Holz and Blenis. 2005, Chiang and Abraham. 2005). Therefore, S6Ks are able to control a diversity of cellular processes, including protein synthesis, cell growth, replication, survival, insulin sensitivity, synaptic plasticity and gene expression, through the regulation of these substrates (Fig. 1.5b, also see Magnuson, Ekim and Fingar. 2012, Ruvinsky and Meyuhas. 2006, Jastrzebski et al. 2007).

4EBPs are translational repressors which compete with eIF4G for binding to eIF4E and thereby disrupt the formation of translation initiation complex (Haghighat et al. 1995). The family of 4EBPs consists of three isoforms: 4EBP1, 4EBP2 and 4EBP3 (Poulin et al. 1998). Upon phosphorylation by mTORC1, 4EBPs release eIF4E and allow the latter to bind eIF4G, resulting in eIF4F complex assembly on the 5' cap structure of mRNA (Brunn et al. 1997, Gingras et al. 1999, Yang, Brunn and Lawrence. 1999, Mothe-Satney et al. 2000, Gingras et al. 2001) (also see section 1.4.1). Like S6Ks, 4EBPs have a *C*-terminal TOS motif, they also contain an *N*-terminal RAIP (letters of amino acids 13-16) motif (absent in 4EBP3) which is required for the phosphorylation of 4EBPs and their release from eIF4E (Tee and Proud. 2002) (Fig. 1.6), because both the TOS motif and RAIP domain of 4EBPs are necessary for the recognition of RAPTOR through the interaction with its RNC domains (Dunlop et al. 2009). Four phosphoryalation sites of 4EBP1 are known to be regulated by extracellular stimuli (Gingras et al. 1999, Gingras et al. 2001) (Fig. 1.6): Thr37/Thr46 are phosphorylated by mTOR (Gingras et al. 1999) and

controlled by the availability of amino acids (Wang et al. 2005), yet they are rapamycin resistant (Gingras et al. 1999), although high dose of rapamycin (μM range) does inhibit Thr37/Thr46 phosphorylation (Yellen et al. 2011); Ser65 (Ser64 in MEFs)/Thr70 phosphorylation are insulin stimulated and rapamycin sensitive (Mothe-Satney et al. 2000, Gingras et al. 2001, Wang et al. 2005), although they are unlikely to be directly phosphorylated by mTOR itself (Wang et al. 2005). Phosphorylation of all these sites and Ser82, a fifth non-regulated constitutive phosphorylation site, is implicated in mRNA translation by influencing 4EBP-eIF4E binding (Mothe-Satney et al. 2000). Gingras *et al*. have shown that phosphorylation of 4EBPs also follows a hierarchical order (Fig. 1.6): Thr37/Thr46 (amino acid sensitive) first, followed by Thr70 and finally Ser65 (mitogen sensitive) (Gingras et al. 2001), although an earlier study from Lawrence's group suggested that Thr70 can still be phosphorylated in the absence of Thr37/Thr46 phosphorylation (Mothe-Satney et al. 2000).

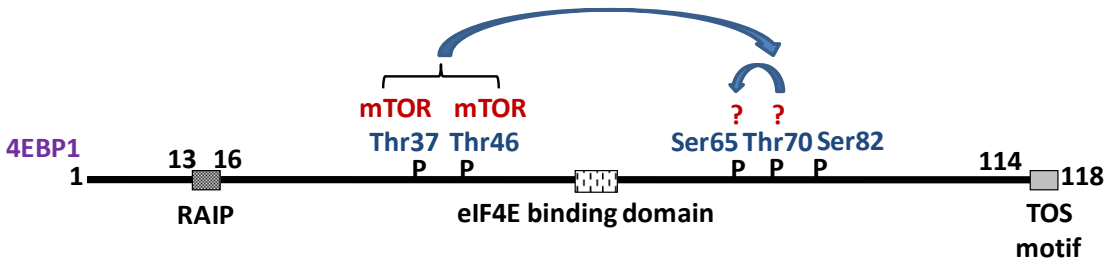


Figure 1.6 Protein structure and phosphorylation sites of 4EBP1. 4EBP1 has an *N*-terminal RAIP domain (Tee and Proud. 2002), an eIF4E binding domain and a *C*-terminal TOS motif (Schalm and Blenis. 2002). It can be phosphorylated on Thr37/Thr46 by mTOR in response to amino acids (Gingras et al. 1999, Wang et al. 2005), and Ser64/Thr70 in response to mitogenic stimuli (Gingras et al. 2001, Wang et al. 2005). Kinase(s) responsible for Ser65/Thr70 phosphorylation is (are) currently unknown. Ser82 is a non-

regulated constitutive phosphorylation site (Mothe-Satney et al. 2000). It has been shown that the phosphorylation of Thr37/Thr46 occurs first, followed by Thr70 and then Ser64 (Gingras et al. 2001).

Besides S6Ks and 4EBPs, a large number of mTORC1 downstream targets have emerged during the last decade (Fig. 1.7). It was discovered that mTORC1 controls the transcription of genes induced by cytokines and oxidative stress, through the regulation of STAT3 (Yokogami et al. 2000) and HIF1α (Zhong et al. 2000, Hudson et al. 2002), respectively, and this has great implications in human diseases such as cancer. Maximal activation of STAT3 (signal transducers and activators of transcription 3) requires its phosphorylation on both Tyr705 by JAKs (Janus kinases) (Darnell, Kerr and Stark. 1994), and Ser727 by mTOR (Yokogami et al. 2000) (Fig. 1.7). STAT3 links mTORC1 to Notch signalling cascade in order to maintain the balance between cell proliferation and differentiation in cancer cells. STAT3 Ser727 phosphorylation is also implicated in the induction of malignant peripheral nerve sheath tumour growth cause by NF-1 deficiency (Banerjee et al. 2010). Therefore, the stimulation of STAT3 provides an explanation to how over-activation of mTOR pathway leads to tumorigenesis (Ma et al. 2010). Also important to mention is that HIF1, the functional heterodimer formed by HIF1α and β, is able to negatively regulate mTORC1 using feedback mechanisms through increasing the expression of mTORC1 upstream inhibitors REDD1 (Wolff et al. 2011, Brugarolas et al. 2004, Sofer et al. 2005, Jin et al. 2011, also see section 1.2.3) and BNIP3 (Bcl2/adenovirus E1B 19-kD protein-interacting protein 3) (Li et al. 2007), the latter directly associates with Rheb to prevent its GTP loading and thereby inhibits mTORC1 (Li et al. 2007).

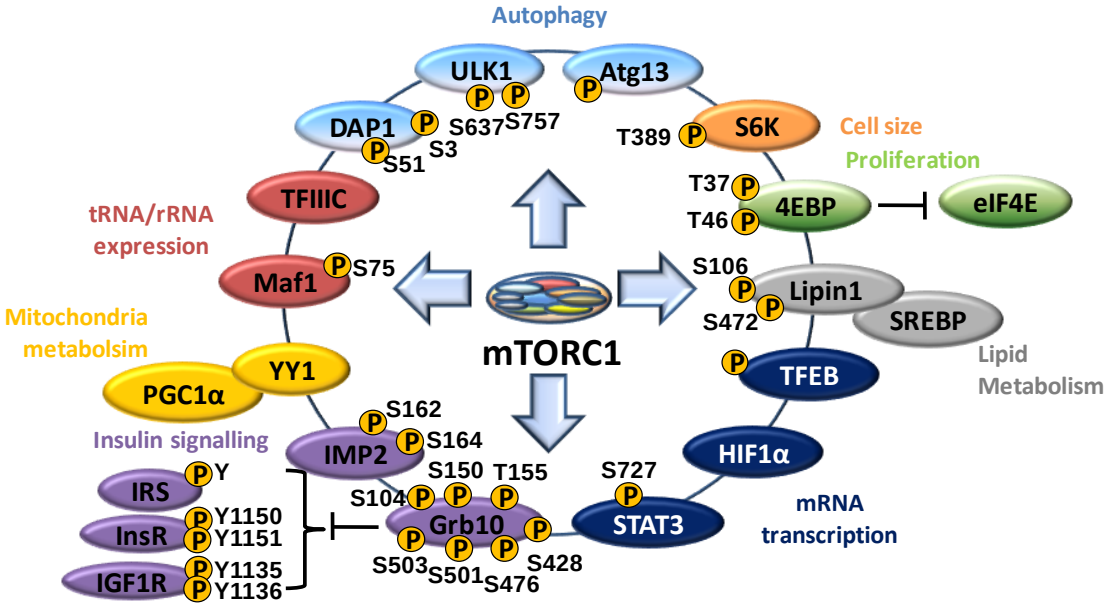


Figure 1.7 Schematic representation of mTORC1 downstream targets. It has been shown that mTORC1 is able to control a large number of proteins through phosphorylation to regulate diverse cellular processes, these include: S6K (Chen et al. 1995, Chung et al. 1992) (orange: cell size); 4EBP (Brunn et al. 1997) (green: proliferation); lipin1/SREBP (Porstmann et al. 2008, Peterson et al. 2011) (grey: lipid metabolism); DAP1 (Koren, Reem and Kimchi. 2010), ULK1 (Jung et al. 2009, Hosokawa et al. 2009, Ganley et al. 2009), ATG13 (Jung et al. 2009, Hosokawa et al. 2009, Ganley et al. 2009) (light blue: autophagy); TFIIIC/Maf1 (Shor et al. 2010, Kantidakis et al. 2010) (dark red: tRNA/rRNA expression); YY1/PGC1 (Cunningham et al. 2007) (yellow: mitochondria metabolism); IMP2 (Dai et al. 2011), Grb10/IRS/InsR/IGF1R (Hsu et al. 2011, Yu et al. 2011) (purple: insulin signalling); STAT3 (Yokogami et al. 2000), HIF1α (Zhong et al. 2000, Hudson et al. 2002), TFEB (Pena-Llopis et al. 2011) (dark blue: mRNA expression).

PI3K-PKB-mTORC1 is a classical insulin/IGF (insulin-like growth factor)-driven signalling pathway which regulates a variety of cellular functions (see section 1.2.1), interestingly, stimulation of mTORC1 also controls various steps of the insulin/IGF

signalling cascade via a series of feedback mechanisms (Fig. 1.7). For instance, mTOR phosphorylates IMP2 (IGF2 mRNA-binding protein 2), the only IMP expressed in β-cells and insulin responsive tissues after birth, at Ser162 and Ser164, which promotes the binding of IMP2 to IGF2 mRNA and enhances its translation by internal ribosomal entry (Dai et al. 2011). Grb10 (growth factor bound-receptor protein 10), a novel mTORC1 substrate identified by Sabatini's (Hsu et al. 2011) and Blenis' (Yu et al. 2011) groups, is a negative regulator of insulin/IGF pathway because it inhibits the autophosphorylation of InsR (insulin receptor) (Tyr1150/Tyr1151)/IGF1R (IGF1 receptor) (Tyr1135/Tyr1136), tyrosine phosphorylation of IRS1/2, and the recruitment of PI3K to IRS (Hsu et al. 2011). Grb10 can be activated by mTORC1 upon direct phosphorylation (Hsu et al. 2011, Yu et al. 2011), on at least seven phosphorylation sites [Sabatini's group (five sites): Ser104, Ser150, Thr155, Ser428, Ser476 (Hsu et al. 2011); Blenis' group (two sites): Ser501, Ser503 (Yu et al. 2011)] of Grb10 have been found to be phosphorylated by mTORC1 *in vitro* or sensitive to the treatment of mTOR inhibitors *in vivo*, the phosphorylation of these sites stabilizes Grb10 which in turn destabilizes IRS (Hsu et al. 2011, Yu et al. 2011). The discovery of Grb10 as a mTORC1 substrate (Hsu et al. 2011, Yu et al. 2011) and IRS as a S6K substrate (Harrington et al. 2004, Tremblay et al. 2007, Tremblay and Marette. 2001, Um et al. 2004, Shah, Wang and Hunter. 2004) are of great importance to the understanding of type 2 diabetes mellitus (T2DM), as abnormal insulin sensing is one of the major characteristics to the development of insulin resistance. It is believed that nutrient overload, as in the case of obese individuals, lead to over-stimulation of mTORC1 and subsequent down-regulation of insulin signalling pathways, which brings

detrimental effects to the maintenance of pancreatic β-cell mass (Tremblay et al. 2007, Um et al. 2004, Elghazi et al. 2010).

The regulation of mTORC1 downstream targets involved in rRNA/tRNA expression (section 1.4.1), autophagy (section 1.4.2), mitochondria and lipid metabolism (section 1.4.4) will be discussed in section 1.4. Recently, more protein substrates of S6K1 (GRP75, CCTβ, PGK1, RACK1) and mTORC1 (ANXA4, PSMA6) have been identified using a phospho-proteomic screen (Jastrzebski et al. 2011), albeit their roles remain to be investigated. Furthermore, oncogenic mTOR signalling, through 4EBP-eIF4E, controls the expression of many genes implicated in proliferation and metastasis (Hsieh et al. 2012).

1.3.2 mTORC2 downstream targets

mTORC2 is responsible for the phosphorylation and activation of several AGC kinases, including PKB (Jacinto et al. 2006, Frias et al. 2006, Sarbassov et al. 2005), SGK1 (Garcia-Martinez and Alessi. 2008) [n.b. an earlier study identified SGK1 as an mTORC1 target (Hong et al. 2008a)], cPKCs (conventional PKC, including α, βI, βII and γ) and ε, a nPKC (novel PKC) (Sarbassov et al. 2004, Ikenoue et al. 2008, Facchinetti et al. 2008) (Fig. 1.8). mTORC2 does not regulate aPKCs (atypical PKCs), however, in breast cancer cells, siRNA knockdown of RICTOR abolishes EGF-induced PKCζ (an atypical PKC isoform) phosphorylation on Thr410 and impairs cancer cell metastasis. Interestingly, PKCζ has been found to be associated with RICTOR but not mTOR upon EGF (epidermal growth factor) treatment (Zhang et al. 2010). This study (Zhang et al. 2010)

and the observation that RICTOR phosphorylation on Thr1135 does not require mTORC2 (Boulbes et al. 2010) strongly suggest that RICTOR has mTORC2-independent functions.

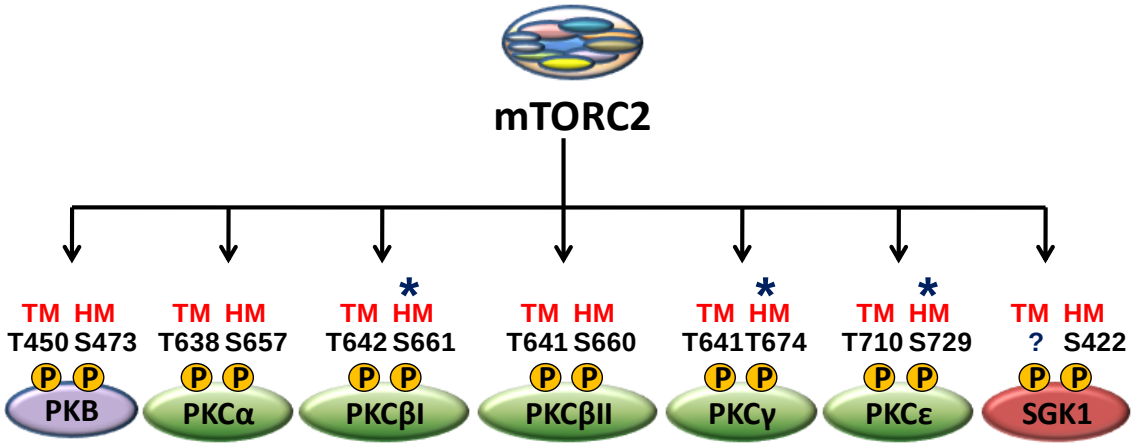


Figure 1.8 Downstream targets of mTORC2. mTORC2 is responsible for the TM (turn motif) and HM (hydrophobic motif) phosphorylation of PKB (Jacinto et al. 2006, Frias et al. 2006, Sarbassov et al. 2005), PKCα, βI, βII, γ, ε (Sarbassov et al. 2004, Ikenoue et al. 2008, Facchinetti et al. 2008) and SGK1 (Garcia-Martinez and Alessi. 2008). *: Numberings of these residues are according to protein alignment against the HM site of PKCα and PKCβII and remain to be confirmed by further experimentation. TM phosphorylation site of SGK1 is yet to be identified.

During synthesis, these AGC kinases are folded and stabilized upon phosphorylation of their turn motif (TM) by mTORC2. For the full stimulation, mTORC2 further phosphorylates sites within their hydrophobic motif (HM) (Ikenoue et al. 2008, Facchinetti et al. 2008). However, PKB HM can also be phosphorylated by other kinases such as DNA-PK (DNA-dependent protein kinase) (Feng et al. 2004, Bozulic et al. 2008) or TBK1 {TANK [TRAF (TNF receptor-associated factor) family member-associated

NF-κB activator]-binding kinase 1} (Xie et al. 2011b), and PKC can also undergo autophosphorylation on its HM (Behn-Krappa and Newton. 1999).

PKB is one of the best characterized mTORC2 downstream targets. It has been shown that mTORC2 associates with polysomes and phosphorylates nascent PKB polypeptide at Thr450, which allows PKB to escape from being ubiquitinated (Oh et al. 2010). Upon stimulation, PKB is translocated to the plasma membrane and is phosphorylated by PDK1 at Thr308 (activation-loop) (Alessi et al. 1997), and Ser473 (HM) by mTORC2 (Sarbassov et al. 2005). In addition, mTORC2 activation evokes negative feedback mechanisms resulting in the ubiquitination and degradation of SGK1 and PKB (Gao et al. 2010, Wu et al. 2011b). Given the fact that specific mTORC2 substrates can be activated upon stimulation of different upstream signals, it is plausible that mTORC2 achieves its specificity due to the existence of a variety of mSIN1 isoforms (Jacinto et al. 2006, Frias et al. 2006).

Although mTORC1 lies downstream of PKB (Gingras et al. 1998, Scott et al. 1998), it is believed that mTORC2 and PKB Ser473 phosphorylation do not contribute to the activation of mTORC1 because RICTOR or mSIN1 null cells have impaired PKB Ser473 phosphorylation yet the activation of mTORC1 downstream targets were unaffected (Jacinto et al. 2006, Frias et al. 2006, Guertin et al. 2006). Indeed, only a few PKB downstream targets including FoxO (Guertin et al. 2006), and in some cases (Case et al. 2011, Barlow et al. 2012) but not all (Guertin et al. 2006), GSK3β, has been shown to be controlled by PKB Ser473 phosphorylation. Therefore, it is plausible that Thr308 and

Ser473 phosphorylations determine PKB substrate specificity. However, in primary human fibroblasts, mTORC2 does lie upstream of PKB-TSC2-Rheb-mTORC1 and PKB-PRAS40-mTORC1 pathways to control cell size and G_1/S cell cycle progression (Rosner et al. 2009).

1.4 Physiological roles of mTOR

1.4.1 mTOR and mRNA translation

mTOR pathway is tightly linked with almost every step of mRNA translation starting from ribosome biogenesis. This is a highly coordinated multistep process involving the synthesis of around eighty ribosomal proteins (RP) and four ribosomal RNAs (rRNA), which is controlled by three nuclear RNA polymerases: PolI (rRNA synthesis except 5S rRNA), PolII (RP gene transcription) and PolIII (production of the 5S rRNA) (for reviews, see Connolly and Culver. 2009, Lempiainen and Shore. 2009, Cisterna and Biggiogera. 2010). mTOR pathway is able to regulate the transcriptional activity of all these three classes of RNA polymerases (Mayer and Grummt. 2006). In yeast, TOR is translocated into the nucleus in response to nutrient supply, and is associated with ribosomal DNA (rDNA) promoter chromatin to regulate the transcription of RP genes and 35S rRNA synthesis (Li et al. 2006). Recent studies have further revealed the molecular mechanisms by which mTOR regulates rRNA and tRNA expression in mammalian cells (Shor et al. 2010, Kantidakis et al. 2010, Wei and Zheng. 2009, Tsang, Liu and Zheng. 2010, Iadevaia et al. 2011). For example, mTOR also associates with TFIIIC (transcription factor 3C) which is a transcription factor for PolI and PolIII

(Kantidakis et al. 2010), this positioning allows mTOR to directly phosphorylate Maf1, a repressor of PolIII, at Ser75 and thereby abolishes the inhibitory effect of Maf1 {MFP1 [MAR (matrix attachment region) binding filament–like protein 1] associated factor 1} on PolIII transcription (Shor et al. 2010, Kantidakis et al. 2010). (Fig. 1.7). The regulation of RP/rRNA by mTOR is likely critical for the cell to meet the demand for global protein synthesis in response to rapid changes in extracellular nutrient supply.

The canonical mechanism of translation consists in three steps termed as initiation, elongation and termination (for reviews, see Proud. 2007, Sonenberg and Hinnebusch. 2009, Jackson, Hellen and Pestova. 2010). In eukaryotes, translation initiation is the rate-limiting phase of protein synthesis, which relies on a subset of highly organized protein-protein interactions between the eIFs (eukaryotic translation initiation factors), such as the recruitment of mRNA to the 43S preinitiation complex (comprised of 40S ribosome subunit, eIF2-GTP-Met-tRNA$_i$ ternary complex, eIF3 and the eIF2 specific GAP eIF5) which is controlled by the eIF4 group of factors. 4EBP-eIF4E bind to the m^{7}GTP (7-methylguanosine triphosphate)-containing "cap" structure of mRNA and upon phosphorylation of 4EBPs by mTORC1, 4EBPs are released from eIF4E (Brunn et al. 1997, Gingras et al. 1999, Yang, Brunn and Lawrence. 1999, Mothe-Satney et al. 2000, Gingras et al. 2001) and thereby allows the constitution of the eIF4F complex (comprised of eIF4E, the RNA helicase eIF4A and the large scaffold protein eIF4G). The association of eIF4F to mRNA promotes the recruitment of mRNA to the 43S preinitiation complex and subsequently begins the 5' to 3' scanning for initiation codon recognition [reviewed in (Sonenberg and Hinnebusch. 2009, Jackson, Hellen and Pestova. 2010)].

mTORC1 downstream target S6K (Chen et al. 1995, Chung et al. 1992), or its upstream regulator RSK (Carriere et al. 2008, Ma et al. 2005, Roux et al. 2004), can phosphorylate eIF4B, a RNA-binding protein that enhances eIF4A helicase activity, on the same residue (Ser422) (Raught et al. 2004, Shahbazian et al. 2006). Inactive S6K1 binds to eIF3, a scaffold protein which provides the platform for the association of mRNA and the 43S preinitiation complex (Holz et al. 2005). Upon phosphorylation by mTORC1, S6K1 is released from eIF3 to phosphorylate eIF4B and thereby promotes translation initiation (Holz et al. 2005).

Newly synthesized mRNAs contain a large amount of non-coding segments termed introns which interrupt coding regions, these introns are to be removed by the splicing machinery upon nucleocytoplasmic mRNA export (Schellenberg, Ritchie and MacMillan. 2008). Exon (translatable sequence of mRNA) junction complex (EJC), a multi-subunit complex which is assembled concomitantly with splicing, binds to, is transported with, and remains associated to the mature mRNA until the end of the "pioneer" round of translation which is characterized by the presence of CBP80/CBP20 rather than eIF4E (Ishigaki et al. 2001). S6K1 substrate SKAR (Richardson et al. 2004) has been shown to co-localize with EJC and recruits active S6K1 to mRNA for the enhancement of translation productivity (Ma et al. 2008). CBP80 can also be phosphorylated by S6K1 on Ser7 which results in the augmentation of its mRNA-cap binding activity (Wilson, Wu and Cerione. 2000).

Eukaryotic translation elongation is controlled by the coordination of two eukaryotic elongation factors (eEFs): eEF1 and eEF2. eEF1 mediates the aminoacyl-tRNA entrance into the free ribosomal A site while eEF2 regulates the translocation of mRNA by one codon and the movement of aminoacyl-tRNA from A into P site (for reviews, see Proud. 2009, Rodnina and Wintermeyer. 2011). The activity of eEF2 is inhibited upon the phosphorylation of Thr56 (Ovchinnikov et al. 1990, Price et al. 1991, Redpath et al. 1993) by the highly specific Ca^{2+}/Calmodulin-dependent enzyme eEF2K (Nairn, Bhagat and Palfrey. 1985, Nairn and Palfrey. 1987, Ryazanov. 1987, Ryazanov, Shestakova and Natapov. 1988, Ryazanov et al. 1988). eEF2K also undergoes inhibitory phosphorylations on Ser78, Ser359 and Ser366 (Wang et al. 2001, Browne and Proud. 2004, Knebel, Morrice and Cohen. 2001). Ser366 phosphorylation, which can be detectable under low Ca^{2+} concentrations, is catalysed by S6K1 (Fig. 1.5b) or RSK1 (Wang et al. 2001); Ser78 (Browne and Proud. 2004) and Ser359 (Knebel, Morrice and Cohen. 2001) phosphorylations can also be regulated by mTOR, although p38δ MAPK (Knebel, Morrice and Cohen. 2001) and the cdc2/cyclin B complex (Smith and Proud. 2008) have been identified as the Ser359 kinase. The Ser78 kinase(s) is(are) yet to be determined. The phosphorylation of Ser359 and Ser366 severely impairs eEF2K kinase activity while the phosphorylation of Ser78 prevents the recruitment of calmodulin to eEF2K and thereby inhibits eEF2K activation (Wang et al. 2001, Browne and Proud. 2004, Knebel, Morrice and Cohen. 2001).

1.4.2 mTOR and autophagy

Macroautophagy (herein referred to as autophagy) and protein synthesis are two opposing cellular processes required for the maintenance of metabolic balance within a cell. Autophagy is a catabolic process in which cytoplasmic proteins or whole organelles are sequestrated and degraded by autolysosomes (fusion of autophagosomes and lysosomes) under stress conditions such as cell damage and nutrient restriction (for reviews, see Lum, DeBerardinis and Thompson. 2005, Jung et al. 2010). Protein degradation by autophagosomes generates high flux of amino acids which can then be utilized for the translation machinery, it has been therefore proposed that the coupling of mTOR and autophagosome/lysosome can facilitate the handling of protein turnover and improve mRNA translation efficiency (He and Klionsky. 2009). Under nutrient-rich conditions, mTORC1 is activated and translocated to peripheral lysosomes close to its upstream signalling molecules. At the beginning of nutrient deprivation, mTORC1 and lysosomes are clustered to perinuclear areas driven by increased intracellular pH, which facilitates autolysosome formation and the induction of autophagy, consequently the activity of mTORC1 is inhibited (Yu et al. 2010, Korolchuk et al. 2011). However, prolonged starvation may reactivate mTORC1 and attenuate autophagy, resulting in the formation of tubular structures containing lysosomal membrane proteins, which ultimately transform to mature lysosomes for the repletion of intracellular lysosome storage (Yu et al. 2010). During senescence, endomembrane reorganization can produce special secretory compartments termed TASCC (TOR-autophagy spatial coupling compartment) in which the core components are mTORC1 and autophagosomes (Narita et al. 2011). Newly synthesized proteins rapidly accumulate to the TASCC, and this swift protein turnover

enhances the production and secretion of senescence-messaging secretomes (Narita et al. 2011). In non-senescent cells, the formation of TASCC also plays roles in the augmentation of protein secretion and the coupling of anabolic and catabolic machinery to increase the efficiency of both processes (Narita et al. 2011).

The building of pre-autophagosomal structure in budding yeast is initiated upon series of interactions among the autophagy-inducing proteins named as ATGs (autophagy-related proteins) (Klionsky et al. 2003). Yeast TORC1 is responsible for the regulation of a number of ATGs (for a review, see Chang et al. 2009), and recently it has been reported by several groups that mTORC1 also controls their highly conserved mammalian homologues via direct phosphorylation (Jung et al. 2009, Hosokawa et al. 2009, Ganley et al. 2009). These authors have identified that a trimeric 3 MDa protein complex, formed by ULK1 (mammalian ATG1 homolog), ATG13 and FIP200 [FAK (focal adhesion kinase) family kinase-interacting protein of 200 kDa], is regulated by mTORC1 (Jung et al. 2009, Hosokawa et al. 2009, Ganley et al. 2009) (Fig. 1.7). The following studies have particularly focused on the regulation of ULK1, which lead to the discovery of twenty-seven ULK1 phosphorylation sites (Shang et al. 2011, Egan et al. 2011, Kim et al. 2011, Bach et al. 2011, Dorsey et al. 2009, Alers et al. 2012). Two of these sites (Ser637 and Ser757) are mTOR phosphorylation sites [(Shang et al. 2011, Kim et al. 2011) and Fig. 1.7]. ULK1 kinase activity is also controlled by the mTORC1 upstream regulator AMPK (Shang et al. 2011, Egan et al. 2011, Kim et al. 2011, Bach et al. 2011, Lee et al. 2010, Loffler et al. 2011). Upon nutrient deprivation, AMPK is activated and in turn phosphorylates ULK1 on Ser317 (Kim et al. 2011), Ser467 (Egan et al. 2011), Ser555

(Egan et al. 2011), Thr574 (Egan et al. 2011), Ser637 (Shang et al. 2011, Egan et al. 2011) and Ser777 (Kim et al. 2011), preventing the binding of mTORC1 to ULK1 and consequently provokes autophagy. Conversely, AMPK is replaced by mTORC1 upon growth factor and nutrient stimulation, which results in the inhibition of ULK1 and autophagy restriction (Shang et al. 2011, Egan et al. 2011, Kim et al. 2011, Bach et al. 2011, Lee et al. 2010, Loffler et al. 2011). Of interest, upon activation, ULK1 also triggers negative feedback towards AMPK and mTORC1 via direct phosphorylation of all three AMPK subunits (Loffler et al. 2011) and RAPTOR (Dunlop et al. 2011, Alers et al. 2012, also see table 1.1 and Fig. 1.1), it implies that the initiation of autophagy may switch off further autophagy-inducing signals exerted by AMPK, and simultaneously shut down mTORC1 to prevent the undergoing of highly energy-demanding processes such as protein synthesis.

As summarized above, most of the literature has pointed out the inhibitory nature of mTORC1 on autophagy, yet surprisingly, DAP1, a repressor of autophagy, is inactivated upon phosphorylation at Ser3 and Ser51 by mTORC1 (Koren, Reem and Kimchi. 2010) (Fig. 1.7). As a result, mTORC1 inhibition may also promote DAP1 activity to reduce excessive autophagy induction, and thereby prevent unrestrained protein degradation (Koren, Reem and Kimchi. 2010).

Under normal nutrient conditions, the repression of autophagic machinery can also be driven by several mTORC1-independent mechanisms which converge to inhibit hVps34, an inducer of autophagy (Lipinski et al. 2010). Another mTORC1-independent

autophagy-inhibiting pathway was described by Rubinsztein's group, the authors studied the effect of various Ca^{2+} and K^{+} channel openers/blockers and discovered that an elevation in intracytosolic Ca^{2+} levels evoked by cAMP inhibits autophagy and retards the clearance of aggregate-prone proteins such as the mutant huntingtin (Williams et al. 2008), which causes the neurodegenerative Huntington's disease (The Huntington's Disease Collaborative Research Group. 1993). More intriguingly, an exceptional proteomic study has revealed that many core systems of the complex autophagic network remained unaltered upon mTORC inhibition, implying that post-translational modifications may be crucial for the induction of autophagy (Behrends et al. 2010).

1.4.3 mTOR and cell mass

After being identified in yeast (Heitman, Movva and Hall. 1991), the first physiological roles designated to TOR are the control of cell proliferation and growth. Rapamycin is known as an immunosuppressant and blocker to lymphocyte (B and T cells) proliferation, especially at the G_1/S phase boundary (Kay et al. 1991). Indeed, mTORC1 controls the expression and activation of various regulatory proteins which govern the G_1/S entry, including CDK/cyclin complexes (cyclin D, E, A, CDK4, CDK2) and CDK inhibitors ($p27^{kip1}$, $p21^{cip1/waf1}$) [reviewed in (Wang and Proud. 2009)]. Increasing evidence has also revealed the role of mTORC1 in the up-regulation of G_2/M progression via promoting the activity of cdc2 (Smith and Proud. 2008, Heesom et al. 2001, Ramirez-Valle et al. 2010), and as a positive feedback mechanism, cdc2/cyclin B complex also phosphorylates TSC1 at Thr417, Ser584 and Ser1047 in cells arrested in G_2/M by nocodazole, which results in the inhibition of TSC1 and subsequent mTORC1 activation (Astrinidis et al. 2003) (Fig.

1.2). On the other hand, genetic ablation of dTOR (*Drosophila* TOR) in *Drosophila* has revealed that TOR plays a fundamental role in the control of cell growth (changes in cell size) (Zhang et al. 2000, Oldham et al. 2000). Later studies have demonstrated that mTORC1 mediated cell growth (Fingar et al. 2002) and proliferation (Fingar et al. 2004) in mammalian cells are controlled by at least two mTORC1 downstream targets: S6K and 4EBPs. Indeed, it has been found previously by the end of the last century that S6K is crucial for the maintenance of cell size in *Drosophila melanogaster* (Montagne et al. 1999) and mice (Shima et al. 1998, Pende et al. 2000), but dispensable for cell proliferation (Montagne et al. 1999, Shima et al. 1998), whereas ectopic expression of d4EBPs (*Drosophila* 4EBPs) caused a reduction in wing size as a result of a decrease in both cell size and number (Miron et al. 2001). Recently Sonenberg's group has developed mouse embryonic fibroblasts (MEFs) in which 4EBP1 and 2 (MEFs do not express 4EBP3) were genetically ablated (4EBPDKO MEFs) (Dowling et al. 2010). When the activity of mTORC1 was inhibited upon RAPTOR deletion using shRNA, 4EBPDKO MEFs were able to escape cell cycle arrest, yet they were unable to recover the decrease in cell size (Dowling et al. 2010). This observation implies that as an evolutionary phenomenon, mammalian cells apply distinct signalling mechanisms to regulate growth and division, because unlike d4EBPs which also control cell size (Miron et al. 2001), mammalian 4EBPs are only responsible for cell proliferation (Dowling et al. 2010).

In some cell types rapamycin is cytotoxic and causes apoptotic cell death, implying a crucial role of mTOR in maintaining cell survival. Because rapamycin is first used as an immunosuppressant, cells within the immune system, such as B-cells, are the first found

to be susceptible to rapamycin-induced cell death (Muthukkumar, Ramesh and Bondada. 1995), rapamycin also enhances cytokine-mediated apoptosis in T-cells (Shi et al. 1995). Examples of other cell systems that have been found to be sensitive to rapamycin-induced apoptosis include renal tubular cells (Lieberthal et al. 2001), dendritic cells (Woltman et al. 2003), and of the greatest importance for this thesis, pancreatic β-cells (Bell et al. 2003). These observations also prompted scientists to investigate whether rapamycin is cytotoxic to cancer cells. Early reports have discovered that rapamycin treatment led to increased cell death in various cancer cell lines lacking functional p53 (Ahn et al. 1997, Hosoi et al. 1999, Thimmaiah et al. 2003), and increasing number of studies pointed out that the inhibition of mTOR can be a promising drug target for cancer treatment [reviewed in (Zoncu, Efeyan and Sabatini. 2011, Guertin and Sabatini. 2009)]. Interestingly, although treatment of rapamycin in nanomolar (nM) concentrations is sufficient to inhibit the phosphorylation of certain mTOR downstream targets such as S6Ks, it is necessary to increase its dose to μM range in order to induce cell death in cancer cells (Chen, Zheng and Foster. 2003, Chen, Rodrik and Foster. 2005, Gadir et al. 2008), a recent study (Yellen et al. 2011) suggests that this is probably caused by the differences in levels of mTOR complex disassembly, subsequent 4EBP dephosphorylation and activation upon low/high doses of rapamycin treatment, as low concentration (nanomolar range) of rapamycin only causes partial dissociation of mTORC1 and has little effect on 4EBP1 phosphorylation in human breast cancer cell lines (Yellen et al. 2011). Paradoxically, in some cell types rapamycin protects against apoptosis [reviewed in (Castedo, Ferri and Kroemer. 2002)]. Recent studies have suggested that the resistance to rapamycin-induced cell death in certain types of cancer

cells can be caused by: 1) EGFR (EGF receptor) transactivation and the induction of MAPK pathway (Chaturvedi et al. 2009); 2) high levels of DEPTOR expression (Peterson et al. 2009); or 3) the fact that rapamycin is unable to completely inhibit mTORC1 (Thoreen et al. 2009, Carayol et al. 2010) and 4) mTORC2 is rapamycin insensitive in many cancer cell lines [reviewed in (Sparks and Guertin. 2010)]. However, novel and potentially more effective mTOR inhibitors have emerged in the last few years that inhibit both mTORC1 and 2 [reviewed in (Guertin and Sabatini. 2009, Benjamin et al. 2011)].

mTOR is essential for embryonic stem cell (ESC) development, it plays a critical role in the growth and proliferation of ESCs (Hentges et al. 2001, Murakami et al. 2004, Gangloff et al. 2004), and therefore disruption of mTOR in *Drosophila* (Zhang et al. 2000, Oldham et al. 2000), *Caenorhabditis elegans* (Long et al. 2002) and mice (Hentges et al. 2001, Murakami et al. 2004, Gangloff et al. 2004) is embryonically lethal. Extracellular stimuli that activate mTOR, such as glucose and amino acids, play permissive roles in the differentiation of trophoblasts (Naeslund. 1979), which forms the outer epithelial layer of blastocysts and are the first to differentiate during mammalian development. Indeed, mTORCs plays an important role in cell differentiation. This can either be positive as in: osteoblasts (mTORC1) (Singha et al. 2008), adipocytes (mTORC1) (Zhang et al. 2009), amniotic fluid stem cells (mTORC1 and 2) (Valli et al. 2010, Siegel et al. 2010), myeloblasts (mTORC2) (Yamada et al. 2010) and T-cells (mTORC1 and 2) (Delgoffe et al. 2011); or negative, as in vascular smooth muscle cells (mTORC1) (Martin et al. 2004), pancreatic β-cells (mTORC1) (Rachdi et al. 2012) and

murine embryonic fibroblasts (mTORC1) (Ma et al. 2010). Paradoxically, mTOR knockdown using siRNA severely impairs myocyte differentiation whereas RAPTOR and Rheb knockdown have the opposite effect (Ge, Yoon and Chen. 2011). Therefore, the exact mechanism by which mTORC1 and 2 control cell differentiation is far from understood.

1.4.4 mTOR and metabolism

mTORC1 can regulate metabolism via multiple mechanism through the up-regulation of transcription factors HIF1α (Zhong et al. 2000, Hudson et al. 2002) and SREBPs (sterol regulatory element-binding proteins) (Porstmann et al. 2008).

Murphy and Manning's groups revealed that mTORC1 controls a variety of metabolic pathways, including glycolysis, pentose phosphate pathway and lipid biosynthesis, in a S6K1-HIF1α/SREBP dependent manner (Duvel et al. 2010). Moreover, in a mouse tumour model lacking LKB1, mTORC1-HIF1α pathway drives the expression of genes involved in glycolysis [hexokinase II and GLUT-1 (glucose transporter 1)] (Shackelford et al. 2009). The activation of SREBPs is regulated by lipin1 (Peterson et al. 2011), a Mg^{2+}-dependent PA phosphatase (Han, Wu and Carman. 2006). mTORC1 phosphorylates lipin1 at Ser106 and Ser472, which promotes its nuclear entry and subsequent stimulation of SREBPs (Peterson et al. 2011) (Fig. 1.7). mTORC1-mediated lipo- and sterolgenesis is dependent upon SREBP1 (Porstmann et al. 2008), but insulin-mediated gluconeogenesis in liver is mTORC1-independent (Li, Brown and Goldstein. 2010). Other studies have further unravelled the role of mTORC1 in the regulation of

triglyceride and ketone bodies. mTORC1 activation reduces the expression of adipose triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL), and thereby suppresses lipolysis and promotes triglyceride storage (Chakrabarti et al. 2010). Furthermore, mTORC1 is suppresive to PPARα (peroxisome proliferator activated receptor α) (Sengupta et al. 2010), a transcription activator of genes implicated in ketogenesis (Issemann and Green. 1990). Liver-specific RAPTOR knock down releases PPARα from the inhibitory binding of NcoRI (nuclear receptor co-repressor 1) and enhances ketone body production, and this is sufficient to prevent the reduction in ketone synthesis caused by aging, implying that aging-induced defects in PPARα function and ketogenesis is mediated by mTORC1 (Sengupta et al. 2010).

mTORC1 plays a crucial role in the regulation of mitochondria metabolism. In a study performed by Schleke *et al*., both mTOR and RAPTOR have been found to localize in the mitochondria, and siRNA knockdown of mTOR or RAPTOR reduces oxygen consumption and oxidative capacity. Conversely, TSC2 or RICTOR knockdown increases oxygen consumption and oxidative capacity (Schieke et al. 2006). Interestingly, mTORC1 controls the expression of a number of mitochondria genes, including *Atp5g1* (encodes mitochondrial ATP synthase lipid-binding protein), *Cox5a* (encodes cytochrome C oxidase subunit 5a), *cytochrome C*, *Idh3a* [encodes mitochondrial isocitrate dehydrogenase subunit α] and others, by directly targeting the transcription factor YY1 (ying-yang 1) (Cunningham et al. 2007). Active mTORC1 recruits PGC1α (peroxisome proliferator-activated receptor 1α), which controls mitochondria function through the interaction with other transcription factors, to YY1 to promote mitochondrial gene

transcription (Cunningham et al. 2007) (Fig. 1.7). shRNA knockdown of YY1 leads to a reduction in mitochondria DNA levels and oxygen consumption (Cunningham et al. 2007).

1.5 The role of mTOR in diabetes Mellitus

1.5.1 Regulation of pancreatic β-cell mass by mTOR

Islets of Langerhans, which form 1-2% of the human pancreas, was first discovered by the German scientist Paul Langerhans in the year 1869 (for a historical review, see Kloppe. 1969). Islets of Langerhans are comprised of many types of endocrine cells including: α-cells which produce glucagon; δ-cells which produce somatostatin; ε-cells which yield ghrelin; PP cells which yield pancreatic polypeptide, and most importantly β-cells, which synthesize and secrete insulin and amylin [also known as IAPP (islet amyloid polypeptide)], both of which synergistically promote glucose clearance after meals (Bouwens and Rooman. 2005, Ackermann and Gannon. 2007). Failure in maintaining pancreatic islet/β-cell mass and/or function results in insulin secretion deficiency and/or insulin resistance, leading to the development of a metabolic disorder known as type 2 diabetes mellitus (T2DM) (Polychronakos and Li. 2011, Chatenoud. 2011, Pozzilli. 2011, Chen, Magliano and Zimmet. 2011, Kolb and Eizirik. 2011, Wilson. 2011, Diaz. 2011).

T2DM causes metabolic changes that lead to the damage and functional impairment of organs and tissues resulting in increased morbidity and mortality. However, the majority

of individuals who have insulin resistance do not develop diabetes due to a compensatory increase in insulin secretion in response to an increase in insulin demand. This adaptive response is sustained by an increase in both β-cell function and mass (for reviews see Lingohr, Buettner and Rhodes. 2002, Prentki and Nolan. 2006). Importantly, there is increasing evidence that mTOR plays a key role in the regulation of β-cell mass and therefore likely plays a critical role in β-cell adaptation (reviewed in Zoncu, Efeyan and Sabatini. 2011, Howell and Manning. 2011).

Many growth factors, hormones and nutrients have also been shown to play an important role in stimulating increases in β-cell mass and many, if not all, activate mTORC1. For example, glucose, a potent *in vivo* stimulator of β-cell mass in rodents (Bonner-Weir et al. 1989, Bernard et al. 1998, Paris et al. 2003, Topp, McArthur and Finegood. 2004), acutely up-regulates mTORC1 activity in isolated rat islets and rodent β-cell lines (Gomez et al. 2004, Kwon et al. 2004, Bartolome, Guillen and Benito. 2010). Moreover, it has been demonstrated that *in vitro* glucose can stimulate β-cell proliferation (Kwon et al. 2004, Bartolome, Guillen and Benito. 2010) and protein synthesis (Xu et al. 1998b, Gomez et al. 2008), an important hypertrophic stimuli (Srivastava. 2002), via a rapamycin sensitive pathway. The mechanism by which glucose activates mTORC1 in islets and β-cell lines has been reported to be mediated via the autocrine action of insulin (Xu et al. 1998b). This is likely via the activation of PKB (El Sayed NM, Moore CE and Herbert TP, unpublished data) and the inactivation of AMPK (Gleason et al. 2007). However, it has recently been reported, in a β-cell line derived from insulin receptor knock-out mice, that glucose-stimulated mTORC1 activation is mediated via the MAPK

pathway independently of PKB (Bartolome, Guillen and Benito. 2010). Conversely, the inhibition of mTOR by rapamycin causes loss of β cell function and viability in pancreatic β-cell lines and murine and human islets (Bell et al. 2003, Fabian et al. 1993, Zhang et al. 2006), indicating that the maintenance of mTOR activity is critical for the integrity of the β-cell.

1.5.2 Transgenic mice models

Although *in vitro* studies have revealed important insights into the role and regulation of mTORC1 in β-cells, transgenic mouse models of upstream regulators and downstream effectors of mTOR have provided unequivocal evidence demonstrating that mTORC1/2 plays a critical role in the regulation of β-cell mass *in vivo*. TSC1/2 is an upstream negative regulator of mTORC1 (Inoki et al. 2002, Tee et al. 2002), and loss of TSC2 leads to the constitutive activation of mTORC1 (Tee et al. 2002, Goncharova et al. 2002, Zhang et al. 2003). Importantly, the generation of β-cell specific TSC2 knock-out mice ($\beta TSC2^{-/-}$), from two independent groups, has revealed that TSC2 plays in important role in the regulation of β-cell size and mass (Shigeyama et al. 2008, Rachdi et al. 2008). Rachdi *et al.* reported that in 8 weeks old $\beta TSC2^{-/-}$ mice, β-cell mass is increased by over 2 fold due to a doubling in β-cell size and proliferation (Rachdi et al. 2008) and that this increase in β-cell mass is maintained for up to 52 weeks of age (Rachdi et al. 2008). Shigeyama *et al.* reported similar increases in β-cell size and mass in 6 weeks old $\beta TSC2^{-/-}$ mice but, in contrast to Rachdi *et al.*, β-cell mass had decreased dramatically (80% reduction compared to control) by 40 weeks of age likely due to an increase in apoptosis (Shigeyama et al. 2008). This was accompanied by hypoinsulinemia and, as a

consequence, hyperglycemia. The positive and negative effects of TSC2 knock-out on β-cell mass are likely mediated by mTORC1 as rapamycin treatment of young βTSC2$^{-/-}$ mice causes a reduction in β-cell mass (Rachdi et al. 2008), whereas rapamycin treatment (18 to 40 weeks) of Shigeyama *et al.*'s βTSC2$^{-/-}$ mice resulted in the maintenance of β-cell mass and improved glyceamic control (Shigeyama et al. 2008). The age-related decrease in β-cell mass observed in βTSC2$^{-/-}$ mice is likely due to the prolonged hyper-activation of mTORC1 resulting in feedback inhibition, possibly through S6K-dependent phosphorylation of IRS2.

The over-expression of Rheb, the downstream target of the TSC1/2 complex, also results in the constitutive activation of mTORC1 (Nobukuni et al. 2005, Smith et al. 2005). β-cell specific over-expression of Rheb in mice enhances β-cell mass by approximately 50% (Hamada et al. 2009). β-cell size was increased by up to 30%, whereas cell proliferation and cell viability is unaffected. This augmentation in β-cell mass correlates with improved glucose tolerance in oral glucose tolerance test and increased late phase GSIS *in vivo* compared to their wild-type littermates. These improvements in glucose tolerance and enhancement of GSIS were reversed upon rapamycin treatment, indicating that that these functional effects are likely mediated via the activation of mTORC1 (Hamada et al. 2009).

S6K1/2 are downstream targets of mTORC1 and S6K1 knock-out (S6K1$^{-/-}$) mice have reduced β-cell mass due to a reduction in β-cell size (a 24% decrease in comparison with the WT) (Pende et al. 2000). In addition, the islets from these mice had decreased islet

insulin content and the amount of insulin secreted per cell in response to glucose was significantly reduced. Conversely, the over-expression of a constitutively active form of S6K1 in mouse β-cells ($S6KCA^{RIP}$) results in an increase in β-cell size by approximately 50% (Elghazi et al. 2010). These reports indicate that S6K1 is a positive effector of β-cell size and function. The effects of S6K1 on β-cell size may be mediated through the phosphorylation of rpS6, as non-phosphorylatable rpS6 knock-in mice ($rpS6^{p-/-}$) have smaller β-cells (a 35% decrease compared to WT) (Ruvinsky et al. 2005). Yet, β-cell mass is unaffected due to a compensatory increase in β-cell number. Interestingly, β-cells from $rpS6^{p-/-}$ mice are smaller than those from $S6K1^{-/-}$ mice, indicating that other rpS6 kinases may be involved. However, there is no reduction in β-cell size or mass in S6K2 knock-out ($S6K2^{-/-}$) mice (Pende et al. 2004), although S6K2 is considered to be the major *in vivo* rpS6 kinase (Pende et al. 2004). It is therefore possible that the difference in cell size between $rpS6^{p-/-}$ and $S6K1^{-/-}$ mice is mediated by an alternative S6K such as p90 ribosomal S6 kinase (RSK) or protein kinase A (PKA) (Roux et al. 2007, Moore et al. 2009).

β-cell specific knock-out of RICTOR (βRicKO), an essential component of the mTORC2 complex, in mice, results in a reduction in β-cell mass due to an impairment in proliferation (Gu et al. 2011). However, no changes in β-cell size or the rate of cell death were detected. These mice also had decreased pancreatic insulin content, moderate hyperglycemia and glucose intolerance. In islets isolated from βRicKO mice, the phosphorylation of PKB at Ser473, a target for mTORC2 and an important site for PKB activation, was not surprisingly compromised. Moreover, this correlated with an increase

in FoxO1 nuclear localisation, which is known to be inhibited by PKB-dependent phosphorylation on Ser473. However, the phosphorylation of PKB at Thr308, another important site for PKB activation and which is mediated by a PDK1, was enhanced. Therefore, it is possible that the increase in the phosphorylation of PKB at Thr308 may compensate for the loss of Ser473 phosphorylation and that PKB activity towards specific subset of substrates is maintained. Indeed, no change in mTORC1 activity was detected in islets isolated from βRicKO mice. Interestingly, in β-cell specific PTEN (it inhibits the activation of PKB through promoting the dephosphorylation of PIP3 and thereby prevents plasma membrane translocation of PDK-1 and PKB) and RICTOR double knock-out mice (βPtenRicKO), Thr308 phosphorylation on PKB was dramatically enhanced and this correlated with an increase in β-cell size (Gu et al. 2011). Therefore, the authors concluded that phosphorylation of PKB at Thr308 (by PDK-1) drives cell size, whereas the phosphorylation of PKB on Ser473 (by mTORC2) drives cell proliferation (Gu et al. 2011).

1.6 Thesis aims

A large number of studies have indicated that both mTORC1 and 2 play crucial roles in the regulation of pancreatic β-cell mass and function (Pende et al. 2004, Ruvinsky et al. 2005, Pende et al. 2000, Shigeyama et al. 2008, Rachdi et al. 2008, Hamada et al. 2009, Gu et al. 2011). Hormones like glucagon-like peptide-1 (GLP-1) that augment intracellular cAMP ($[cAMP]_i$) have been shown to activate mTORC1 in β-cells (Moore et al. 2009, Kwon et al. 2004), and it has been shown that GLP-1 and its analogs are able to enhance β-cell proliferation *in vitro* [reviewed in (Doyle and Egan. 2007, Yu and Jin.

2010)]. Therefore, the first aim of this thesis is to determine the role of mTORC1 in the regulation of β-cell function and mass.

On the other hand, rapamycin analogs have been used as immunosuppressive drugs in islet transplantation during the last decade (Shapiro et al. 2000), however, several studies have suggested that rapamcyin has detrimental effect on β-cell survival and islet engraftment (Bell et al. 2003, Fabian et al. 1993, Zhang et al. 2006). Interestingly, previous work performed by Barlow AD from our lab has demonstrated that prolonged rapamycin treatment not only inhibits mTORC1 but also mTORC2 in β-cells, and the exogenous expression of constitutively active PKB rescues β-cells from rapamycin-induced cell death. Therefore, the second aim of this thesis is to investigate the role of mTORC2 in rapamycin islet toxicity.

Previously we (Moore et al. 2009) and others (Kwon et al. 2004) have shown that an increase in $[cAMP]_i$ is stimulatory to mTORC1 in pancreatic β-cells. However, in many other cell types such as hepatocytes, neurons and cancer cell lines, increases in $[cAMP]_i$ lead to the inhibition of mTORC1 which coincides with an impairment of cell replication (Scott and Lawrence. 1998, Mothe-Satney et al. 2004, Kimball, Siegfried and Jefferson. 2004, Rocha et al. 2008, Baum, Kimball and Jefferson. 2009, Baum, Kimball and Jefferson. 2009, Kim et al. 2001). Therefore, the third and final aim of this thesis is to study how cAMP negatively regulates mTOR complexes in cancer cell lines.

Chapter 2. Materials and Methods

2.1 Chemicals

All Chemicals, unless stated, are purchased from Sigma-Aldrich (St. Louis, MO, USA). Solvents (ethanol, ethanol, isopropanol, acetic acid, HCl and acetone) were obtained from Fisher Scientific (Loughborough, UK). Forskolin and rapamycin were purchased from Calbiochem (Nottingham, UK). Foetal bovine serum, 1X trypsin/EDTA (ethylenediaminetetraacetic acid) (0.5%) and lipofectamine 2000^{TM} were purchased from Invitrogen (Carlsbad, CA, USA). Rapamycin was purchased from Calbiochem (Darmstadt, Germany). [γ^{32}P] ATP was purchased from GE Healthcare (Piscataway, NJ, USA). Collagenase was purchased from Serva (Heidelberg, Germany). Tris base, glycine, agarose and bacterial cell culture reagents, were from Melford (Suffolk, UK). Torin1 (Thoreen et al. 2009) was kindly provided by David Sabatini (Whitehead Institute for Biomedical Research, Cambridge, MA, USA).

2.2 Cell culture and treatments

2.2.1 Cell lines

TSC2$^{+/+}$/p53$^{-/-}$ MEFs, TSC2$^{-/-}$/p53$^{-/-}$ MEFs, kindly provided by David J. Kwiatkowski (Harvard Medical School, Boston, MA) (Zhang et al. 2003); MIN6 (mouse insulinoma cell line 6) cells, kindly provided by Jun-Ichi Miyazaki (Osaka University Medical School, Japan) (Miyazaki et al. 1990), INS1E (rat insulinoma cell line 1E) cells (Asfari et

al. 1992) and HEK293 (human embryonic kidney 293) cells (Graham et al. 1977) were grown to approximately 80% confluence prior to treatments.

2.2.2 Maintenance and propagation

INS1E cells were grown in RPMI (Roswell Park Memorial Institute) 1640 media containing 11 mM glucose, supplemented with 5% FBS (foetal bovine serum) (Invitrogen, Carlsbad, CA), P/S/N (100 μg/ml streptomycin, 100 units/ml penicillin sulphate, 100 units/ml neomycin), 1 mM sodium pyruvate, 10 mM HEPES [4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] an 55 μM β-mercaptoethanol (INS1E growing media). MIN6 cells were maintained in DMEM containing 25 mM glucose, supplemented with 15% (all serum used in this thesis are heat-inactivated, and the % given are always v/v) FBS, P/S/N, 40 mM $NaHCO_3$ and 75 μM β-mercaptoetanol (MIN6 growth media). MEFs were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 10% (v/v) FBS, P/S/N, 50 μM β-mercaptoetanol and 1X non-essential amino acids (MEF growth media). HEK293 cells were grown in DMEM containing 25 mM glucose, supplemented with 10% FBS and P/S/N (HEK293 growth media).

All cells were equilibrated with 5% CO_2, 95% air at 37 ºC cell incubators (all cells used in this thesis have been incubated under these conditions unless specified). The media were changed every two to three days. After reaching 80% confluency, cells were washed once with 1X phosphate buffered saline (PBS) followed by incubation with 1X trysin/EDTA (0.5%) for 1-5 min at 37 ºC. Cells were resuspended in their respective

growing media as soon as they started to detach from the plate. Resuspended cells were plated out for maintenance, or as required for the experiments. Please see figure legends for treatment details.

2.2.3 Cryopreservation of cells

Cells were collected in growing media after trypsinization, and spun at 200 x *g* for 5 min, cell pellet was resuspended in freezing down buffer comprised of 10% steril DMSO and 90% FBS. Cell tubes were then temporally kept at -80 °C insulated with tissue, before storage under liquid nitrogen. To prepare cells for maintenance and experimentation from frozen cell stocks, cells were rapidly defrosted at 37 and added to pre-warmed growing media. Media were changed after 24 h to remove DMSO and unattached cells.

2.3 Islets isolation and dispersion

Pancreatic islets were isolated from male Sprague-Dawley rats weighing 200-250g by collagenase digestion and Histopaque density gradient centrifugation using a modified method previously described (Guest, Rhodes and Hutton. 1989). Briefly, abdomen of newly sacrificed animals was opened and the ampulla located in the duodenum was clamped using curved hemostatic forceps. The joint site of hepatic duct, cystic duct and common bile duct was cut by surgery scissors in order to insert the needle to slowly inject 6 ml RPMI 1640 media containing 11.1 mM glucose, 1 mg/ml freshly dissolved collagenase (in a 10 ml syringe). Inflated and distended pancreata was removed from the duodenum and incubated at 37ºC in 10 ml RPMI 1640 media (without FBS and P/S/N)

for 17 min. The pancreata was then resuspended by hand shaking for 1 min and centrifuged for 3 min at 200 x *g*, 4 ºC. Supernatant was discarded and the pellet was again resuspended with RPMI 1640 media (without FBS and P/S/N) and spun for 3 min at 200 x *g*, 4 ºC to further wash off undigested tissues. The pellet was resuspended in RPMI 1640 media containing 5% FBS and filtered through a 6.5 cm-diameter plastic tea strainer. Filtrate was centrifuged for 3 min at 200 x *g*, 4 ºC, pellets were resuspended using 10 ml histopaque 1077 and slowly over-layered with 10 ml RPMI 1640 (without FBS and P/S/N). The tube was centrifuged for 20 min at 1600 x *g*, 4 ºC, 0 break/acceleration and islets were recovered from the interface and washed once with RPMI 1640 media containing 5% FBS. Islets were hand-picked under a stereomicroscope and were cultured in RPMI 1640 media containing 10% FBS and P/S/N (rat islet growth media). Following treatment, islets were collected by centrifugation for 1 min at 200 x *g* and lysed in ice-cold triton lysis buffer as described above.

Islets were dispersed 1 h post-isolation as previously described (Moore et al. 2011). Briefly, islets were washed once with Ca^{2+}-free KRB [138 mM NaCl, 5.6 mM KCl, 12 mM $MgCl_2$, 5 mM HEPES, pH 7.4, 1 mM EGTA, 0.02% (w/v) BSA (bovine serum albumin)], and then incubated with Ca^{2+}-free KRB for 10 min, when they were dispersed by gentle vortexing and pipetting, islets were collected by centrifugation for 4 min at 200 x *g*, resuspended in RPMI 1640 media containing 10% FBS and P/S/N, and plated on a poly-D-lysine pre-coated 24-well plate.

2.4 Buffers and bacterial culture media

- 0.3% (w/v) CHAPS {3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate hydrate} lysis buffer: 1M HEPES pH7.5, 120 mM NaCl, 1 mM EDTA pH8, 10 mM $Na_2H_2P_2O_7$, 10 mM β-glycerolphosphate, 50 mM NaF, 0.5 mM sodium orthovanadate, 0.3% CHAPS, 1 mM benzamidine-HCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 µg/ml each of leupeptin and pepstatin
- 100 mM $CaCl_2$-glycerol mix: 1 ml 1M $CaCl_2$ stock + 2.665 ml 50% glycerol + 6.335 ml ddH_2O (double distilled water)
- 1X ADB (assay dilution buffer): obtained by diluting 5X ADB (Cat No. 20-145, Millipore, Watford, UK) 1/5 in ddH_2O
- 1X PBST: 10% (v/v) 10X PBS (100 g NaCl, 14 g Na_2HPO_4, 3 g KCl and 3 g KH_2PO_4 in 1 L ddH_2O, pH 7.4) and 0.1% (v/v) tween-20
- 1X Laemmli sample buffer: 62.5 µM Tris pH6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 2.5% β-mercaptomethanol and 5 µg/ml bromophenol blue
- 1X tris-glycine buffer: 10X tris-glycine stock, which is prepared by dissolving 30 g tris base and 144 g glycine in 1 L ddH_2O
- 2X HBS (HEPES-Buffered Saline): 8 g NaCl, 6.5 g HEPES, 0.2 g Na_2HPO_4·7H2O, pH to 7.0 and bring up to 500 ml with ddH_2O
- 2X Laemmli sample buffer: 0.125 M Tris pH6.8, 2% (w/v) SDS, 20% (v/v) glycerol, 5% β-mercaptomethanol and 10 µg/ml bromophenol blue
- 4X Laemmli sample buffer: 0.25 M Tris pH6.8, 4% (w/v) SDS (sodium dodecyl sulphate), 40% (v/v) glycerol, 10% β-mercaptomethanol and 20 µg/ml bromophenol blue

- PKB kinase assay lysis buffer: 50 mM HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA, 0.5 mM Na_3VO_4, 0.1% (v/v) β-mercaptoethanol, 1% triton X-100, 50 mM NaF, 5 mM $Na_2H_2P_2O_7$, 10 mM β-glycerophosphate, 1 mM benzamidine-HCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin and pepstatin
- PKB kinase assay buffer: 50 mM Tris-HCl, pH 7.5, 0.03% (w/v) Brij-35, 0.1 mM EGTA and 0.1% (v/v) β-mercaptoethanol
- KRB (Krebs-Ringer bicarbonate) buffer: 115 mM NaCl, 5 mM KCl, 10 mM $NaHCO_3$, 2.5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 20 mM HEPES, pH 7.4, supplemented with 0.5X MEM amino acids solution (50X stock), 0.5X MEM non-essential amino acids solution (100X stock) and 0.5X L-glutamine (100X stock). 0.5X concentration of amino acids was defined as (in mM): L-arginine 0.36, L-cystine 0.1, L-glutamine 1.0, L-histidine·HCl·H_2O 0.1, L-isoleucine 0.2, L-leucine 0.2, L-lysine HCl 0.25, L-methionine 0.05, L-phenylalanine 0.1, L-threonine 0.2, L-tryptophan 0.025, L-tyrosine 0.1, L-valine 0.2, L-alanine 0.05, L-asparagine 0.05, L-aspartic acid 0.05, L-glutamic acid 0.05, glycine 0.05, L-proline 0.05 and L-serine 0.05
- LB (Luria-Bertani)-agar: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl, 1.5% (w/v) granulated agar in ddH_2O
- LB media: 1% (w/v) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl in ddH_2O
- Mg^{2+}-[γ-32P]ATP mix: 1 portion of 10 mCi/ml [γ-32P]ATP and 9 portions of Mg^{2+}/ATP cocktail (500 μM cold ATP and 75 mM $MgCl_2$ in 1X ADB), so the final concentration of [γ-32P]ATP is 1 μCi/μl
- Propidium iodide-RNase-PBS mix: 20 μg/ml propidium iodide, 100 μg/ml RNase in 1X PBS

- Transfer buffer: 1X tris-glycine buffer, 0.01% (w/v) SDS and 20% methanol
- Triton lysis buffer: 1% (v/v) triton X-100, 10 mM β-glycerophosphate, 50 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1 mM EGTA (ethylene glycol tetraacetic acid), 1 mM sodium orthovanadate, 1 mM benzamidine-HCl, 0.2 mM phenylmethylsulfonyl fluoride, 1 μg/ml each of leupeptin and pepstatin, 0.1% (v/v) β-mercaptoethanol and 50 mM NaF

2.5 Molecular biology techniques

2.5.1 Making of competent cells

DH5α *Escherichia coli* cells (Invitrogen, Carlsbad, CA, USA) were grown in LB media at 37 °C until OD_{600} (optical density measured at 600 nm) reached between 0.3 and 0.4. Cells were spun for 15 min, 200 x *g* at 4 °C. Cell pellets were resuspended in ice-cold 100 mM $MgCl_2$ and incubated on ice for 20 min. Cells were again spun for 15 min, 200 x *g* at 4 °C and pellets were resuspended in 25 ml ice-cold 100 mM $CaCl_2$ and incubated on ice for another period of 20 min. Cells were then subjected to another centrifugation for 15 min, 200 x *g* at 4 °C. Cell pellets were resuspended in 10 ml 100 mM $CaCl_2$-glycerol mix and snap-frozen in dry ice-IMS (industrial methylated spirit) bath and stored at -80 °C until use.

2.5.2 Transformation

For heat-shock transformation, DH5α competent cells obtained as described in section 2.5.1, or original DH5α competent cells (Invitrogen, Carlsbad, CA, USA) (for ligation only) were thawed on ice for 5 min, and added gently to approximately 100 ng DNA, followed by 30 min incubation on ice. Cells were heat-shocked at 42 °C for 40 s, and incubated again on ice for 2 min. If the transformed plasmid contains a DNA sequence that confers ampicillin resistance, the cells were then immediately plated on LB-agar containing 100 μg/ml ampicillin. If the transformed plasmid contains a DNA sequence that confers kanamycin resistance, cells were further incubated in LB media at 37 ºC for 45 min, and then spun at 200 x *g* for 1 min, cell pellets were resuspended in LB media before plated on LB-agar containing 50 μg/ml kanamycin. LB-agar plates were then incubated upside down at 37 ºC for 16 h, before subsequent plasmid DNA purification (section 2.5.3).

2.5.3 Plasmid DNA purification

Plasmid DNA obtained from transformation (see last section) were maxipreped. A bacterial colony was picked from the LB-agar plate and grown in LB culture media containing the corresponding antibiotic (100 μg/ml ampicillin or 50 μg/ml kanamycin) at 37 ºC for 16 h, before the plasmid DNA being purified using the PurElute™ IEX Plasmid Maxiprep kit (EdgeBio, Gaithersburg, MD, USA), according to manufacturer's instructions. Typically, a DNA purification process (either mini- or maxiprep) involves six steps: resuspension of the bacterial culture, cell lysis, neutralization, plasmid DNA

extraction, column washes and elution of the product. Plasmid DNA was dissolved in TE (Tris-EDTA) buffer provided within the maxiprep kit.

2.6 Adenoviral techniques

2.6.1 Adenovirus amplification

Recombinant adenovirus expressing dominant negative AMPK was kindly provided by Ian Salt (University of Glasgow, UK). Adenovirus expressing 4EBP1-5A (non-phosphorylable 4EBP1, all five phosphorylation sites mentioned in Fig. 1.6 are mutated to alanine) is a kind gift of Shile Huang (Louisiana State University Health Sciences Center, USA). For the amplification of adenoviruses, two T75 flasks of HEK293 cells per virus were grown to 80-90% confluent and infected by the adenoviruses. Cells were harvested when approximately 70-90% of cells were floating (3-5 days post-infection). Cells were washed off the flasks, transferred to conical tubes and spun at 1800 x *g* for 5 min at 4 ºC. Pellets were resuspended in 1X PBS, kept at -80 ºC prior to cell lysis. To harvest the virus, resuspended pellets were thawed in a 37 ºC water bath, vortexed and then frozen in a dry ice/IMS bath. This thaw/vortex/freeze cycle was repeated four times. Samples were centrifuged at 3200 x *g* for 10 min at 4 ºC, and the viral supernatant was collected and stored at -80 ºC.

2.6.2 Adenovirus infection

Prior to adenoviral infection, cells were cultured on 4 cm-diameter dishes until approximately 70% confluent, growth media were then removed and replaced by 0.4 ml DMEM (minus FBS) with indicated amount of adenovirus within the figures or figure legends. Cells were incubated at 37 °C for 1 h, and then 1.2 ml growth media were added per plate and cells were further incubated at 37 ºC for 48 h prior to experimentation. Adenoviral infection efficiency was monitored by assessing GFP (green fluorescent protein) expression levels using a fluorescence microscope fitted with a mercury lamp (Nikon, Surrey, UK).

2.7 Transfection

2.7.1 Calcium phosphate transfection

HA (hemagglutinin) tagged S6K1 (HA-S6K1) pRK7 vector was kindly provided by Prof. John Blenis (Harvard Medical School, Boston, MA, USA) (Cheatham et al. 1995), FLAG tagged Rheb (FLAG-Rheb) (plasmid 19996, Addgene, Cambridge, MA) and myc (myelocytomatosis) tagged mTOR (myc-mTOR) (plasmid 1861, Addgene, Cambridge, MA) were subcloned into the pRK7 vector by Andrew R. Tee (Cardiff University, UK). $RagB^{GTP}$Q99L and $RagC^{GDP}$S75L were described previously (Dunlop et al. 2009).

HEK293 cells were transfected by the calcium phosphate method as previously described (Herbert, Tee and Proud. 2002). Briefly, HEK293 cells were plated 6 h prior to transfection to obtain 20-30% confluency, transfection reagents were prepared as stated

in table 2-1. Solution in tube 2 was transferred to tube 1 drop by drop and mixed up by pipetting before adding directly to the cells cultured in growing media. Media were changed 16 h post-transfection, and cells were further incubated until experimentation.

Table 2-1 Preparation for calcium phosphate transfection. Reagents with larger volume were added first. ddH_2O: filtered sterilized double distilled water. 2X HBS: HEPES-Buffered Saline. 2X HBS was aliquoted into Eppendorfs (2 ml each) and stored at -20 °C, after defrosted and used, remaining 2X HBS was discarded.

Dish size (cm-diameter)		***10***	***6***	***4***
Tube 1	2X HBS	500 µl	180 µl	120 µl
Tube 2	ddH_2O	Up to 500 µl	Up to 180 µl	Up to 120 µl
	$CaCl_2$	61 µl	22 µl	18 µl
	DNA plasmid	10 µg	5 µg	4 µg

2.7.2 Lipofectamine transfection for siRNA

Rat islets of Langerhans were dispersed in a 24-well plate as described in section 2.3, 18-20 h prior to transfection using Lipofectamine^{TM} 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For InsR, IGF1R, RAPTOR or RICTOR knock-down, the cells were transfected for 48 h with 200 nM on-target plus siRNA (Dharmacon, Epsom, UK) against InsR (J-080102-10-0010), IGF1R (J-091936-10-0010), RICTOR (L-087724-00-0005) or RAPTOR (L-086862-00-0005). 200 nM siGENOME non-targeting siRNA (Dharmacon, Epsom, UK) (scrambled) was used as a

control. siRNA powder was dissolved in DEPC (diethylpyrocarbonate)-treated water (Invitrogen, Carlsbad, CA, USA).

Before transfection, growing media were first replaced by 400 µl Opti-MEM® I + Glutamax^{TM} -I (MEM stands for Eagle's minimum essential media, abbreviated as Opti-MEM from now on) media (Invitrogen, Carlsbad, CA, USA) per well, and then the following microtubes of mixtures were prepared:

1. 1 µl lipofectamine + 49 µl Opti-MEM media;
2. 2.5 µl of 20 µM siRNA stock + 49.5 µl Opti-MEM media.

Tubes were incubated at room temperature for 5 min, and then the content of tube 1 was transferred to tube 2. The mixture was further incubated for 20 min, before adding to their respective wells. Opti-MEM media were replaced by rat islet growing media 5-6 h post-transfection. Cells were then incubated for 48 h before harvest for their corresponding analysis.

2.8 Immunoprecipitation

The immunoprecipitation of mTORC1 and 2 was essentially performed as previously described (Dunlop et al. 2009). Briefly, HEK293 cells (10 cm dishes) were transfected with myc-mTOR as mentioned in section 2.7.1 and treated as described in the figures. After treatment, cells were lysed in 0.3% (w/v) CHAPS lysis buffer. Protein lysates were then centrifuged for 10 min at 16000 x *g*. The supernatants were kept, and total protein concentrations were determined by the Bradford assay (see section 2.10.1). Lysates containing 1.2 mg of protein were incubated with anti-myc antibody for 16 h at 4°C,

followed by the incubation with protein-G sepharose beads for a further period of 2 h at 4 °C. Beads were washed three times with 0.3% CHAPS buffer, and then resuspended in 2X Laemmli sample buffer before subjected to SDS-PAGE (see section 2.10) and western-blotting (see section 2.11).

2.9 7-methyl GTP pull down

After treatment, MIN6 cells were lysed in ice-cold triton lysis buffer. Protein lysates were then spun for 10 min at 16000 x *g*. The supernatants were kept, and total protein concentrations were determined by the Bradford assay (see section 2.10.1). 15 µl of 7-methyl GTP sepharose^{TM} 4B (Amersham Biosciences, Piscataway, NJ, USA) beads were washed once with triton lysis buffer before the addition of cell lysates. Beads and lysates were incubated for 1 h at 4 °C. Beads were then washed twice with triton lysis buffer and resuspended in 2X Laemmli sample buffer before subjected to SDS-PAGE (see section 2.10.2 and 2.10.3) and western-blotting (see section 2.11).

2.10 Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

2.10.1 Bradford assay

To determined the protein content of cell lysates, 1 ml Bradford assay reagent (Bio-Rad, Hercules, CA, USA) at 1:5 dilution was added to of either BSA (bovine serum albumin) standards or lysate samples with unknown protein content. The mixture was incubated for

5 min at room temperature, and then the absorbance at 595 nm was measured by the UV1101 Biotech photometer (Biochrom WPA, Cambridge, UK). Protein content of the lysates was determined by linear regression against the standard curve established using the BSA standards. Samples were then normalized to the lowest protein concentration with triton lysis buffer.

2.10.2 SDS-PAGE gel preparation

Polyacrylamide gels were prepared in either an ATTO (Tokyo, Japan) mini PAGE (polyacrylamide gel electrophoresis) system (14-well) or Bio-Rad (Hercules, CA, USA) mini-PAGE system using solutions listed in table 2-2.

Table 2-2 Solutions used in the preparation of SDS-PAGE gels, for two running and three (ATTO) or four (Bio-Rad) stacking gels. Tris: tris(hydroxymethyl)aminomethane; ddH_2O: double distilled water; SDS: sodium dodecyl sulphate; TEMED: N, N, N', N'-tetramethylethylenediamine; APS: ammonium persulfate.

Solution (ml)	***Running gels***						***Stacking gel***
ATTO	**7.5%**	**10%**	**12.5%**	**15%**	**17.5%**	**20%**	
40% acrylamide	2.925	3.825	4.725	5.625	6.525	7.275	1.24
2% bisacrylamide	1.56	2.04	2.52	3	3.48	3.75	0.65
1.5 M Tris-HCl pH 8.8	3.75	3.75	3.75	3.75	3.75	3.75	-
1M Tris-HCl pH 6.8	-	-	-	-	-	-	1.25
ddH_2O	6.54	5.165	3.78	2.4	1.02	-	6.7

10% SDS	0.15	0.15	0.15	0.15	0.15	0.15	0.1
TEMED	0.0075	0.0075	0.0075	0.0075	0.0075	0.0075	0.01
10% APS	0.11	0.11	0.11	0.11	0.11	0.11	0.075
Solution (ml)	**Running gels**						**Stacking gel**
Bio-Rad	**7.5%**	**10%**	**12.5%**	**15%**	**17.5%**	**20%**	
40% acrylamide	1.95	2.55	3.15	3.75	4.35	4.85	1.24
2% bisacrylamide	1.04	1.36	1.68	2	2.32	2.5	0.65
1.5 M Tris-HCl pH 8.8	2.5	2.5	2.5	2.5	2.5	2.5	-
1M Tris-HCl pH 6.8	-	-	-	-	-	-	1.25
ddH_2O	4.36	3.44	2.52	1.6	0.68	-	6.7
10% SDS	0.15	0.15	0.15	0.15	0.15	0.15	0.1
TEMED	0.005	0.005	0.005	0.005	0.005	0.005	0.01
10% APS	0.05	0.05	0.05	0.05	0.05	0.05	0.075

2.10.3 Running a SDS-PAGE gel

1 portion of 4X Laemmli sample buffer was added to 3 portions of normalized lysate samples, samples were then boiled for 3 min at 100 ºC before loaded on the SDS-PAGE gel alongside prestained protein marker (5-175 kDa) (New England Biolabs, Hitchin, Herts, UK).

ATTO or Bio-Rad gels were assembled in the gel tank from their respective manufacturers, the running buffer of the tank is comprised of 1X tris-glycine buffer and

0.1% (w/v) SDS. Gels were run at 180 V for 70-120 min depending on the nature of the gel, room temperature and the protein of interest.

2.11 Western-blotting

After SDS-PAGE, proteins were transferred on to Immobilon-P PVDF filter membranes (Millipore, Watford, UK) using a Semi-Dry or wet transfer system (both from Bio-Rad, Hercules, CA, USA). For a semi-dry transfer, membrane was soaked in 100% methanol for 1 min. Six Whatman® 3MM papers (GE Healthcare, Piscataway, NJ, USA) per gel, the membrane and the gel were then equlibrated in transfer buffer for 1 min. The paper/gel/membrane "sandwich" was then prepared as (from the bottom to the front): three 3MM papers, membrane, gel and another three 3MM papers. Air bubbles were removed after each layer of the "sandwich" was added. The transfer was then performed at 15 V for 38 min.

For a wet-transfer, membrane was soaked in 100% methanol for 1 min, two Whatman® 3MM papers per gel, the membrane and the gel were then equlibrated in transfer buffer. As the orientation of the electrodes is opposite to the semi-dry transfer tank, the paper/gel/membrane "sandwich" was prepared as (from the bottom to the front): one 3MM paper, gel, membrane and another 3MM paper. Air bubbles were removed after each layer of the "sandwich" was added. The transfer was then performed at 100 V for 1 h.

Following transfer, the membrane was blocked in 5% skimmed milk (Morrisons or ASDA, Leicester, UK)/1X PBST for 1 h at room temperature on the orbital shaker (Stuart Scientific, Chelmsford, Essex, UK). If the primary antibody was diluted in 5% milk/1X PBST, the blocking step was followed by the incubation at 4°C for 16 h with the specific antibody. If the primary antibody was diluted in 5% BSA in 1X PBST, the membrane was washed thrice for 5 min in 1X PBST on the orbital shaker prior to the 16 h priod of incubation. Dilution factors of the primary antibodies (table 2-3) were according to manufacturer's instructions, the abundance of the actual (phospho-) protein within the cell, and empirical experience. After 16 h incubation, the membrane was washed thrice for 5 min in 1X PBST. The membrane was then incubated with the appropriate secondary antibody (New England Biolabs, Hitchin, Herts, UK) typically at 1/3333 dilution, made in 5% milk/1X PBST, for 2 h at room temperature. The membrane was then washed thrice for 10 min with 1X PBST. Detection of proteins was performed by enhanced chemiluminescence (ECL) reactions using ECL reagents obtained from either Amersham Biosciences (Piscataway, NJ, USA) or Cheshire sciences (Chester, UK). Membranes were treated with the ECL reagent mixture for 1 min before exposed to X-ray film [obtained from either Genetic Research Institute (Essex, UK) or Wolf laboratories (Pocklington, York, UK)] in the dark room. The film was developed by a hyper-processor™ (Amersham Biosciences, Piscataway, NJ, USA) to produce images shown in the figures.

Table 2-3 List of primary antibodies used in this thesis. Dilution ratios given in the third lane are for western blotting. Abbreviations: ART: Andrew R Tee, Cardiff University; CB: Cambridge Biosciences,

Cambridge, UK; IP: immunoprecipitation; P-: phosphorylated; NEB: New England Biolabs, Hitchin, Herts, UK; SCB: Santa Cruz Biotechnology, Santa Cruz, CA, USA; WB: western blotting.

1ry antibody	***Obtained from***	***Dilution***	***2ry antibody***	***Application***
P-S6K1 Thr389	NEB	1:1000	Rabbit	WB
S6K1	NEB	1:1000	Rabbit	WB
P-rpS6 Ser240/Ser244	NEB	1:10000	Rabbit	WB
rpS6	NEB and SC	1:2000	Mouse	WB
P-4EBP1 Ser64	NEB	1:1000	Rabbit	WB
4EBP1	NEB	1:1000	Rabbit	WB
P-PKB Ser473	NEB	1:1000	Rabbit	WB
P-PRAS40 Thr246	NEB	1:1000	Rabbit	WB
P-ERK1/2 Thr202/Tyr204	NEB	1:1000	Rabbit	WB
P-AMPK Thr172	NEB	1:1000	Rabbit	WB
AMPK	NEB	1:1000	Rabbit	WB
Rheb (exogenous only)	SC	1:1000	Goat	WB
P-rpS6 Ser235/Ser236	NEB	1:2000	Rabbit	WB
RAPTOR	NEB	1:500	Rabbit	WB
RICTOR	NEB	1:500	Rabbit	WB
mTOR	NEB	1:500	Rabbit	WB
mLST8	NEB	1:1000	Rabbit	WB
PRAS40	NEB	1:1000	Rabbit	WB
Myc (9E10)	Sigma-Aldrich	-	-	IP
P-mTOR Ser2481	NEB	1:1000	Rabbit	WB

P-mTOR Ser2448	NEB	1:1000	Rabbit	WB
P-PKCα/βII Thr638/Thr641	NEB	1:1000	Rabbit	WB
PKB	NEB	1:1000	Rabbit	WB
GAPDH	SC	1:10000	Rabbit	WB
P-PKB Thr308	NEB	1:500	Rabbit	WB
Cleaved caspase 3	NEB	1:500	Rabbit	WB
HA	ART	1:1000	Mouse	WB
GFP	CB	1:1000	Rabbit	WB
eIF4E	NEB	1:1000	Rabbit	WB

2.12 PKB kinase assay

After treatment, MIN6 cells (10 cm dishes) were lysed in PKB kinase assay lysis buffer. Protein lysates were then spun for 10 min at 16000 x *g*. The supernatants were kept, and total protein contents were determined by the Bradford assay (see section 2.10.1). For each immunoprecipitation, 4 µg of anti-PKB antibody (Millipore, Watford, UK) were added to 15 µl protein-G sepharose beads (previously equilibrated in PKB kinase assay lysis buffer) and 500 µl PKB kinase assay lysis buffer, the mixture was incubated at 4 ºC for 30 min with rotation. 500 µg cell lysates were added to the beads/antibodies and were further incubated at 4 ºC for 1 h with rotation. Beads were then washed thrice with PKB kinase assay lysis buffer containing 0.5 M NaCl before being washed twice with PKB kinase assay buffer and once with 1X ADB.

The activity of PKB determined using crosstide (GRPRTSSFAEG) as a substrate peptide (30 mM) (Millipore, Watford, UK) as previously described (Cross et al. 1995). Briefly, the following solutions were added to the beads per sample: 10 µl of ice-cold 1X ADB, 10 µl of 40 µM PKA inhibitor peptide, 10 µl of 100 µM crosstide and 10 µl Mg^{2+}-[γ-32P]ATP mix (1 µCi/µl final concentration of [γ-32P]ATP). The mixture was incubated for 10 min at 30 ºC and then pulse spun, 30 µl of the supernatant fraction was spotted onto the centre of a 2cm x 2cm P81 phosphocellulose paper square. Paper squares were washed thrice with 75 mM phosphoric acid for 5 min per wash at room temperature before being washed once (5 min, room temperature) in acetone. Paper squares were then transferred to a scintillation counting vial followed by the addition of 3 ml emulsifier-safe™ (PerkinElmer Inc, Waltham, Massachusetts, USA). Vials were read by a LS6400 multi-purpose scintillation counter (Beckman Coulter, Fullerton, CA) to detect the CPM (counts per minute) of each vial.

2.13 Protein synthesis measurements

Cells were pre-incubated in KRB supplemented with 0.5X amino acids for 1 h, before treated as described in the figure legends in the presence of 20 µCi [^{35}S]methionine per sample. Cells were then lysed in ice-cold triton lysis buffer, protein content was determined using Bradford assay (see section 2.10.1), samples were normalized to the lowest concentration. 20 µg of proteins were spotted onto the centre of a 2cm x 2cm P81 phosphocellulose paper square. Paper squares were washed by boiling them twice in 5% TCA (trichloroacetic acid) containing 0.1 g/L L-methionine for 1 min, and washed once with 5% TCA containing 0.1 g/L L-methionine and once with absolute ethanol, both

under room temperature. Paper squares were dried out at 80 ºC for 1 h, and then transferred to a scintillation counting vial followed by the addition of 3 ml emulsifier-safe™. Vials were read by a LS6400 multi-purpose scintillation counter to detect the CPM of each vial.

2.14 Flow cytometry

Following treatment, the media was removed and kept. The cells were then typsinized using 1X trypsin/EDTA (0.5%). Growing media was added and the cells were gently dispersed by pipetting, combined with the reserved media and centrifuged at 200 x *g* for 5 min at room temperature. The cell pellet was gently resuspended in 100 µl ice cold 1X PBS, fixed upon drop by drop addition of 900 µl methanol and kept at -20 ºC for 16-24 h before processing. Prior to flow cytometry analysis, cells were pelleted by centrifugation at 200 x *g* for 5 min at room temperature and the media were removed, precipitates were resuspended in propidium iodide-RNase-PBS mix and incubated for 30 min at 37 ºC. Quantification of staining was performed using a FACSCalibur flow cytometer and Cellquest software (BD Biosciences, San Jose, CA, USA), 10000 events were recorded per sample.

2.15 Cell death detection ELISA

Following treatment as described in the figure legends, evaluation of cell death was performed using the Cell Death Detection ELISA^{PLUS} kit (Roche, Burgess Hill, UK), as per manufacturer's instructions. Absorbance was measured at 405 nm against 2,2'-azino-

bis(3-ethylbenzothiazoline-6-sulphonic acid) (ABTS) solution and ABTS stop solution as blank using the Novostar plate reader (BMG Labtech, Aylesbury, UK). The results are expressed in arbitrary units of oligonucleosome-associated histone.

2.16 Insulin secretion assay

Following treatment, islets were incubated in KRB buffer supplemented with 1 mM glucose for 60 min at 37°C. The incubation continued in KRB buffer containing 1 or 20 mM glucose for a further 60 min at 37°C. The supernatants were collected. Insulin concentration in the supernatants were assayed using anti-rat insulin ELISA kit (DRG Instruments GmbH, Marburg, Germany) with rat insulin as a standard in accordance with manufacturer's instructions. The absorbance was read at 450 nm on the Novostar plate reader.

2.17 Proliferation assay using [^{3}H]thymidine

10000 INS1E cells or 75 rat islets of Langerhans per treatment were used for proliferation assay. INS1E or islet cells were starved in RPMI media supplemented with 0.2% FBS (for islets only) and 2 or 7.8 mM glucose in the absence or presence of 10 nM GLP-1 or exendin-4 for 96 h. Inhibitors (200 nM rapamycin or 10 μM AKTi) and 10 μCi [^{3}H]thymidine per sample were added during the last 24 h. Cells were then collected and washed thrice with 1X PBS. DNA were precipitated on ice upon the addition of 5% TCA for 30 min. Samples were spun at 16000 x *g*, 4°C for 10 min, DNA pellets were then washed twice with 5% TCA and then solubilised by adding 0.1 N NaOH and incubated

for 30 min at room temperature. [^{3}H]thymidine incorporation was determined by scintillation counting using the LS6400 multi-purpose scintillation counter to detect DPM (disintegrations per minute) in each sample.

2.18 Quantification and statistical analysis

Immunoblot band intensities were quantified using the ImageJ (version 1.44) software. Statistical analyses were performed as indicated in the figure legends using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA).

Chapter 3. Role of mTORC1 in the regulation of β-cell mass

3.1 Introduction

3.1.1 mTORC1 and pancreatic β-cell mass

A large number of studies have revealed that mTORC1 plays a key role in the regulation of β-cell mass and is critical to β-cell adaptation. For instance, glucose is a potent *in vivo* stimulator of β-cell mass in rodents (Bonner-Weir et al. 1989, Bernard et al. 1998, Paris et al. 2003, Topp, McArthur and Finegood. 2004), and *in vitro* glucose can stimulate β-cell proliferation (Kwon et al. 2004, Bartolome, Guillen and Benito. 2010) and protein synthesis (Xu et al. 1998b, Gomez et al. 2008) in a rapamycin-sensitive fashion. Transgenic mouse models of upstream regulators and downstream effectors of mTOR have also provided evidence demonstrating that mTORC1 plays a critical role in the regulation of β-cell mass *in vivo* (Pende et al. 2004, Ruvinsky et al. 2005, Pende et al. 2000, Shigeyama et al. 2008, Rachdi et al. 2008, Hamada et al. 2009). S6Ks and 4EBPs are two of the best characterized mTORC1 downstream targets (see section 1.3.1). S6K1 is a positive effector of β-cell size and function (Pende et al. 2000), yet it has not been reported whether it controls β-cell proliferation. In contrast, the role of 4EBPs in the regulation of β-cell mass is currently unknown. Interestingly, it has been suggested that in mammalian cells S6Ks control cell size whereas 4EBPs regulate proliferation (Dowling et al. 2010). Therefore, it is plausible that 4EBPs play roles in the stimulation of pancreatic β-cell replication.

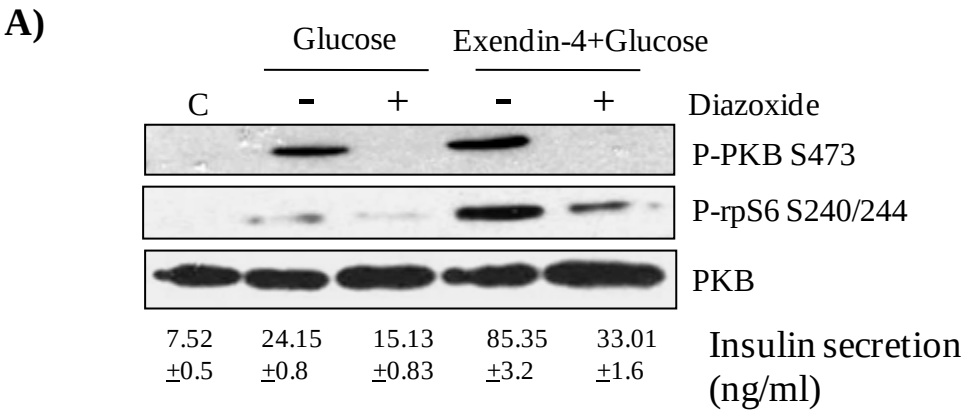
3.1.2 GLP-1 and pancreatic β-cell mass

GLP-1 is a hormone secreted in the gut to enhance postprandial insulin release and glucose uptake (McIntyre, Holdsworth and Turner. 1965), when binding to its receptor GLP-1R (GLP-1 receptor), a Gs-protein coupled receptor (GsPCR), it results in an increase in $[cAMP]_i$, which in turn activates a number of intracellular signalling pathways that leads to the augmentation of insulin release from β-cells [reviewed in (Doyle and Egan. 2007, Estall and Drucker. 2006)]. In the presence of glucose, GLP-1 is also a potent stimulator of β-cell mass *in vivo* (Buteau et al. 1999, Xu et al. 1999, Tourrel et al. 2001, Stoffers et al. 2000) and has been shown to potentiate glucose-stimulated mTORC1 activation in isolated islets and clonal cell lines via a PI3K dependent mechanism (Moore et al. 2009, Kwon et al. 2004). Therefore, GLP1-stimulated β-cell replication is likely to be mediated, at least in part, through the activation of mTORC1.

3.1.3 Chapter aims

Previous results from our lab have indicated that the activation of mTORC1 by exendin-4, a GLP-1 analog isolated from the lizard (*Heloderma suspectum*) venom, which unlike GLP-1, are non-degradable upon the action of dipeptidyl peptidase IV (DPP-IV) (Parker et al. 1984, Eng et al. 1992), correlated with an increase in PKB phosphorylation, both of which can be inhibited by diazoxide, a potassium channel activator, which subsequently led to a reduction in intracellular calcium levels and thereby blocked insulin/IGF1/2 secretion (Howell and Taylor. 1966). This observation implies that the activation of

mTORC1 by GLP-1/exendin-4 is dependent upon the autocrine effect of either insulin or IGF1/2 and the subsequent stimulation of PKB (Fig. 3.1, performed by El Sayed NM). Therefore, the aims of this chapter are to further elucidate how mTORC1 is activated by GLP-1/exendin-4 and whether the up-regulation of β-cell replication by GLP-1/exendin-4 is mediated through the stimulation of mTORC1, and ultimately unravel the molecular mechanism by which mTORC1 controls β-cell cycle progression.



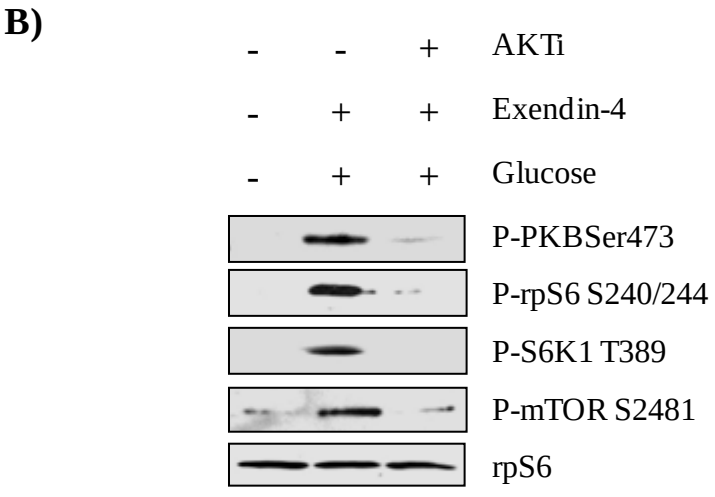


Figure 3.1 Activation of mTORC1 by GLP-1 is dependent upon the autocrine effect of insulin and the stimulation of PKB. **A)** Rat islets of Langerhans were serum starved over night in CMRL media supplemented with 1X L-glutamine, and then incubated in KRB buffer containing 2.8 mM glucose. Cells were treated with glucose (7.8 mM) or Exendin-4 (20 nM) plus glucose (7.8 mM) in the presence or absence of 250 μM diazoxide for 30 min. Insulin secretion was determined using insulin ELISA kit following manufacturer's instructions. **B)** Islets were treated as in A) in the presence or absence of AKTi (1 μM) for 30 min. Cells lysates were separated on a SDS-PAGE gel and immunoblotted for phosphorylated (P)-PKB Ser473 (S473), P-rpS6 Ser240/Ser244 (S240/244), P-S6K1 Thr389 (T389), P-mTOR Ser2481 (S2481), total PKB or rpS6 were used as loading controls. Experiments performed by El Sayed NM.

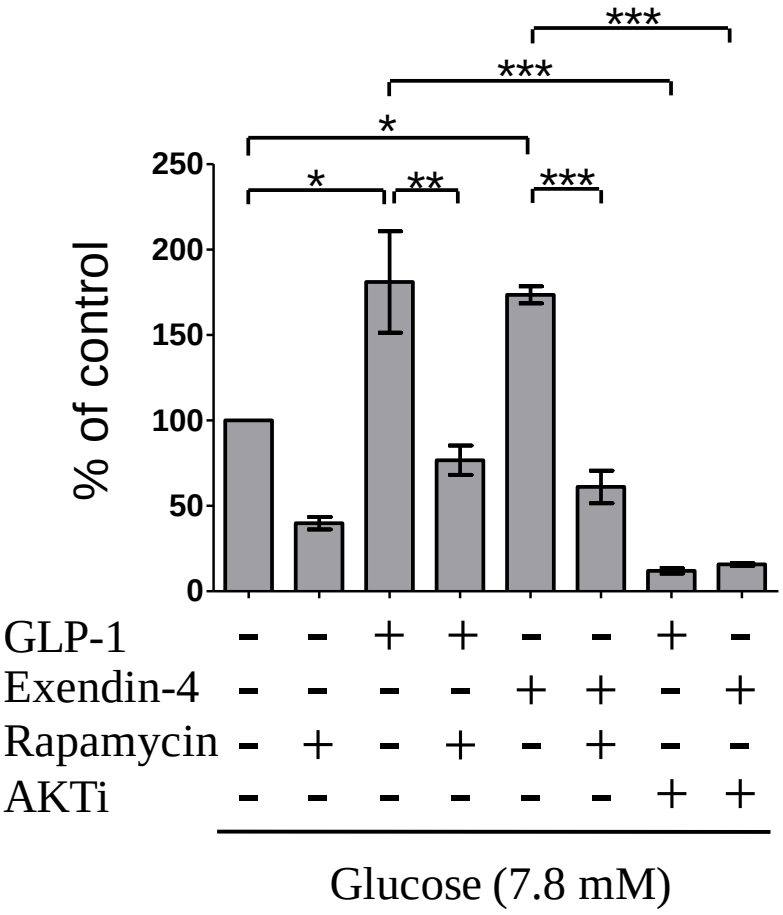
3.2 Results

3.2.1 GLP-1/exendin-4 regulates β-cell replication in a PKB and mTOR dependent manner

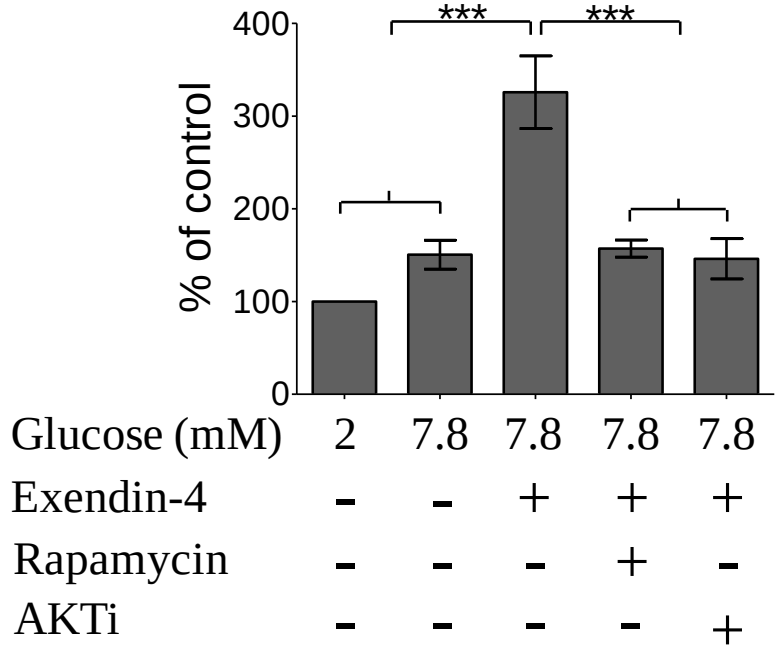
Previous studies have indicated that the proliferative effect of GLP-1/exendin-4 on pancreatic β-cells involves a number of factors including the transactivation of EGFR (INS1 cells and rat islets) (Buteau et al. 2003) or IGF1R (MIN6 cells and mouse islets) (Cornu et al. 2010), the stimulation of IRS2 (mouse islets) (Park et al. 2006), PI3K (INS1 cells) (Buteau et al. 1999), PKB (INS1 cells) (Wang et al. 2004), FoxO (INS832/13 cells and mouse islets) (Buteau, Spatz and Accili. 2006), PKCζ (INS832/13 cells) (Buteau et al. 2001), β-catenin/TCF7L2 (transcription factor 7-like 2) (INS1 cells) (Liu and Habener. 2008) and Pdx1 (pancreatic duodenal homeobox 1) (mouse islets) (Buteau et al. 1999, Li et al. 2005) and the inhibition of SirT1 (silent information regulator two protein 1) (INS832/13 cells and mouse islets) (Bastien-Dionne et al. 2011). However, it remains to be investigated whether GLP-1/exendin-4 induces β-cell proliferation via PKB and

mTOR in primary rodent islets. To investigate this, I performed a cell proliferation assay using a modified protocol to the one established by McDaniel's group (Kwon et al. 2006). Briefly, either rat insulinoma cell line INS1E (Fig. 3.2A) or primary rat islets of Langerhans (Fig. 3.2B) were cultured in RPMI media supplemented with 2 or 7.8 mM glucose in the presence or absence of GLP-1 or exendin-4. During the final 24 h of the 96 h incubation, [^{3}H]thymidine and PKB inhibitor AKTi (Barnett et al. 2005) or rapamycin were added and the incorporation of [^{3}H]thymidine into the cell DNA were determined. As shown in Fig. 3.2, cells incubated with GLP-1 and exendin-4 represented increased rates of replication (80% increase for GLP-1, 70% increase for exendin-4 treated INS1E cells and 110% increase for exendin-4 treated rat islets compared to 7.8 mM glucose treated control) as determined by [^{3}H]thymidine incorporation, which is effectively blocked by AKTi and rapamycin. Interestingly, rates of [^{3}H]thymidine incorporation in INS1E cells treated with AKTi or rapamycin were dramatically reduced to 20-40% of basal levels (Fig. 3.2A), implying that the inhibition of PKB and mTOR may also severely impair cell viability in INS1Es.

A)


***
***
*
*
**
***
250
200
150
100
50
0
% of control
GLP-1 - - + + - - + -
Exendin-4 - - - - + + - +
Rapamycin - + - + - + - -
AKTi - - - - - - + +
Glucose (7.8 mM)

B)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400
300
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0
% of control
Glucose (mM) 2 7.8 7.8 7.8 7.8
Exendin-4 - - + + +
Rapamycin - - - + -
AKTi - - - - +

Figure 3.2 GLP-1 and exendin-4 enhance β-cell proliferation via PKB and mTOR. **A)** INS1E cells were incubated in RPMI containing 0.2% FBS and 7.8 mM glucose for 96 h in the presence or absence of 10 nM GLP-1 or 10 nM exendin-4, 10 μCi [^{3}H]thymidine, 200 nM rapamycin and 10 μM AKTi were added during the last 24 h. DNA from the samples were extracted by TCA precipitation and [^{3}H]thymidine incorporation was determined using a scintillation counter. **B)** Proliferation assays were performed in rat islets of langerhans as in A). *P* values were obtained using a one-way ANOVA followed by Bonferroni adjustment. Data are shown as means ± SE, $n = 3$. *P = 0.05-0.01, ***P* = 0.01-0.001, ****P* < 0.001.

To confirm that under the experimental conditions established in the proliferation assays (Fig. 3.2), PKB and mTORC1 are activated upon exendin-4 treatment, rat islets of Langerhans were starved in RPMI media supplemented with 0.2% FBS and 2 mM glucose for 16 h, and then treated with 7.8 mM glucose alone or plus exendin-4 for 2 h in the presence or absence of rapamycin. As anticipated, 7.8 mM glucose alone induces PKB and rpS6 (as readout of mTORC1 activity) phosphorylation on Ser473 and Ser240/Ser244, respectively, and exendin-4 greatly enhances the phosphorylation of both proteins (Fig. 3.3), furthermore, rapamycin pretreatment blocked exendin-4-stimulated rpS6 phosphorylation on Ser240/Ser244 (Fig. 3.3). These results indicated that under experimental conditions applied for the proliferation assays, exendin-4 indeed activates PKB and mTORC1.

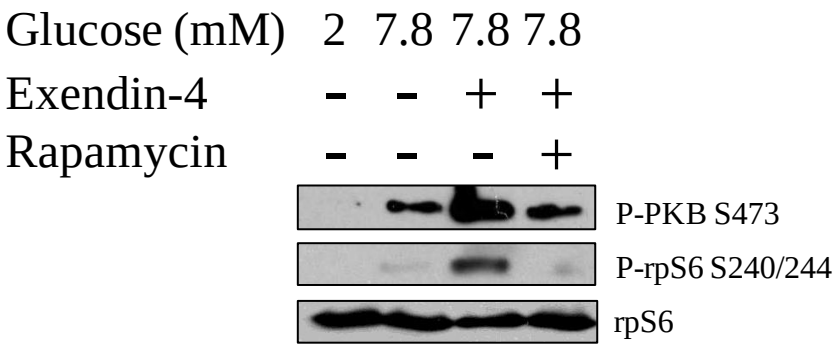


Figure 3.3 Exendin-4 enhances glucose-induced PKB and rpS6 phosphorylation. Rat islets of Langerhans were starved in RPMI media containing 0.2% FBS and 2 mM glucose for 16 h, followed by the treatment of 7.8 mM glucose alone or 7.8 mM glucose plus 10 nM exendin-4, in the presence or absence of rapamycin for 2 h. Cells were then lysed in 1X Laemmli sample buffer and proteins were separated on a SDS-PAGE gel, and then western blotted using antisera against phospho (P)-PKB Ser473 (S473) and P-rpS6 Ser240/Ser244 (S240/244). rpS6 was used as a loading control. Immunoblots are representative of two independent experiments.

3.2.2 Exendin-4 activates mTORC1 via transactivation of IGF1R

Given that both PKB and mTOR are responsible for the proliferative effect of GLP-1 in pancreatic β-cells, I next sought to find out how mTORC1 is regulated by GLP-1. Previous studies have demonstrated that GLP-1 can induce the transactivation of EGFR (Buteau et al. 2003) and IGF1R (Cornu et al. 2010) to enhance β-cell replication, EGFR inhibitors are able to abrogate the augmentation of PI3K activity elicited upon GLP-1 treatment in INS832/13 cells (Buteau et al. 2003). However, data from our lab have indicated that EGFR inhibitors are unable to block the GLP-1-mediated PKB and mTORC1 activation in INS1E, MIN6 and rat islets of Langerhans (El Sayed NM, Herbert

TP, unpublished data). On the other hand, IGF1R knockdown using siRNA in MIN6 cells abolishes GLP-1-induced PKB activation (Cornu et al. 2009), therefore, I hypothesized that the transactivation of IGF1R is responsible for the stimulation of mTORC1 by GLP-1.

For this purpose, I transfected dispersed rat islets with siRNA against either InsR or IGF1R and treated them with exendin-4 for 1 h. As shown in Fig. 3.4, GLP-1 treatement led to an increase in the phosphorylation of PKB (Ser473) and, as a readout of mTORC1 activation, rpS6 (Ser240/Ser244). Both siRNAs were able to effectively block the expression of their respective receptors. InsR knockdown has no effect on the phosphorylation of PKB and rpS6, whereas IGF1R knockdown abolished both phosphorylations (Fig. 3.4). This observation indicates that GLP-1/exendin-4 stimulates PKB and mTORC1 via IGF1R in rat islets of Langerhans.

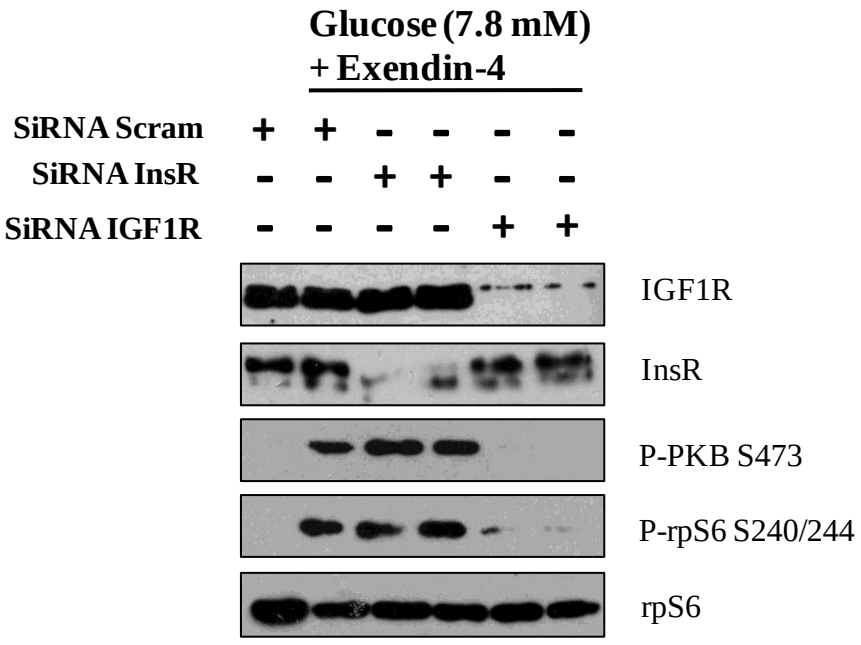


Figure 3.4 Exendin-4 activates PKB and mTORC1 via IGF1R. Dispersed rat islets of Langerhans were transfected with InsR or IGF1R siRNA for 48 h, cells were serum starved in CMRL media supplemented with 0.5X L-glutamine for 16 h, followed by an incubation in KRB buffer supplemented with 0.5X amino acids for 30 min in the presence or absence of 7.8 mM glucose and 10 nM exendin-4. Cells were then lysed in 1X Laemmli sample buffer and lysates were separated on a SDS-PAGE gel, followed by western blotting for phospho (P)-PKB Ser473 (S473), P-rpS6 Ser240/Ser244 (S240/244), as well as InsR, IGF1R and rpS6. Immunoblots are representative of three independent experiments.

3.2.3 Characterization of adenovirus expressing hypophosphorylated 4EBP1

S6Ks and 4EBPs are the best characterized mTORC1 downstream targets. Pancreatic β-cells isolated from S6K1 knockout mice represent a 24% reduction in size compared to their wild-type countermates (Pende et al. 2000), yet changes in β-cell proliferation haven't been reported in S6K1 knockout (Pende et al. 2000) or S6K1/2 double knockout (Pende et al. 2004) animal models. Of interest, it has recently been reported that mTORC1 activation increases β-cell replication by controlling the synthesis and stability of cell cycle regulators (Balcazar et al. 2009). In addition, Sonenberg's group has reported that in mammalian cells the effect of mTORC1 on cell growth and proliferation are mediated by S6Ks and 4EBPs, respectively (Dowling et al. 2010), therefore, it is plausible that the effect of mTORC1 on β-cell proliferation is mediated by the 4EBPs.

To investigate this, a HA and GFP tagged, non-phosphorylatable 4EBP1 in which all five phosphorylation sites mentioned in Fig. 1.6 are mutated to alanine (Ad 4EBP1-5A), were expressed by adenoviral infection in MIN6 cells. As demonstrated in Fig. 3.5, as little as

1 µl of the control virus (Ad GFP) produces a similar level of GFP expression as 20 µl Ad 4EBP1-5A, therefore, 1 µl Ad GFP and 20 µl Ad 4EBP1-5A were applied in subsequent experiments.

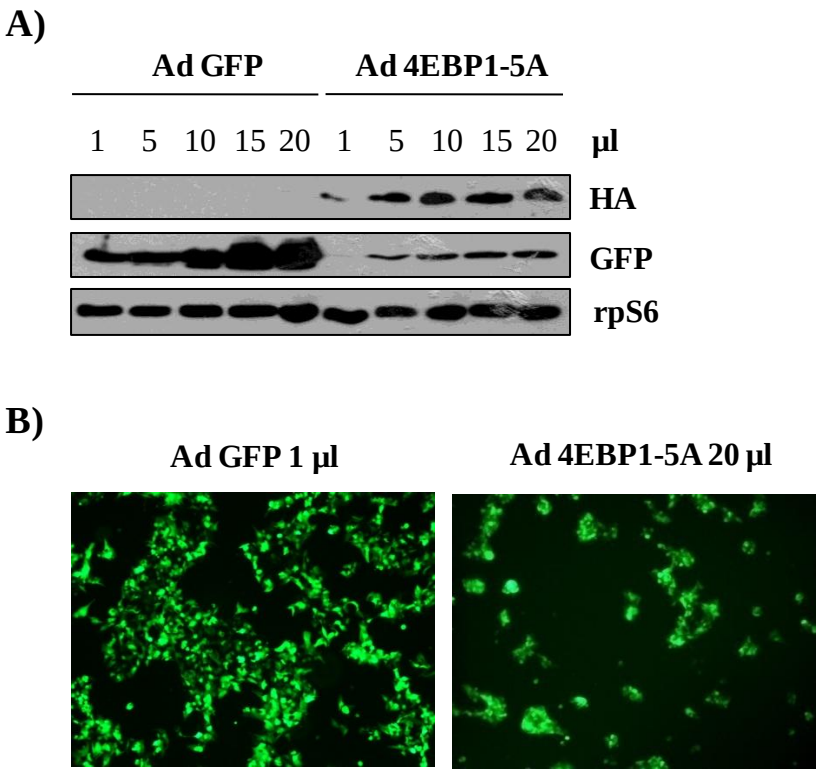


Figure 3.5 Titration of Ad 4EBP1-5A. **A)** MIN6 cells were infected with indicated amount of adenovirus over-expressing HA tagged non-phosphorylated form of 4EBP1 in which five phosphorylable serine and threonine residues were replaced by alanine (Ad 4EBP1-5A, the plasmid also expresses GFP), cells infected with Ad GFP were used as controls, cells were lysed 48 h post infection. Lysates were subjected to SDS-PAGE followed by western blotting for total HA and GFP, rpS6 was used as a control. Immunoblots are from one experiment. **B)** Green fluorescence images of MIN6 cells infected with 1 µl of Ad GFP and 20 µl of Ad 4EBP1-5A.

3.2.4 Expression of hypophosphorylated 4EBP1 increases its binding to eIF4E

The activation of mRNA for cap-dependent mRNA translation initiation involves eIF4F complex (comprised of eIF4A, eIF4E and eIF4G) formation and its attachment to the mRNA cap-proximal region. The binding of unphosphorylated 4EBPs to eIF4E inhibits eIF4F complex formation and cap-dependent translation [reviewed in (Sonenberg and Hinnebusch. 2009, Jackson, Hellen and Pestova. 2010)]. To investigate whether exogenously expressing 4EBP1-5A increases the association of 4EBP1 and eIF4E, I infected MIN6 cells with either Ad GFP or Ad 4EBP1-5A and precipitated cap-interacting proteins using m^7GTP sepharose beads, which mimic the 5'mRNA cap, from the cell lysates, and as a positive control, cells were also treated with the allosteric mTOR inhibitor rapamycin (Fig. 3.6). Ad 4EBP1-5A effectively increased the binding of 4EBP1 to eIF4E in cells to even a greater extent compared to rapamycin treated ones. 4EBP1-5A had no effect on the phosphorylation of rpS6, indicating that the S6K-rpS6 arm of mTORC1 was unaffected upon Ad 4EBP1-5A infection, whereas rapamycin completely blocked rpS6 phosphorylation (Fig. 3.6).

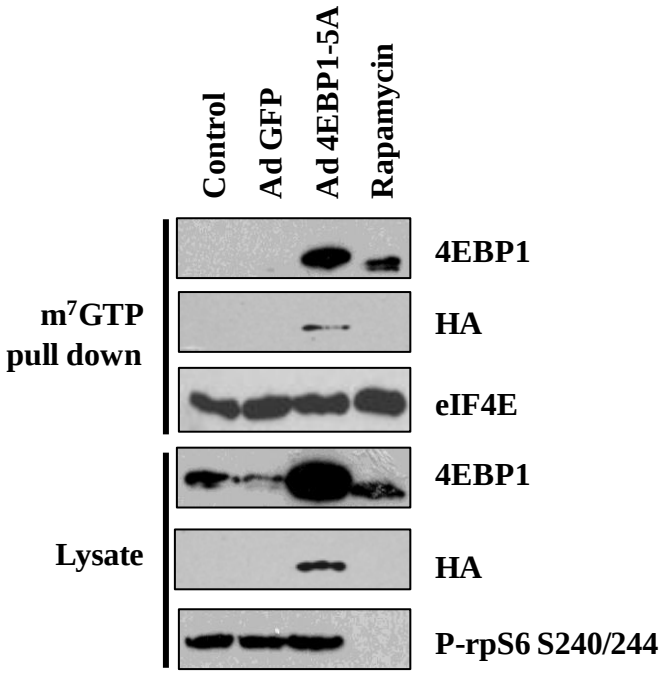


Figure 3.6 Over-expression of hypophosphorylated 4EBP1 increases 4EBP1-eIF4E binding. MIN6 cells were infected with 1 µl of Ad GFP or 20 µl of Ad 4EBP1-5A, 4EBP1 cap binding activity was determined by m^7GTP pulldown assay 48 h after infection. Mock infected cells treated with rapamycin (200 nM, 24 h) were used as a control. Proteins were lysed and resolved on SDS-PAGE followed by immunoblotting using antisera against 4EBP1, HA, eIF4E and phosphorylated (P)-rpS6 Ser240/Ser244 (S240/244). Immunoblots are representative of two independent experiments.

3.2.5 Hypophosphorylated 4EBP1 impairs cell size and survival

Several studies have indicated that the effect of mTORC1 on mammalian cell proliferation is mediated through the 4EBP-eIF4E pathway (Fingar et al. 2004, Dowling et al. 2010, Lynch et al. 2004, Barnhart et al. 2008), over-expression of hypophosphorylated 4EBP1 blocks G_1 to S cell cycle progression whereas over-

expression of eIF4E has the opposite effect in a variety of cell types (Fingar et al. 2004, Lynch et al. 2004, Barnhart et al. 2008). To determine the effect of expressing hypophosphorylated 4EBP1 on β-cell proliferation, MIN6 cells were infected with Ad GFP or Ad 4EBP1-5A and then maintained in growing media for 48 h before subjected to flow cytometry analysis. Surprisingly, 4EBP1-5A expression led to a dramatic increase in the sub G_1 population (around 25%), indicative of cell death, which is accompanied by decreases in the number of cells within the G_0/G_1 (15% decrease) and G_2/M phase (10% decrease), whereas the percentage of cells in S phase was similar between Ad GFP and Ad 4EBP1-5A infected cells (Fig. 3.7A and B). It is possible that the increase in sub G_1 population induced by Ad 4EBP1-5A was resulted from blocking G_1 to S cell cycle progression.

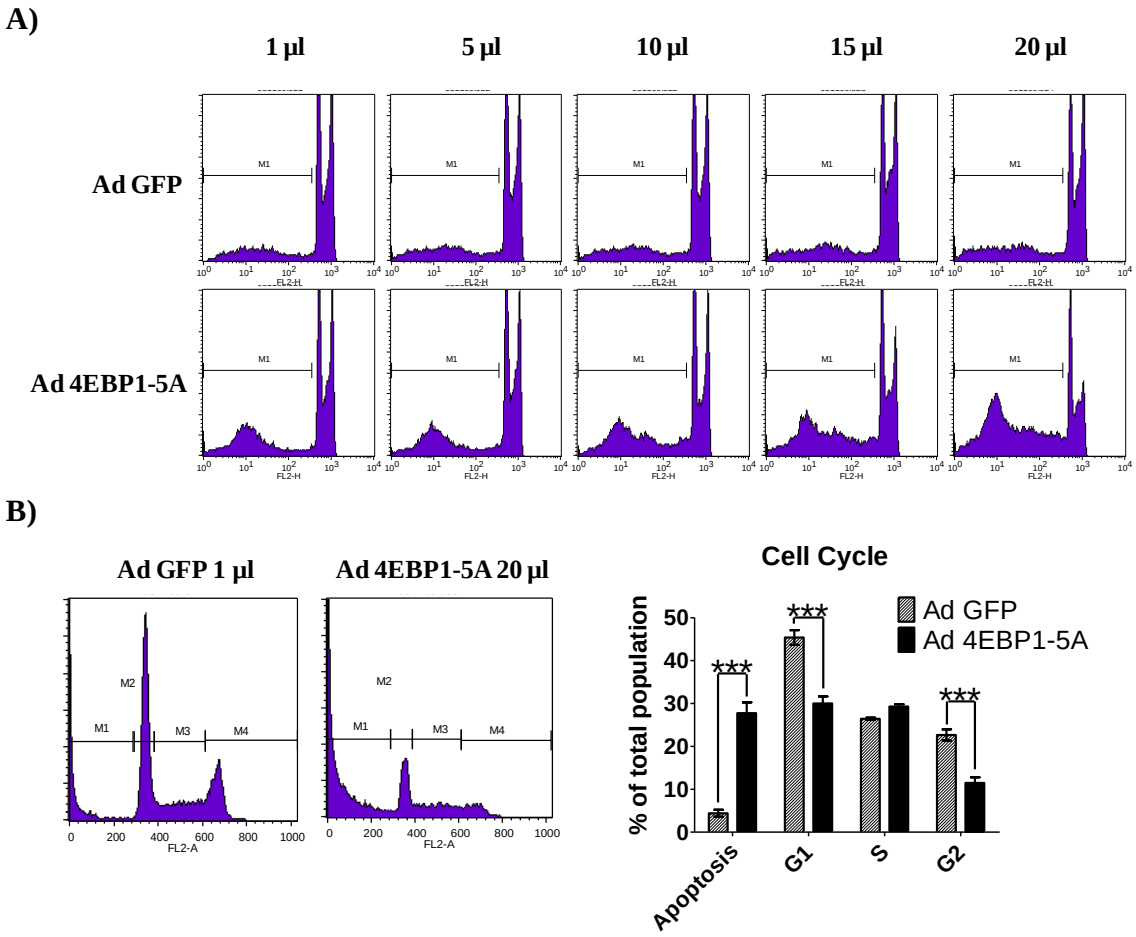


Figure 3.7 Over-expression of 4EBP1-5A induces MIN6 cell death. **A)** MIN6 cells were infected with increasing concentrations of Ad GFP or Ad 4EBP1-5A and then maintained in growing media for 48 h, before being trypsinized and subjected to flow cytometry analysis to determine cell cycle distribution (log scale). *X* axis: FL2-H, *Y* axis: cell numbers; M1 indicates sub G1 population. **B)** Cell cycle distribution of MIN6 cells infected (48 h) with 1 µl of Ad GFP or 20 µl of Ad 4EBP1-5A was determined on a linear scale. *X* axis: FL2-A, *Y* axis: cell numbers; M1: sub G1, M2: G0/G1, M3: S, M4: G2/M. Results are presented as % of total cell population and as means±SE.

mTORC1 is a positive regulator of cell size. To investigate whether hypophosphorylated 4EBP1 has any effect on β-cell size, MIN6 cells infected with either Ad GFP or Ad

4EBP1-5A were also subjected to cell size analysis using flow cytometry. As shown in Fig. 3.8, 4EBP1-5A expression caused a reduction in overall cell size and within individual cell cycle phases for approximately 10-15%, as can be judged by the shift detected on the FSC-H (forward scatter-height) curve. Therefore, this provides evidence that hypophosphorylated 4EBP1 severely impairs cell growth and survival in MIN6 cells.

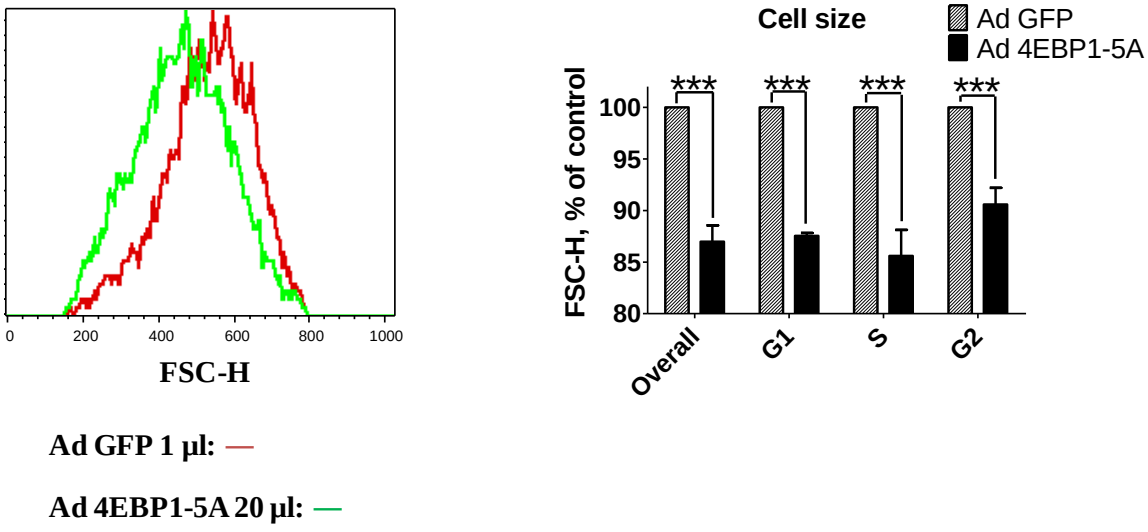


Figure 3.8 MIN6 cells were infected with increasing concentrations of Ad GFP (1 µl) or Ad 4EBP1-5A (20 µl), 48 h after infection, cells were trypsinized and subjected to flow cytometry analysis to determine cell size. G0/G1 phase cell size was compared using two-dimensional FSC analysis and represented on the left. *X* axis: FSC-H, *Y* axis: cell numbers. Overall, G0/G1, S or G2/M cell size was also quantified as shown on the right. Data is presented as % of control (Ad GFP, 1 µl) and as means ± SE, $n = 3$. *P* value is obtained by two-way ANOVA with Bonferroni post-test. ***$P < 0.001$.

3.2.6 Exendin-4 does not cause the dissociation of 4EBP1 from eIF4E

In section 3.2.1 and 3.2.2, it has been demonstrated that exendin-4 was able to induce rpS6 phosphorylation on Ser240/Ser244, in rat islets of Langerhans, indicative of mTORC1 activation. To further investigate whether exendin-4 can inactivate 4EBP1, I treated rat islets with exendin-4 in the presence or absence of rapamycin, and precipitated cap-interacting proteins using m^7GTP sepharose beads from the cell lysates. As anticipated, exendin-4 treatment led to an increase of rpS6 phosphorylation, yet surprisingly, it had no effect on the association of 4EBP1 to eIF4E. In contrast, rapamycin caused an increase in 4EBP1-eIF4E binding. This result indicated that 4EBP1 may not be implicated in the induction of β-cell proliferation by GLP-1/exendin-4.

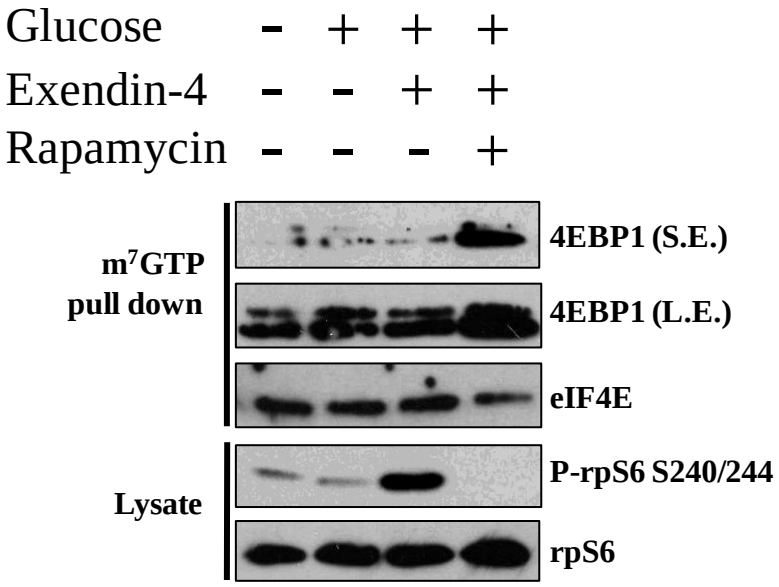


Figure 3.9 Exendin-4 does not affect 4EBP1-eIF4E binding. Rat islets of Langerhans were starved in RPMI media containing 0.2% FBS and 2 mM glucose for 16 h, followed by the treatment of 7.8 mM glucose

alone or 7.8 mM glucose plus 10 nM exendin-4, in the presence or absence of rapamycin for 2 h. Cells were then lysed in triton lysis buffer. 4EBP1 cap binding activity was determined by m^7GTP pulldown assay. Proteins were lysed and resolved on SDS-PAGE followed by immunoblotting using antisera against 4EBP1, eIF4E, phosphorylated (P)-rpS6 Ser240/Ser244 (S240/244), and as a loading control, rpS6. S.E.: short exposure; L.E.: long exposure. Immunoblots are representative of two independent experiments.

3.3 Discussion

A large number of studies have provided strong evidence that mTORC1 plays a critical role in the maintenance of pancreatic β-cell mass (Pende et al. 2004, Ruvinsky et al. 2005, Pende et al. 2000, Shigeyama et al. 2008, Rachdi et al. 2008, Hamada et al. 2009). GLP-1, an insulin secretagogue which augments $[cAMP]_i$, is able to activate mTORC1 in β-cells (Kwon et al. 2004). Here in this chapter, I have demonstrated that GLP-1 and its analog exendin-4 can increase cell proliferation in INS1E cells and primary rat islets of Langerhans, and this is mediated by the mTOR pathway.

Cornu *et al*. reported that augmentation in $[cAMP]_i$ can increase the expression of IGF1R mRNA in pancreatic β-cells (Cornu et al. 2010), moreover, the autocrine secretion of IGF2 and transactivation of IGF1R are crucial for the proliferative (Cornu et al. 2010) and anti-apoptotic (Cornu et al. 2009) effect of GLP-1. Importantly, IGF1R transactivation stimulates PKB (Cornu et al. 2010, Cornu et al. 2009). Of interest, the stimulation of IRS2 (Park et al. 2006), PI3K (Buteau et al. 1999) and PKB (Wang et al. 2004) have all been shown to play important roles in the regulation of β-cell replication. Based on these studies (Buteau et al. 1999, Cornu et al. 2010, Park et al. 2006, Wang et al.

2004, Cornu et al. 2009) and results I obtained in this chapter, one possible mechanism by which GLP-1 stimulates β-cell replication is via the autocrine activation of IGF1R via IGF2, which in turn induces the activation of IRS2, PI3K, PKB and mTOR (Fig. 3.10). Therefore, my data has further revealed the molecular mechanism upon which GLP-1 elicits mTORC1 stimulation and increases β-cell proliferation.

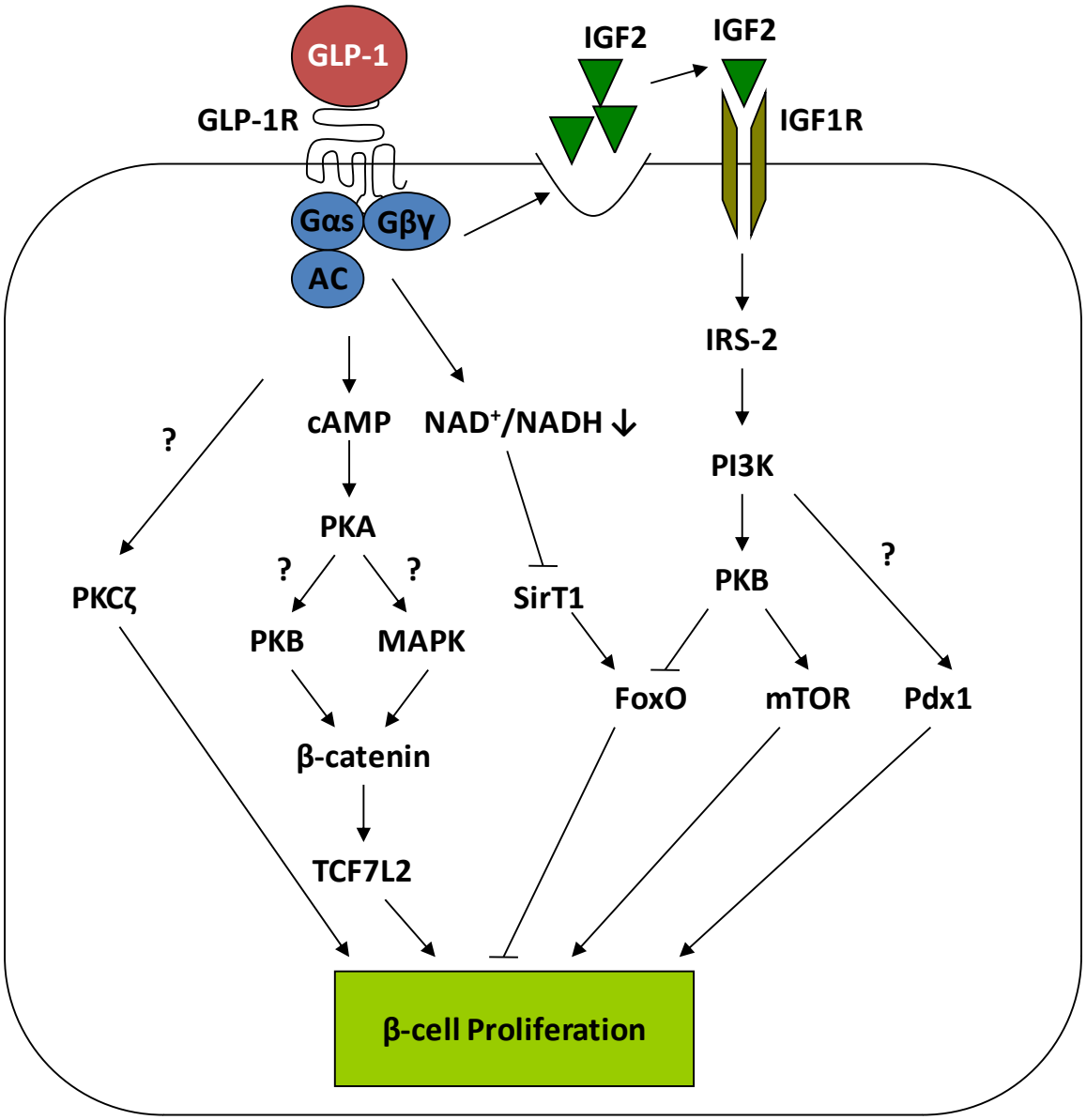


Figure 3.10 Signalling pathways implicated in GLP-1-induced β-cell proliferation. A number of signalling pathways are involved in the proliferative effect of GLP-1, including the activation of PKCζ (Buteau et al. 2001), PKA/PKB/MAPK/β-catenin/TCF7L2 (Liu and Habener. 2008), PI3K/Pdx1 (Buteau et al. 1999, Li et al. 2005), IGF2/IGF1R/IRS2/PI3K/PKB/mTOR [(Cornu et al. 2010, Park et al. 2006, Cornu et al. 2009), El-sayed NM and Herbert TP, unpublished data and Fig. 3.1-3.3], and the inhibition of SirT1/FoxO through a reduction in NAD^+ (nicotinamide adenine dinucleotide)/NADH (NAD plus hydrogen) ratios (Bastien-Dionne et al. 2011). This diagram is based on results obtained from different β-cell lines and/or primary islet cells as followed: IGF1R: MIN6 cells, mouse islets (Cornu et al. 2010) and rat islets (Fig. 3.4); IRS2: mouse islets (Park et al. 2006); PI3K: INS1 cells (Buteau et al. 1999); PKB: INS1 cells (Wang et al. 2004); FoxO: INS832/13 cells and mouse islets (Buteau, Spatz and Accili. 2006); PKCζ: INS832/13 cells (Buteau et al. 2001); β-catenin/TCF7L2 (transcription factor 7-like 2): INS1 cells (Liu and Habener. 2008); Pdx1 (pancreatic duodenal homeobox 1): mouse islets (Buteau et al. 1999, Li et al. 2005) and SirT1 (silent information regulator two protein 1) : INS832/13 cells and mouse islets (Bastien-Dionne et al. 2011).

Other signalling pathways also parallel IGF1R-PKB-mTORC1 pathway to promote β-cell replication in response to GLP-1 treatment. For instance, Wnt/β-catenin/TCF7L2 pathway has been shown to be activated by GLP-1, it is required for both basal and GLP-1-induced proliferation in INS1 cells (Liu and Habener. 2008). The stimulation of Wnt pathway by GLP-1 are PKA, PKB and MEK dependent, yet surprisingly, PI3K inhibitor LY294002 and the expression of dominant negative PI3K has no effect on GLP-1-induced Wnt activation. Although in many cell types, such as cardiomyocytes (Naito et al. 2005) and osteoblasts (Almeida et al. 2005), the stimulation of Wnt by PKB requires PI3K activity, PKB can also activate Wnt independently of the stimulation of PI3K (Fukumoto et al. 2001), nevertheless, stimulation of mTORC1 by GLP-1 in INS1E cells requires both the activation of class I PI3K and PKB (El-Sayed NM, Herbert TP,

unpublished data, Fig. 3.1B). Additionally, PI3K/Pdx1 (Buteau et al. 1999), SirT1/FoxO (Bastien-Dionne et al. 2011) and PKCζ (Buteau et al. 2001) also contribute to the proliferative effect of GLP-1 (Fig. 3.10).

4EBP1 is highly expressed in most of the tissues and the ratio of 4EBP1 to 4EBP2 and 3 is higher in pancreas than in many other tissues (Tsukiyama-Kohara et al. 2001). Transgenic animal studies have revealed that 4EBP1 knockout in mice only has mild effect on glucose homeostasis (Tsukiyama-Kohara et al. 2001), although 4EBP1/2 double knockout mice represent accelerated adipocyte differentiation and increased susceptibility to the development of insulin resistance (Le Bacquer et al. 2007). However, whether 4EBP1 regulates β-cell mass in these models is unknown.

In this chapter, I've tested whether exogenously expressing the non-phosphorylable form of 4EBP1 (4EBP1-5A) has any impact on β-cell mass. 4EBP1-5A expression in MIN6 cells caused a severe reduction in cell size (Fig. 3.8) and an induction of cell death (a 20% increase compared to control, see Fig. 3.7). However, not all the five phosphorylable sites mutated in 4EBP1-5A are controlled by mTOR or sensitive to rapamycin. Thr37/Thr46 are mTOR phosphorylation sites (Gingras et al. 1999) but rapamycin resistant (Gingras et al. 1999); Ser64/Thr70 phosphorylation are rapamycin sensitive (Mothe-Satney et al. 2000, Gingras et al. 2001, Wang et al. 2005), yet they are not directly phosphorylated by mTOR (Wang et al. 2005), Ser82 is a constitutive phosphorylation site (Mothe-Satney et al. 2000). Therefore, it would be of interest to find out whether the induction of cell death by 4EBP1-5A is caused by the

hypophosphorylation of a specific site, or some (or all) of these sites, and this can be studied by exogenously expressing individual site specific 4EBP1 mutant, a better model to confirm the observations in this chapter is the development of 4EBP1 β-cell specific knock-in mice (4EBP1-5A or individual phosphorylation site specific).

Surprisingly, I was unable to detect any effect of exendin-4 on the binding of 4EBP-1 on eIF4E, although exendin-4 can activate the S6K-rpS6 arm of mTORC1 (Fig. 3.3, 3.4 and 3.9), however, it remains to be determined whether exendin-4 can induce the phosphorylation of 4EBP1 on each of the mTORC1-controlled sites. Nevertheless, because of the fact that exendin-4 was unable to cause the dissociation of 4EBP1 from the eIF4E, it is unlikely that the proliferative effect of GLP-1/exendin-4 is mediated through 4EBP1. Therefore, other mTORC1 downstream targets should be responsible for mediating the proliferative effect of rapamycin, one possibility is S6K and rpS6, which is activated by exendin-4 in islet cells (Fig. 3.3), proteins that control mRNA transcription (STAT3, HIF1α and TFEB) or ribosome biogenesis (TFIIIC, Maf1) may also be involved (see section 1.3.1).

In conclusion, I have shown here that GLP-1/exendin-4 increases β-cell proliferation in an mTOR dependent fashion, exendin-4 activates mTORC1 through the transactivation of IGF1R. The effect of GLP-1/exendin-4 on β-cell proliferation is unlikely to be mediated through the inactivation of 4EBP1, although the expression of unphosphorylable 4EBP1 severely impaires β-cell viability.

Chapter 4. Role of mTORC2 in the regulation of β-cell mass

4.1 Introduction

4.1.1 Rapamycin islet toxicity in transplantation

For more than three decades, islet transplantation has been considered as a possible curative treatment to patients suffering type 1 diabetes mellitus (T1DM), which is characterized by a complete loss of β-cells as a result of autoimmune destruction (Polychronakos and Li. 2011, Chatenoud. 2011, Pozzilli. 2011). However, the success of islet transplantation has been considerably limited due to a number of factors, including the fact that old immunosuppressive drugs, such as calcineurin inhibitors and glucocorticoids, severely damage islet engraftment (Liu and Herold. 2000, Winter and Schatz. 2003, Burke, Ciancio and Sollinger. 2004). At the beginning of this century, rapamycin has been introduced as the primary immunosuppressive drug for islet transplantation in a landmark study carried out by Shapiro *et al*. in Edmonton, Canada (Shapiro et al. 2000). The initial results were promising, until it was described in a five-year follow-up report that only a minority (approximately 10%) of those recipients remained insulin independent (Ryan et al. 2005). The cause is non-immunological according to the post-mortem examination of a 52-year-old female patient who gradually lost insulin independency and passed away because of hypertensive stroke two years after her second islet transplant under Edmonton's protocol (Smith et al. 2008). On the other hand, increasing evidence suggests that rapamycin has detrimental effects on the survival

and function of pancreatic β-cells in a variety of β-cell lines, murine and human islets (Bell et al. 2003, Fabian et al. 1993, Zhang et al. 2006), indeed, a large number of studies have indicated that mTOR plays a critical role in the maintenance of β-cell mass and function (Pende et al. 2004, Ruvinsky et al. 2005, Pende et al. 2000, Shigeyama et al. 2008, Rachdi et al. 2008, Hamada et al. 2009, Mori et al. 2009). Therefore, it is most likely that the down-regulation of mTORC pathway by rapamycin results in the deterioration of β-cell survival, and ultimately led to the failure of islet transplantation in Edmonton's protocol.

4.1.2 Chapter aims

Rapamycin acutely inhibits mTORC1 whereas prolonged rapamycin treatment in some cell types also blocks the assembly and activation of mTORC2 (Sarbassov et al. 2006). Interestingly, Barlow AD and Moore CE from our lab have demonstrated that long-term rapamycin treatment not only blocks the activation of mTORC1 but also inhibits the activation of mTORC2 in MIN6 cells and rat islets of Langerhans (Fig. 4.1). Moreover, rapamycin treatment impairs cell viability in MIN6 cells and rat islets, which can be rescued upon exogenous expression of constitutively active PKB (Fig. 4.2, performed by Barlow AD). Therefore, in this chapter I sought to further investigate the molecular mechanism by which rapamycin deteriorates β-cell survival, and discovered that the inhibition of mTORC2-PKB is primarily responsible for rapamycin islet toxicity.

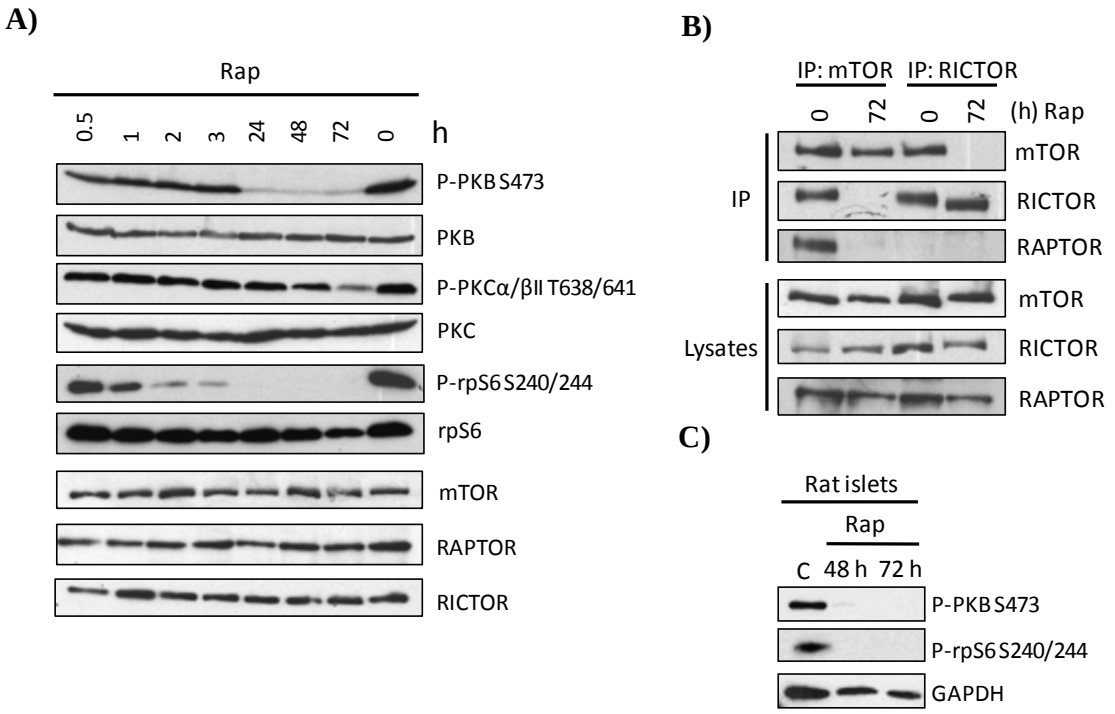


Figure 4.1 Rapamycin disrupts the assembly and activation of mTORC2. **A)** MIN6 cells were treated with 200 nM rapamycin (Rap) for the indicated time periods. **B)** MIN6 cells were treated with 200 nM Rap for 72 h. Cells were lysed in 0.3% CHAPS buffer and immunoprecipitation was performed using anti-mTOR and anti-RICTOR antibodies. **C)** Rat islets of Langerhans were treated with 200 nM Rap for the indicated time periods. Immunoprecipitates (IP) and lysates were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P)-PKB Ser473 (S473), P-rpS6 Ser240/Ser244 (S240/244), P-S6K1 Thr389 (T389), P-PKCα/βII Thr638/Thr641 (T638/641), as well as total levels of mTOR, RAPTOR, RICTOR, PKB, rpS6 and S6K1. Experiments performed by Barlow AD (A and B) and Moore CE (C).

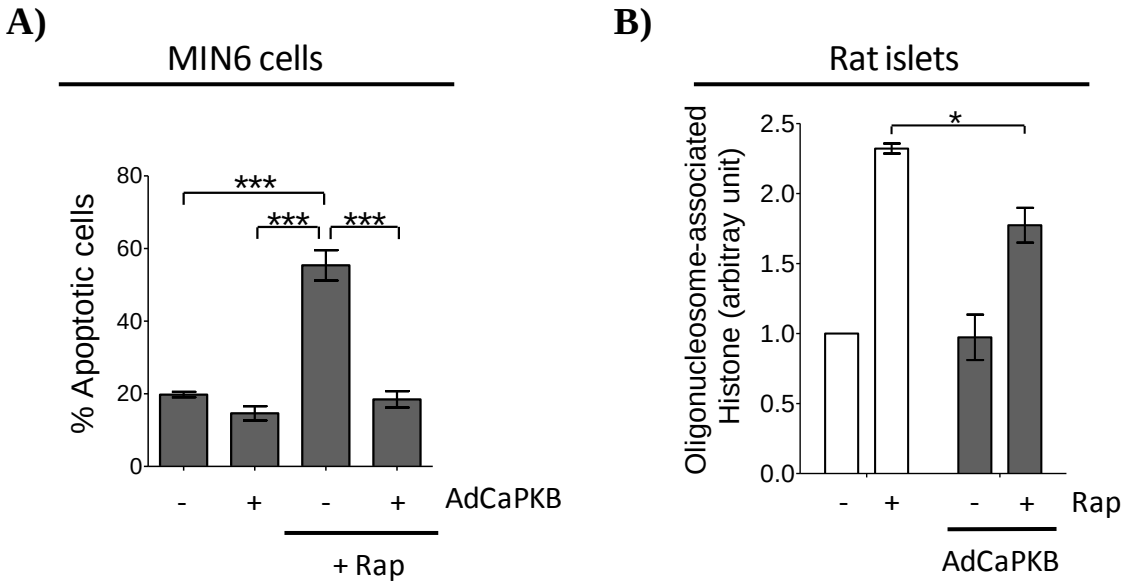


Figure 4.2 Over-expression of constitutively active PKB rescues MIN6 and rat islets from rapamycin-induced apoptosis. MIN6 cells were mock infected or infected with adenovirus producing constitutively active PKB (AdCaPKB) at a multiplicity of infection of 500 for 24 h, followed by incubation for 24 h in the presence or absence of 200 nM rapamycin (Rap). Following treatment, cells were dispersed, stained with annexin V and propidium iodide, and then analysed by flow cytometry. The percentage of apoptotic cells was quantified. Rat islets were infected with Ad CaPKB for 24 h, followed by a further 48 h incubation in the presence or absence of 200 nM Rap. To determine the rate of cell death, internucleosomal DNA fragmentation was analysed. *p* values were obtained using a one-way ANOVA with Bonferroni post-test. All data are shown as means±SE, n=3. $^{*}p$=0.05–0.01 and $^{***}p$<0.001. Experiments performed by Barlow AD.

4.2 Results

4.2.1 Rapamycin causes cell death in rat islets of Langerhans

To determine the effect of rapamycin on mTOR signalling and cell survival in primary cells, isolated rat islets of Langerhans were treated with increasing concentrations (1-200 nM) of rapamycin for 48 h (Fig. 4.3). 1 nM of the drug was sufficient to inhibit the activation of mTORC1, which is evidenced by the decrease in rpS6 phosphorylation at Ser240/Ser244. Interestingly, all doses of rapamycin tested also effectively blocked the phosphorylation of PKB at Ser473, which is phosphorylated by mTORC2 (Fig. 4.3A), and led to an induction of islet cell death, as demonstrated by the increase in cytoplasmic mononucleosome- and oligonucleosome-associated histone accumulated in membrane-intact cells (Fig. 4.3B). The concentration of rapamycin analogs sirolimus and tacrolimus detected in the portal vein of islet transplant recipients is approximately 10 nM (Desai et al. 2003), however, these patients may receive immunosuppressant treatment for weeks/months, therefore, in order to produce robust and maximal effects, 200 nM of rapamycin was used in subsequent experiements.

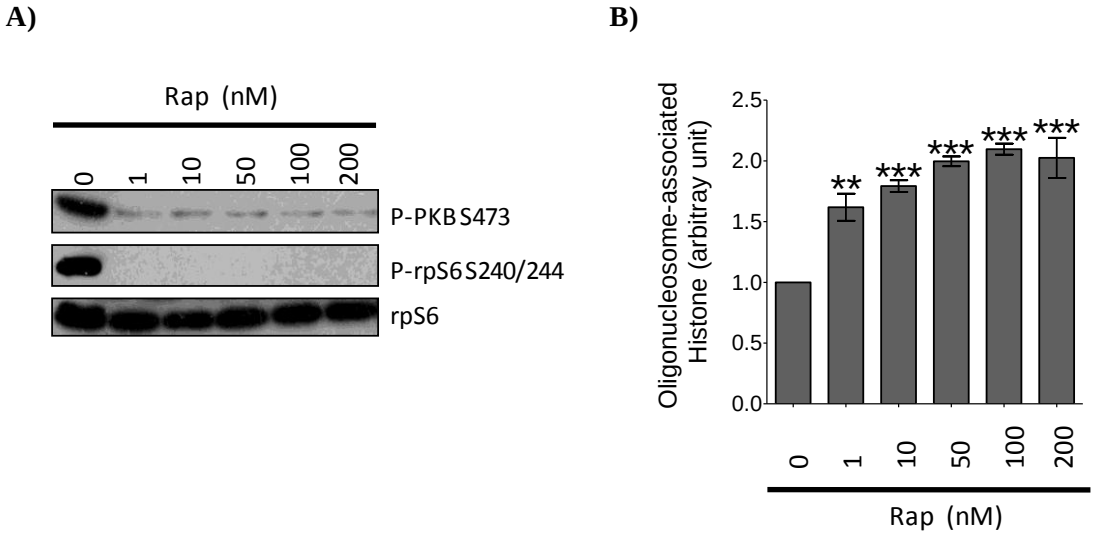


Figure 4.3 Dose-dependent effect of rapamycin on rat islet viability. **A)** Rat islets of Langerhans were treated with increasing concentrations of rapamycin (Rap) for 48 h. Cells were lysed and proteins were separated on SDS-PAGE and Western blotted against phosphorylated (P)-PKB Ser473 (S473), P-rpS6 Ser240/Ser244 (S240/244), and total rpS6 as loading control. **B)** Rat islets were treated as in A), internucleosomal DNA fragmentation was determined as an indicator of cell apoptosis using the Cell Death Detection ELISA kit (Roche). *P* values were obtained using a one-way ANOVA followed by Dunnett's test. Data are shown as means ± SE, $n = 3$. $^{**}P = 0.01\text{-}0.001$, $^{***}P < 0.001$.

4.2.2 Rapamycin decreases PKB kinase activity

In Fig. 4.3, I observed that rapamycin treatment caused a reduction in PKB Ser473 phosphorylation, which prompted me to investigate its effect on PKB kinase activity. For this purpose, MIN6 cells were treated with rapamycin for 72 h. Rapamycin causes a significant decrease in PKB activity (Fig. 4.4A), it also inhibited the activation of both mTORC1 and mTORC2 as determined by a decrease in the phosphorylation status of rpS6 on Ser240/244 and PKB on Ser473, respectively (Fig. 4.4B). The phosphorylation

of PKB on Thr308 and total PKB levels were unaffected. Previous work in our lab (Fig. 4.2, performed by Barlow AD) has suggested that PKB is important for β-cell survival, and indeed, this decrease in PKB activity was coincident with an augmentation in the expression of cleaved caspase 3, indicative of cell apoptosis (Fig. 4.4B).

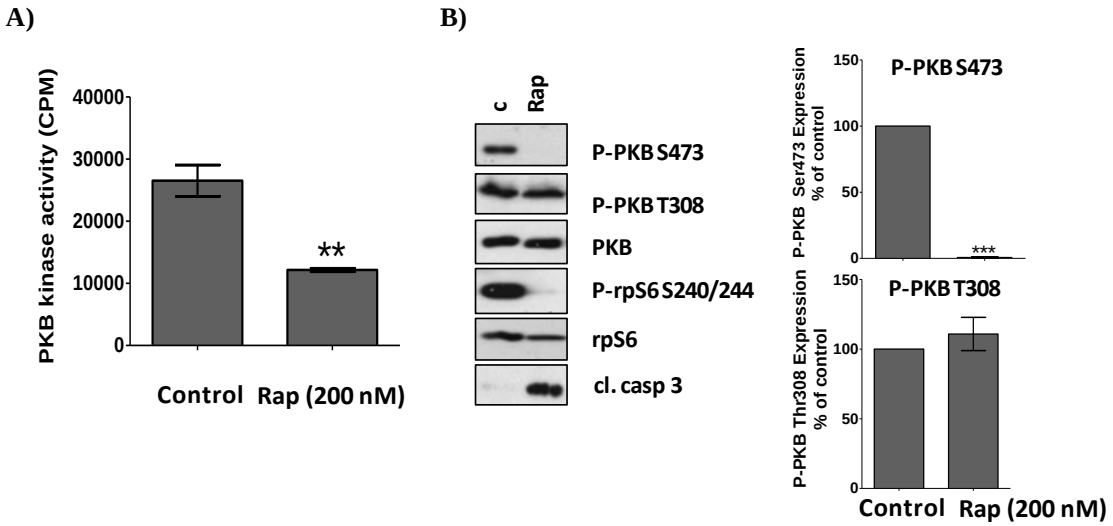


Figure 4.4 Rapamycin inhibits PKB kinase activity in MIN6 cells. **A)** PKB *in vitro* activity assay. MIN6 cells were incubated in growing media in the absence or presence of rapamycin (Rap, 200 nM) for 72 h. Endogenous PKB were immunoprecipitated from the lysates and subjected to PKB kinase assay as described in section 2.12. **B)** Protein lysates were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P)-PKB Ser473 (S473), P-PKB Thr308 (T308), P-rpS6 Ser240/Ser244 (S240/244), as well as total levels of PKB, rpS6 and cleaved caspase 3 (cl. casp 3). Quantification of P-PKB S473 and T308 were performed by densitometry. *P* values were obtained using an unpaired student's *t*-test, $n = 3$. $^{**}P = 0.01\text{-}0.001$. Immunoblots are representative of three independent experiments.

4.2.3 PKB inhibition impairs β-cell survival

To determine whether PKB activity is critical for β-cell survival, MIN6 cells were treated with the selective PKB inhibitor AKTi (Barnett et al. 2005) for 8 to 40 h, AKTi effectively inhibited the phosphorylation of PKB on both Ser473 and Thr308 (Fig. 4.5A) and importantly caused an increase in apoptosis (Fig. 4.5B). The phosphorylation of rpS6 on Ser240/Ser244 was only partially inhibited, indicating that alternative signalling pathways, such as MAPK and AMPK pathway (see sections 1.2.1 and 1.2.3), may also contribute to the activation of mTORC1 under these conditions.

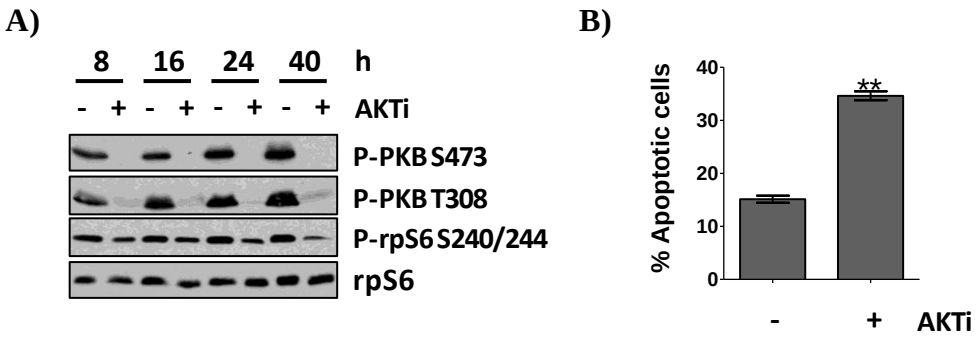


Figure 4.5 PKB is indispensible for MIN6 cell survival. **A)** MIN6 cells were treated with 10 μM AKTi for 8, 16, 24 and 40 h, lysates were collected and separated by SDS-PAGE. Proteins were resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P)-PKB Ser473 (S473), (P)-PKB Thr308 (T308), P-rpS6 Ser240/Ser244 (S240/244), and rpS6 as a loading control. **B)** MIN6 cells were treated with 10 μM AKTi for 40 h, lysates were collected and subjected to cell death analysis by flow cytometry. P values were obtained using a paired student's t-test. Data are shown as means ± SE, n = 3. $^{**}P$ = 0.001-0.01.

To confirm the above finding in primary cells, isolated rat islet of Langerhans were treated for up to 40 h with the PKB inhibitor AKTi (Barnett et al. 2005). AKTi effectively inhibited the phosphorylation of PKB on both Ser473 and Thr308 by 8 h (Fig. 4.6A) and caused a time-dependent increase in apoptosis (Fig. 4.6B) and GSIS (glucose-stimulated insulin secretion) (Fig. 4.6C). These results indicate that PKB activity is essential for the maintenance of β-cell viability.

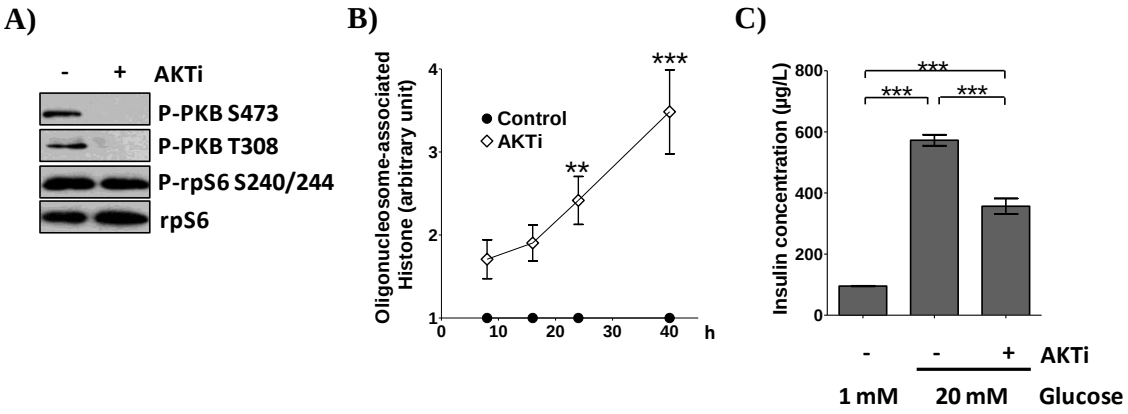


Figure 4.6 PKB is essential for maintaining cell viability in rat islets of Langerhans. **A)** Rat islets of Langerhans were treated with 10 µM AKTi for 8 h, lysates were collected and analysed by SDS-PAGE and western blotting using antisera against phosphorylated (P)-PKB Ser473 (S473), (P)-PKB Thr308 (T308), P-rpS6 Ser240/Ser244 (S240/244), and total rpS6 as loading control. **B)** Rat islets were treated as in *A* for 8, 16, 24 and 60 h, internucleosomal DNA fragmentation was determined as an indicator of cell apoptosis using the Cell Death Detection ELISA. *P* values were obtained using a two-way ANOVA followed by Bonferroni post-test. **C)** Rat islets were incubated in the presence or absence of AKTi for 40 h and insulin secretion assay was performed. *P* values were obtained using a one-way ANOVA followed by Bonferroni post-test. All data are shown as means ± SE, $n = 3$. $^{**}P = 0.01\text{-}0.001$, $^{***}P < 0.001$. All immunoblots are representative of three independent experiments.

4.2.4 Torin1 inhibits the activation of mTORC1 and 2 and causes β-cell death in MIN6 cells

Rapamycin acutely inactivates mTORC1, however, prolonged treatment of rapamycin is required to inhibit mTORC2 in certain cell types (Sarbassov et al. 2006). In contrast, Torin1, a novel ATP-competitive mTOR inhibitor, acutely inhibits both mTORC1 and 2 (Thoreen et al. 2009). Therefore, it is of interest to investigate whether β-cell death can be rapidly induced upon Torin1 treatment and whether this correlates with acute mTORC2 inhibition. To establish the time-dependent effect of rapamycin islet toxicity, MIN6 cells were treated with rapamycin and Torin1 for 1 to 24 h (Fig. 4.7A). As anticipated, mTORC1 activity was rapidly abolished upon rapamycin/Torin1 treatment as indicated by the phosphorylation status of rpS6 on Ser240/Ser244 and the delay in mobility of 4EBP1 on the SDS-PAGE gel. Rapamycin also inhibited mTORC2 activation after 16 h as determined by PKB Ser473 phosphorylation. In contrast, Torin1 completely suppressed mTORC2 by 1 h (Fig. 4.7A).

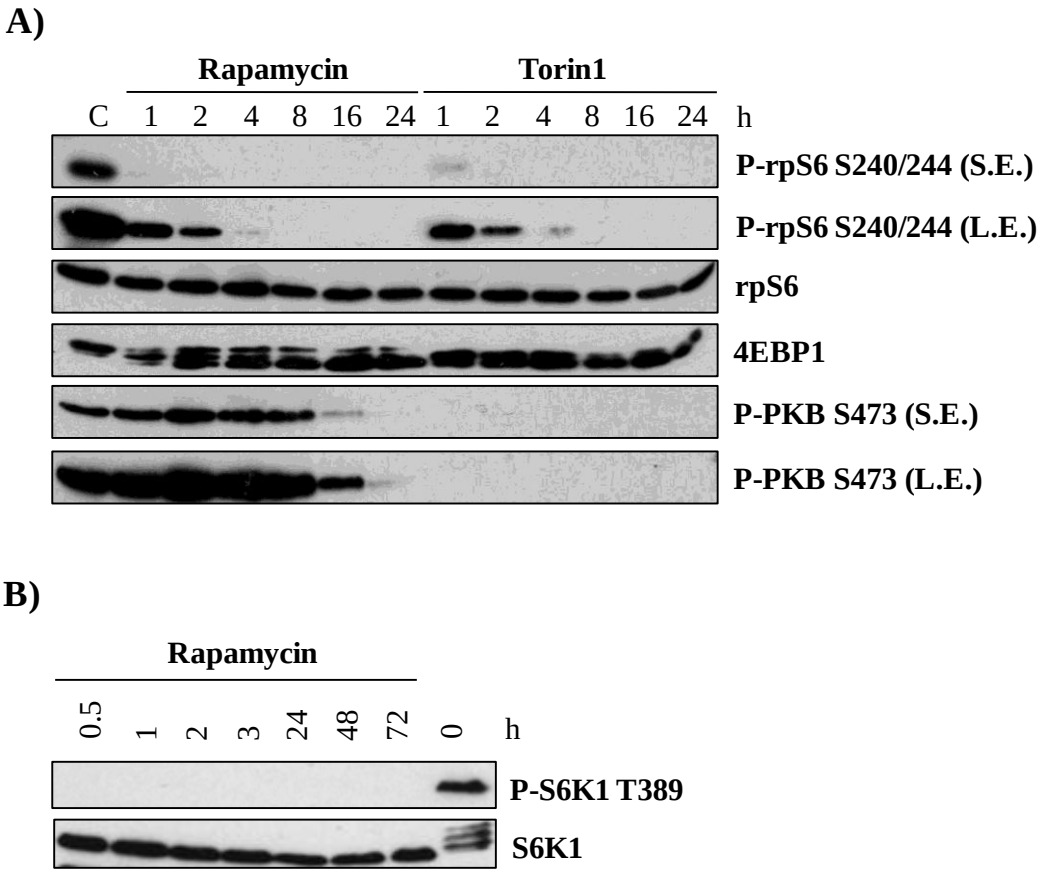


Figure 4.7 Torin1 inhibit mTORC1 and 2 in MIN6 cells. MIN6 cells were treated with rapamycin (200 nM) or Torin1 (200 nM) for up to 24 h [in **A)**] or 72 h [in **B)**]. Cells were lysed and proteins were resolved by SDS-PAGE, followed by western blotting of indicated phospho (P)-rpS6 Ser240/Ser244 (S240/244), P-PKB Ser473 (S473), P-S6K1 Thr389 (T389) as well as total rpS6, 4EBP1 and S6K1. S.E.: short exposure; L.E.: long exposure. Immunoblots are representative of three independent experiments.

Next, I sought to find out whether Torin1 treatment causes cell death as well as rapamycin in MIN6 cells. For this purpose, MIN6 cells were treated with 200 nM rapamycin or Torin1 for up to 72 h without replenishment of the growing media, and the rate of cell death was analyzed by flow cytometry, data was normalized to control. As shown in Fig. 4.8A, Torin1 led to an augmentation in cell death started from 28 h and

peaked at 64 h, rapamycin treated cells represented a delayed but similar pattern, although remained statistically insignificant until 44 h. 24 h rapamycin treatment also caused a reduction in cell size, and this effect was even more pronounced in Torin1 treated cells (Fig. 4.8B). Taken together, the results so far indicate that Torin1 leads to a rapid inhibition of mTORC2 and an accelerated decrease in β-cell survival compared to those treated with rapamycin.

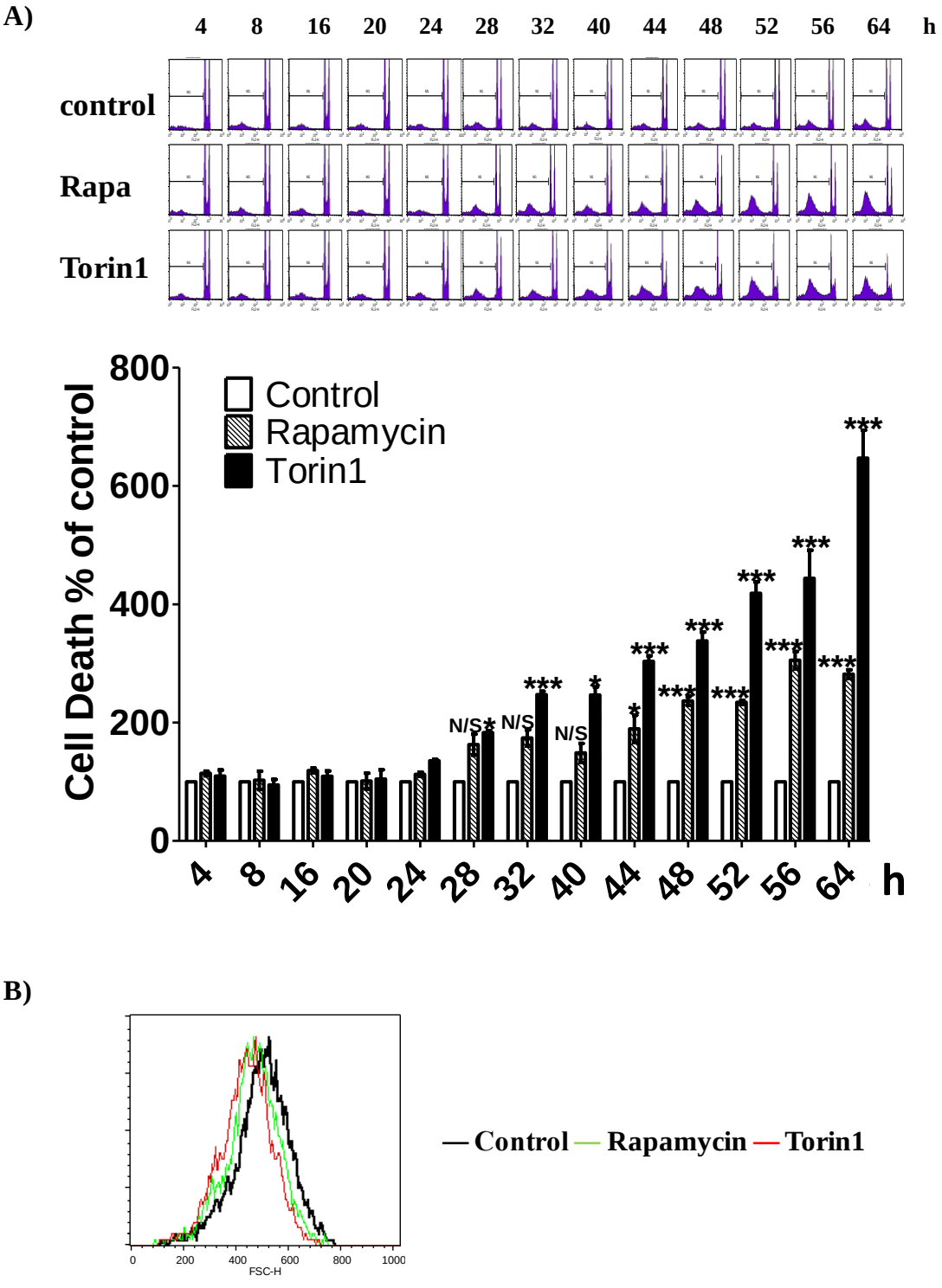


Figure 4.8 Rapamycin and Torin increases cell death and reduce cell size in MIN6 cells**. A)** MIN6 cells (1 x 10^6 cells per plate) were maintained in growing media for 48 h, cells were then treated with rapamycin (200

nM) or Torin1 (200 nM) for up to 64 h, cells were trypsinized and collected for cytometry analysis to determine the rate of cell death at indicated time points (log scale). *X* axis: FL2-H, *Y* axis: cell numbers; M1 indicates sub G1 population. Results were normalized to control and are presented as means ± SE; $n = 3$. $*P = 0.05\text{-}0.01$, $***P < 0.001$ using two-way ANOVA followed by Bonferroni adjustment. **B)** G_0/G_1 cell size of control, rapamycin and Torin1 treated (24 h) cells were compared using two-dimensional FSC analysis. *X* axis: FSC-H, *Y* axis: cell numbers.

4.2.5 Torin1 inhibits the activation of mTORC1 and 2 and causes β-cell death in rat islets of Langerhans

To confirm the above finding in primary cells, isolated rat islets of Langerhans were treated for up to 24 h with rapamycin or Torin1. Similar to the results obtained from MIN6 cells, the inhibitory effects of rapamycin on mTORC2 and PKB were delayed compared to islets treated with Torin1, because Torin1 completely inhibits both mTORC1 and 2 at 8 h whereas the activity of mTORC2 in rapamycin treated cells only started to drop at 16 h and was inhibited to a similar extent as Torin1 treated cells at 24 h (Fig. 4.9A). Torin1 also caused a rapid increase in the rate of cell death (Fig. 4.9B), and a decrease in GSIS (Fig. 4.9C). Moreover, the impairment of cell viability was accelerated in Torin1 treated cells compared to rapamycin treated ones (Fig. 4.9B). Therefore, it is the inhibition of mTORC2, rather than mTORC1, which correlates with decreased islet viability and function.

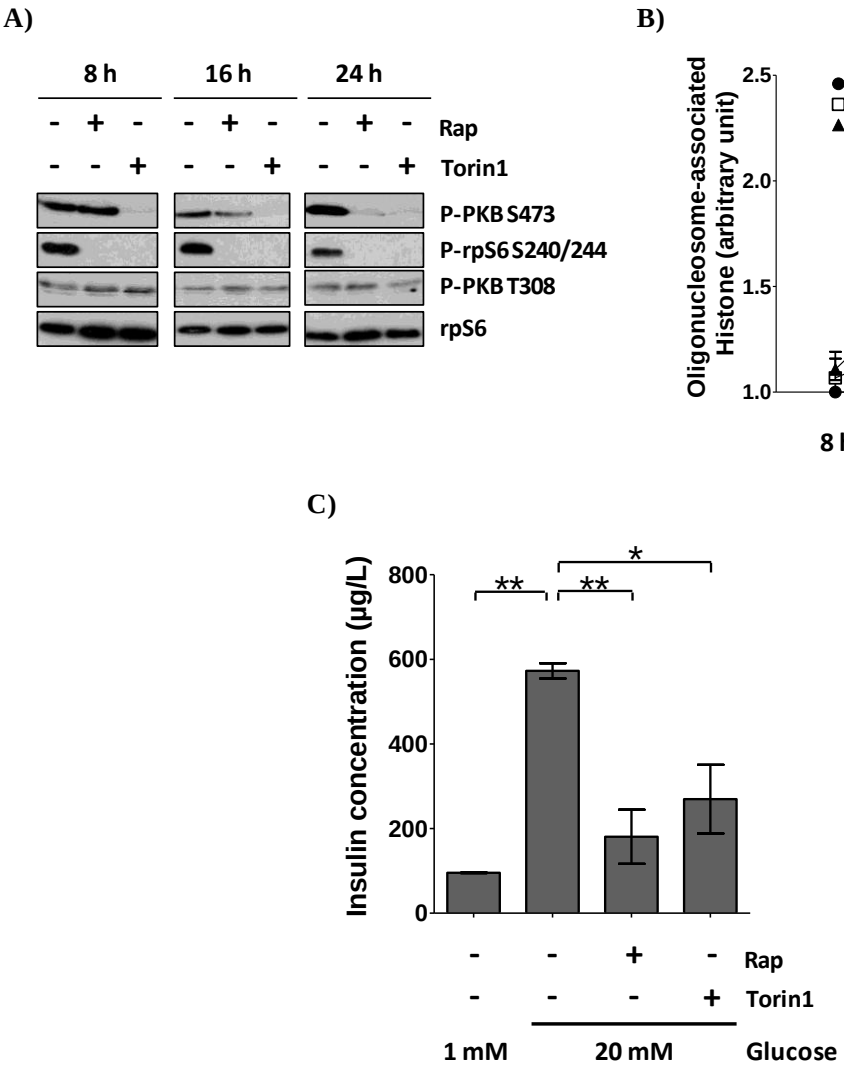


Figure 4.9 Torin1 causes a reduction in the viability and function of islets of Langerhans. **A)** Rat islets were treated with rapamycin (Rap) (200 nM) or Torin1 (200 nM) for the times indicated. Islets were then lysed and resolved on SDS-PAGE and immunoblotted using antisera against phosphorylated (P)-PKB Ser473 (S473), P-PKB Thr308 (T308), P-rpS6 Ser240/Ser244 (S240/244) and rpS6. All immunoblots are representative of three independent experiments. **B)** Rat islets were treated as in A, and internucleosomal DNA fragmentation was determined as an indicator of cell apoptosis using the Cell Death Detection ELISA. P values were obtained using a two-way ANOVA followed by Bonferroni post-test comparing rapamycin and Torin1 treated samples. **C)** Rat islets were treated with rapamycin (Rap, 200 nM) or Torin1 (200 nM)

for 40 h, and then incubated in KRB containing 1 mM glucose for 1 h followed by a further 1 h incubation in KRB containing 20 mM glucose. Supernatants were collected and assayed for insulin concentration using ELISA. P values were obtained using a one-way ANOVA followed by Bonferroni post-test. Data are shown as means ± SE, n = 3. *P = 0.05-0.01, **P = 0.01-0.001.

4.2.6 RAPTOR knockdown does not impair β-cell viability and GSIS in islets

To provide further evidence that the inhibition of mTORC1 is not involved in rapamycin islet toxicity, I transfected dispersed rat islets with on-target plus SMARTpool siRNA against the mTORC1 specific component RAPTOR, which contains four different siRNA sequences for the rat RAPTOR. siRNA RAPTOR effectively blocked the expression of RAPTOR and caused a reduction in the phosphorylation of S6K1 (Thr389) and its downstream target rpS6 (Ser240/Ser244) (Fig. 4.10). Although S6K1 phosphorylation on Thr389 was almost completely abolished, RAPTOR knockdown only partially impeded rpS6 phosphorylation on Ser240/Ser244 (Fig. 4.10), this could be due to: 1) rpS6 phosphorylation signal is much stronger than phospho-S6K1 signal; 2) residual S6K activity was sufficient to maintain basal rpS6 phosphorylation, or 3) there is another rpS6 kinase rather than S6K1/2 responsible for the phosphorylation of these residues (Roux et al. 2007, Moore et al. 2009). The inhibition of RAPTOR also led to a slight increase in PKB phosphorylation on Ser473, which is in agreement with previous observations that mTORC1/S6K1 inhibition facilitates the sensitization of insulin signalling pathway (see section 1.3.1).

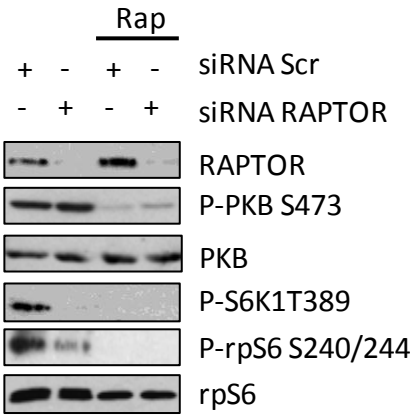


Figure 4.10 Dispersed rat islets were transfected with scrambled (Scr) or RAPTOR siRNAs and incubated for 48 h in the presence or absence of rapamycin (Rap, 200 nM). After cell lysis, proteins were separated on SDS-PAGE and Western blotted using anti-sera against RAPTOR, phosphorylated (P)-PKB Ser473 (S473), P-S6K1 Thr389 (T389), P-rpS6 Ser240/Ser244 (S240/244), PKB and rpS6. Immunoblots are representative of three independent experiments.

Next I tested whether RAPTOR knockdown has any effect on cell viability and GSIS (glucose-stimulated insulin secretion) in rat islets of Langerhans. Dispersed rat islets were transfected with siRNA scramble and RAPTOR in the presence or absence of rapamycin for 48 h, cells were then lysed and the presence of oligonucleosome-associated histone was detected as readout of cell death. As anticipated, rapamycin treated cells have increased cell death (Fig. 4.11A) and decreased GSIS (Fig. 4.11B) compared to non-treated ones, whereas RAPTOR knockdown has no effect on those parameters, implying that mTORC1 is unlikely to be the major cause of rapamycin islet toxicity.

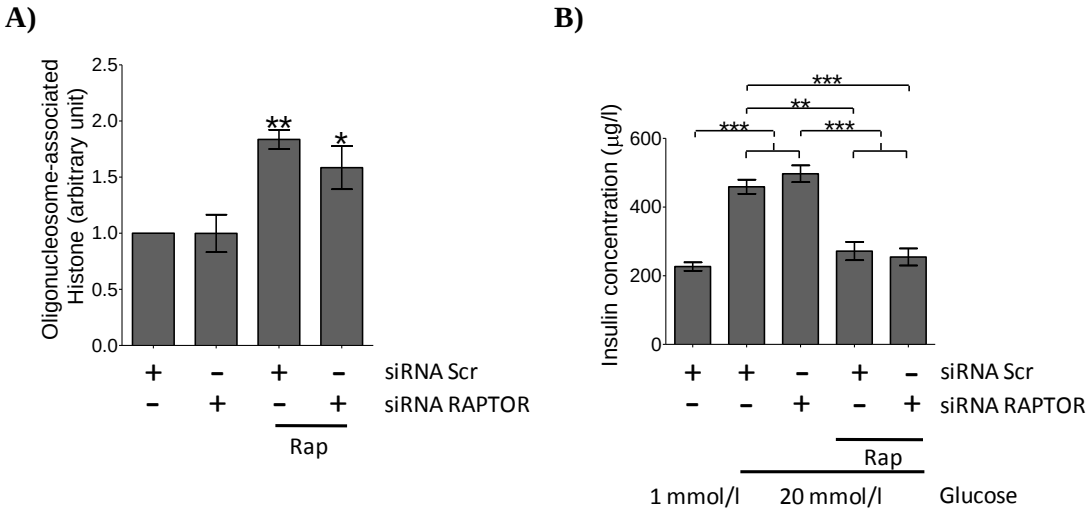


Figure 4.11 **A)** Dispersed rat islets were transfected with scrambled (Scr) or RAPTOR siRNAs and incubated for 48 h in the presence or absence of 200 nM rapamycin (Rap). Apoptosis was determined using the Cell Death Detection ELISA kit (Roche). *P* values were obtained using one-way ANOVA followed by Dunnett's test comparing each column to the first column (SiRNA Scr). *n* = 3. **B)** Rat islets were transfected and treated as in A), and then incubated in KRB containing 1 mM glucose for 1 h followed by a further 1 h incubation in KRB containing 20 mM glucose. Supernatants were collected and assayed for insulin concentration using ELISA. *P* values were obtained using one-way ANOVA followed by Tukey's multiple comparison test, *n* = 3. All data are shown as means ± SE. ***P* = 0.01-0.001, ****P* < 0.001.

4.2.7 RICTOR knockdown by siRNA in dispersed rat islets impairs β-cell survival and GSIS

Results so far suggested that mTORC2 inhibition is most likely the cause of rapamycin islet toxicity. To provide further evidence to support this hypothesis, I transfected dispersed rat islets with on-target plus SMARTpool siRNA against RICTOR, which contains four different siRNA sequences for the rat RICTOR. siRNA RICTOR effectively blocked RICTOR expression and led to a decrease in the phosphorylation of

PKB on Ser473, indicative of decreased mTORC2 activity, without affecting the phosphorylation of rpS6, a marker of mTORC1 activity (Fig. 4.12). This correlated with an increase in apoptosis (Fig. 4.13A) and a decrease in GSIS (Fig. 4.13B). Previously Gu *et al*. has reported that islets isolated from β-cell specific RICTOR knockout (βRicKO) mice represent compensatory increases in PKB phosphorylation on Thr308 (Gu et al. 2011). However, there was no significant change in the phosphorylation of PKB on Thr308 in islets transfected with RICTOR siRNA (Fig. 4.12).

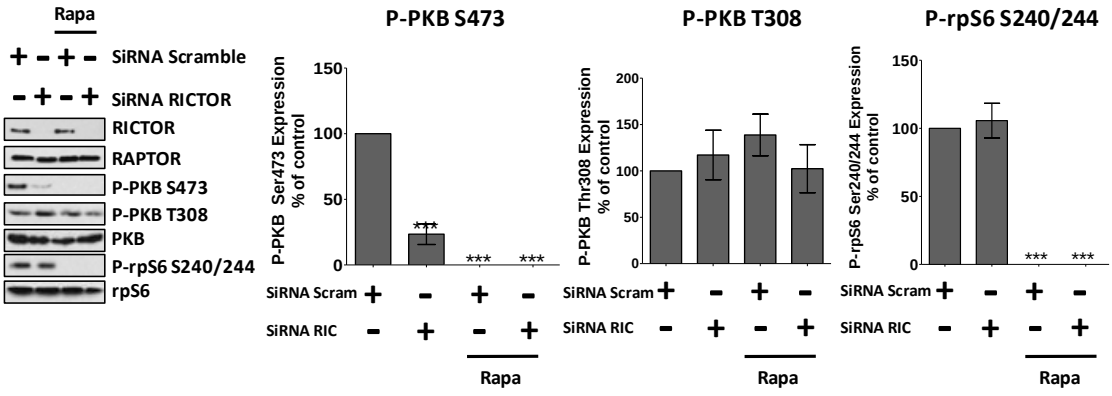


Figure 4.12 siRNA knockdown RICTOR in dispersed rat islets. Dispersed rat islets were transfected with 200 nM siRNA scramble (Scram) or 200 nM RICTOR (RIC) and then incubated for 48 h in the presence or abasence of 200 nM rapamycin (Rapa). Cells were then lysed, proteins were resolved on SDS-PAGE and Western blotted using anti-sera against RICTOR, RAPTOR, phosphorylated (P)-PKB Ser473 (S473), P-PKB Thr308 (T308), P-rpS6 Ser240/Ser244 (S240/244) and rpS6. Levels of P-PKB S473, P-PKB T308 and P-rpS6 S240/244 were quantified by densitometry and expressed as percentage of control (SiRNA scramble). *P* values were obtained using student's *t*-test, $n = 5$, ***$P < 0.001$. Immunoblots are representative of five independent experiments.

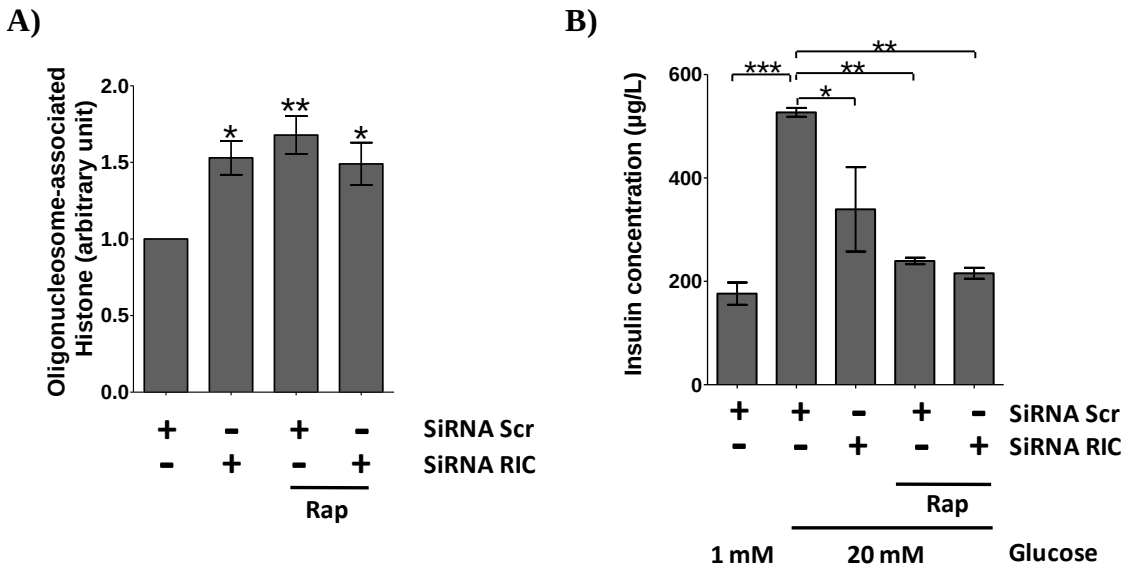


Figure 4.13 RICTOR depletion causes a loss of β-cell viability. **A)** Dispersed rat islets were transfected with 200 nM SiRNA scramble (Scr) or 200 nM RICTOR (RIC) and then incubated for 48 h in the absence or presence of rapamycin (200 nM). Apoptosis was determined using the Cell Death Detection ELISA kit (Roche). Rates of cell death are expressed as enrichment factors as described in manufacturer's instructions using SiRNA scramble as a control. *P* values were obtained using Dunnet test followed by one-way ANOVA. **B)** Rat islets were transfected and treated as in *A*, and then incubated in KRB containing 1 mM glucose for 1 h followed by a further 1 h incubation in KRB containing 20 mM glucose. Supernatants were collected and assayed for insulin concentration using ELISA. *P* values were obtained using one-way ANOVA followed by Tukey's multiple comparison test, $n = 3$. All data are shown as mean ± SE, $n = 3$. *P = 0.05-0.01, **P = 0.01-0.001, ***$P < 0.001$.

4.3 Discussion

Rapamycin has been used as the primary immunosuppressant for islet transplantation over the last decade (Shapiro et al. 2000), as rapamycin is less toxic to pancreatic β-cells than other immunosuppressants such as corticosteroids and tacrolimus. However,

rapamycin and its analogues can cause deleterious effects on β-cell mass and islet engraftment, primarily due to an induction of β-cell apoptosis (Bell et al. 2003, Fabian et al. 1993, Zhang et al. 2006). Prior to this work, the molecular basis for rapamycin islet toxicity had not been revealed.

Although rapamycin is known to inhibit mTORC1 in β-cells, I show that prolonged rapamycin treatment is also able to abolish the activity of mTORC2 in MIN6 cells and rat islets of Langerhans as demonstrated by either a decrease in the phosphorylation of its downstream target PKB on Ser473 (Fig. 4.3) and/or a reduction in PKB kinase activity (Fig. 4.4). Inhibition of PKB using AKTi also results in the loss of cell viability (Fig. 4.5 and 4.6). Unlike rapamycin, Torin1 and AKTi rapidly inhibit PKB phosphorylation on Ser473, and also lead to a more rapid loss in β-cell viability compared to rapamycin (Fig. 4.5-4.9). Moreover, the inactivation of mTORC2, by knocking-down RICTOR expression in rat islets, causes an increase in apoptosis to a similar extent as rapamycin (Fig. 4.12 and 4.13), whereas the inactivation of mTORC1, by knock-down of RAPTOR expression, had no significant effect on β-cell survival (Fig 4.10 and 4.11). Therefore, it can be concluded that the maintenance of mTORC2 activity is critical for β-cell viability and that the inhibition of mTORC2 by rapamycin is likely responsible for rapamycin toxicity to pancreatic islets. Yet it was recently reported that βRicKO mice show no loss in β-cell viability, although a reduction in islet mass and function was observed (Gu et al. 2011). One possible explanation for this apparent contradiction is that although PKB phosphorylation at Ser473 is compromised in the islets isolated from βRicKO mice, the phosphorylation of PKB at Thr308, which is mediated by PDK1 (Alessi et al. 1997), was

enhanced (Gu et al. 2011). This is likely to have a compensatory effect on the activity of PKB. However, the overall kinase activity of PKB in islets from βRicKO mice was not reported. In contrast, I was unable to detect any significant increase in the phosphorylation of PKB at Thr308 in rat islets in which RICTOR expression was acutely knocked-down (Fig. 4.12) or in islets in which mTORC2 activity was inhibited by either rapamycin or Torin1 (Fig. 4.9). Therefore, it is plausible that in βRicKO mice, signalling events downstream of PKB Thr308 phosphorylation rescues β-cells from apoptosis.

Prolonged rapamycin treatment led to an increase in cell death concomitant with a decrease in GSIS (Fig. 4.11 and 4.13). Other studies have also shown that rapamycin long-term treatment inhibits GSIS (Bell et al. 2003, Fabian et al. 1993, Zhang et al. 2006), which is unaffected by short-term treatment (Fabian et al. 1993, McDaniel et al. 2002, Johnson et al. 2009), indicating that the deleterious effects of rapamycin on GSIS are not through the inhibition of mTORC1 but caused by the inhibition of mTORC2. Although mTORC1 regulates protein synthesis, *in vitro*, rapamycin has little effect on either insulin synthesis (Gomez et al. 2004) or insulin content (Shimodahira et al. 2010) in pancreatic β-cells. However, the chronic inhibition of mTORC1 *in vivo* may eventually lead to decreased insulin content which in turn could impact on GSIS. However, the inhibition of GSIS by rapamycin has been reported to be caused by reduced mitochondrial ATP production, an indicative of cell death, through the suppression of carbohydrate metabolism in the Krebs cycle and reduced ketoglutarate dehydrogenase activity (Shimodahira et al. 2010).

PKB integrates upstream survival signals to maintain β-cell viability. It protects β-cells from streptozotocin-induced cell death and mediates the anti-apoptotic actions of insulin, IGF1 and GLP-1 (Cornu et al. 2009, Li et al. 2005). Of note, $Akt2^{-/-}$ mice, unlike $Akt1^{-/-}$ mice (Cho et al. 2001), results in a decrease in β-cell mass, which parallels an increase in β-cell apoptosis (Garofalo et al. 2003). Indeed, $Akt1^{-/-}$ mice are less susceptible to obesity and the development of insulin resistance (Wan et al. 2012). Interestingly, it has recently been reported that the inhibition of PKB phosphorylation on Ser473 by rapamycin in primary rat and human platelets correlates with a decrease in the activity of PKBβ rather than PKBα (Moore, Hunter and Hers. 2011), Therefore, it is tempting to speculate that rapamycin islet toxicity is caused by the specific impairment of PKBβ activity via the loss of Ser473 phosphorylation due to the inactivation of mTORC2. Moreover, decreases in PKB phosphorylation in transgenic mice where components of the IRS-PDK1 pathway are ablated [reviewed in (Elghazi and Bernal-Mizrachi. 2009)], or mice expressing constitutively active S6K in which IRS signalling is impaired (Elghazi et al. 2010), correlate with decreased viability. These anti-apoptotic effects of PKB may be mediated by the nuclear exclusion and degradation of the FoxO family of proteins, which is controlled by the phosphorylation of PKB on Ser473 (Guertin et al. 2006). However, rapamycin was unable to inhibit the phosphorylation of FoxO in MIN6 cells (Barlow AD *et al.*, Diabetologia, 2012, supplementary Fig. 3) and mTORC2 ablation in β-cells from βRicKO mice leads to an increase in protein expression of FoxO1 and its nuclear retention yet it does not result in an increase in apoptosis (Gu et al. 2011). Therefore, it is likely that the positive role of PKB in β-cell viability is mediated by other downstream targets implicated in cell survival, such as BAD (Datta et al. 1997), pro-caspase-9 and

Mdm2. PKB phosphorylates BAD on Ser136 which disrupts its binding to pro-survival Bcl-2 proteins (Datta et al. 1997), it also phosphorylates pro-caspase-9 on Ser196 which impairs its intrinsic protease activity and thereby improves cell survival (Cardone et al. 1998), moreover, PKB phosphorylates Mdm2 on Ser166 and Ser186 which inhibits Mdm2 nuclear import and results in an increase in the expression of anti-apoptotic protein p53 (Mayo and Donner. 2001, Zhou et al. 2001a). However, it remains to be investigated whether these PKB downstream targets are controlled by PKB Ser473 phosphorylation.

In conclusion, I have shown that the molecular basis of rapamycin islet toxicity is through the inhibition of mTORC2, the subsequent reduction in PKB phosphorylation at Ser473 and the suppression of its kinase activity. As a consequence, this work has revealed an important role for mTORC2 in β-cell survival. Therefore, it can be proposed that novel immunosuppressants used in islet transplantation should be tested for their effects on mTORC2-PKB activity.

Chapter 5. Negative Regulation of mTOR complexes by cAMP

5.1 Introduction

5.1.1 Crosstalk between cAMP and mTOR pathways

The second messenger cAMP is yielded in response to a broad range of extracellular stimuli that act upon G-protein coupled receptors (GPCRs) (for reviews, see Millar and Newton. 2010, Lappano and Maggiolini. 2011). cAMP is synthesized by the action of adenylate cyclase (AC) and its degradation is mediated by the action of cAMP phosphodiesterases (PDEs) (Houslay. 2010, Omori and Kotera. 2007). Most of the effects of cAMP are dependent upon the activation of its downstream effectors protein kinase A (PKA) (Taylor, Buechler and Yonemoto. 1990) and exchange protein directly activated by cAMP (EPAC, also referred to as cAMP-GEF) (Bos. 2006). Like mTOR, diverse array of biological processes are regulated by cAMP (Cheng et al. 2008). Interestingly, there is good evidence of crosstalk between these two pathways. For instance, depending upon cell type, cAMP can either stimulate (Blancquaert et al. 2010, Moore et al. 2009, Kwon et al. 2004, Brennesvik et al. 2005, Palaniappan and Menon. 2010, Kim et al. 2010) or inhibit (Scott and Lawrence. 1998, Mothe-Satney et al. 2004, Kimball, Siegfried and Jefferson. 2004, Rocha et al. 2008, Baum, Kimball and Jefferson. 2009, Baum, Kimball and Jefferson. 2009, Kim et al. 2001) mTORC1. This is likely through the activation or suppression of signalling transduction cascades upstream of mTORC1 such as the PKB (Kim et al. 2001, Brennesvik et al. 2005, Filippa et al. 1999, Liu, Xie and

Lou. 2005, Hong et al. 2008b), the MAPK (Dumaz and Marais. 2005) and the AMPK pathways (Kimball, Siegfried and Jefferson. 2004, Baum, Kimball and Jefferson. 2009, Hurley et al. 2006, Djouder et al. 2010). For example, glucagon, a GsPCR agonist that increases in $[cAMP]_i$, inhibits mTORC1 in rat hepatocytes (Kimball, Siegfried and Jefferson. 2004, Baum, Kimball and Jefferson. 2009). This is coincident with an increase in AMPK phosphorylation on Thr172 (Kimball, Siegfried and Jefferson. 2004, Baum, Kimball and Jefferson. 2009), a positive regulator of TSC1/2 (see section 1.2.3). Of particular interest is that cAMP has anti-proliferative effects on cancer cells, which can be mediated by either targeting cell cycle regulatory molecules (Rocha et al. 2008, Chen et al. 2002, Naderi et al. 2005), or cellular signalling pathways pivotal to cell cycle progression, such as the MAPK pathway (Dumaz and Marais. 2005). It is possible that the anti-proliferative effects of cAMP are mediated, at least in part, by the inhibition of mTORC1 (Rocha et al. 2008), as the over-activation of mTORC1 has been implicated in the development and maintenance of tumours (Zoncu, Efeyan and Sabatini. 2011).

5.1.2 Chapter aims

In the third chapter, I have shown that $[cAMP]_i$ elevating agents, such as GLP-1, are able to activate mTORC1 in pancreatic β-cells. Therefore, we turned to HEK293 cells to investigate the mechanism by which cAMP signals to mTORC1. However, we found that forskolin, a diterpene which activates adenylyl cyclase (Seamon, Padgett and Daly. 1981), and IBMX (3-isobutyl-1-methylxanthine), which inhibits PDEs (Houslay. 2010)), inhibited insulin-induced mTORC1 activation in HEK293 cells [Fig. 1A in (Xie et al. 2011a), performed by Moore CE]. This was interesting because forskolin and IBMX are

stimulatory to mTORC1 in pancreatic β-cells (Moore et al. 2009). Therefore, I investigated the molecular mechanism by which increases in $[cAMP]_i$ suppresses mTOR complexes.

5.2 Results

5.2.1 cAMP inhibits insulin stimulated mTORC1 activation in MEFs

To select a cell line to study the inhibitory effect of cAMP on mTORC1 activation, I first tested mouse embryonic fibroblasts (MEFs). MEFs were treated with insulin, which led to an increase in the activity of mTORC1, as evidenced by the augmentation of S6K1 phosphorylation on Thr389, 4EBP1 on Ser64 and rpS6 on Ser240/Ser244, I also observed a decrease in the electrophoretic mobility of S6K1 in insulin-treated cells, indicative of its phosphorylation (Fig. 5.1). In addition, insulin treatment led to an increase in the phosphorylation of PKB on Ser473, an indicator of its activation state (Bozulic and Hemmings. 2009). PKB can activate mTORC1 through the phosphorylation and inactivation of TSC2, an upstream inhibitor of mTORC1 (Huang and Manning. 2008), and the phosphorylation of PRAS40 on Thr246 (Sancak et al. 2007, Wang et al. 2007, Vander Haar et al. 2007). Indeed, insulin stimulated the phosphorylation of PRAS40 on Thr246 (Fig. 5.1). However, in cells pretreated with increasing concentrations of forskolin and IBMX which elevated $[cAMP]_i$ [as shown in Fig. 1D in (Xie et al. 2011a), performed by Ponuwei GA], insulin-stimulated mTORC1 activation was suppressed as determined by the phosphorylation of S6K1 on Thr389, 4EBP1 on Ser64, rpS6 on Ser240/Ser244 and the electrophoretic mobility of S6K1. This paralleled a decrease in the

phosphorylation of PKB at Ser473 and PRAS40 on Thr246 (Fig. 5.1). Therefore, in MEFs, cAMP-dependent inhibition of mTORC1 may be mediated via the inhibition of PKB. The MAPK pathway is unlikely to be involved as insulin treatment of MEFs had no effect on the phosphorylation of ERK (Fig. 5.1).

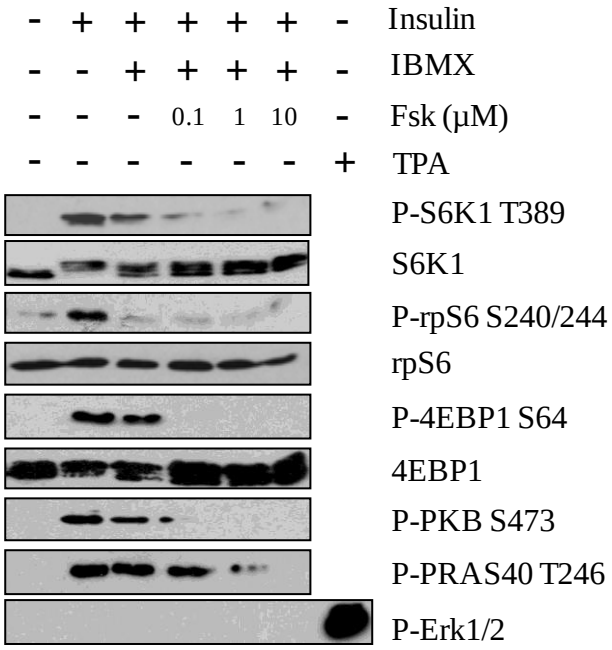
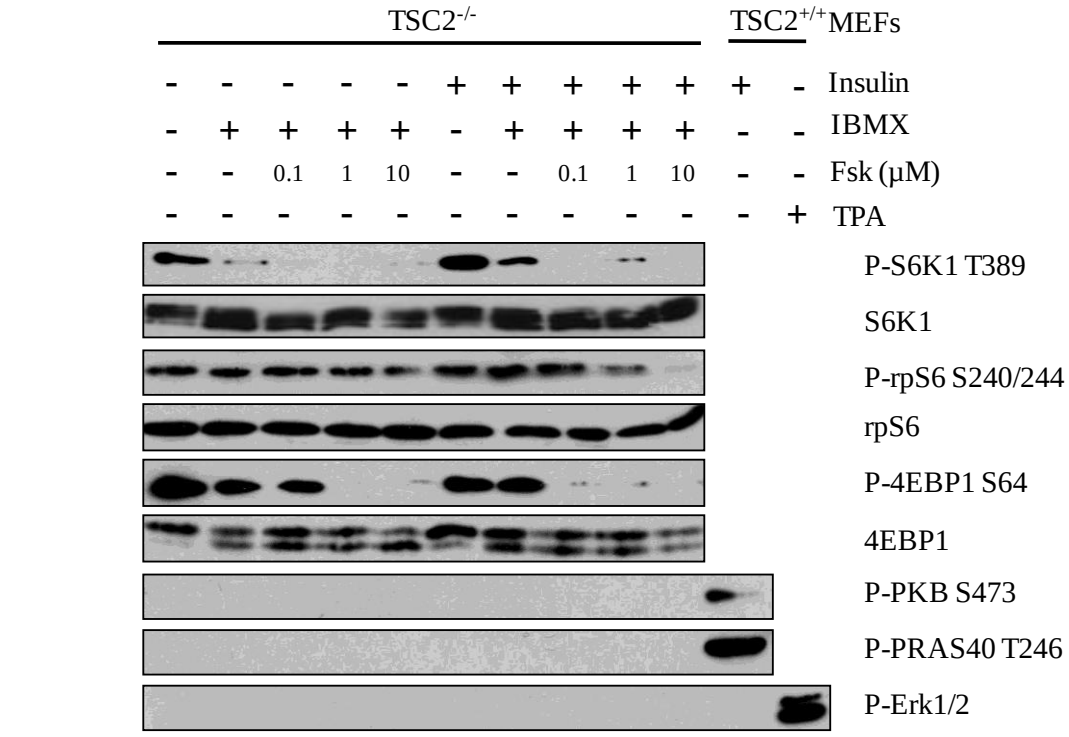


Figure 5.1 cAMP inhibits insulin-induced activation of mTORC1 and 2. MEFs were serum starved for 16 h, and then incubated in KRB buffer supplemented with 0.5X amino acids for 30 min, pre-treated with forskolin (10 μM) and IBMX (1 mM) for 30 min, before stimulated with insulin (100 nM) for another period of 30 min. Cell were lysed and lysates were separated by SDS-PAGE and western blotted for phospho (P)-S6K1 Thr389 (T389), P-rpS6 Ser240/Ser244 (S240/244), P-4EBP1 Ser64 (S64), P-PKB Ser473 (S473), P-PRAS40 Thr246 (T246), P-Erk1/2, as well as total S6K1, rpS6 and 4EBP1. Immunoblots are representative of two independent experiments.

5.2.2 cAMP inhibits mTORC1 independently of TSC2

In order to explore the molecular mechanism by which cAMP inhibits mTORC1, I investigated whether cAMP could inhibit mTORC1 in cells deleted of TSC2. $TSC2^{-/-}$ MEFs were pretreated with increasing concentrations of forskolin plus IBMX in the absence or presence of insulin (Fig. 5.2). mTORC1 is constitutively activated in these cells due to inactivation of the TSC1/2 complex (Inoki et al. 2002, Gao et al. 2002, Goncharova et al. 2002). Therefore, S6K1 and 4EBPs are constitutively hyper-phosphorylated in serum starved cells and insulin treatment is unable to further stimulate mTORC1. Importantly, incubation of these cells with forskolin/IBMX dose-dependently suppressed the phosphorylation of S6K1 (Thr389) and 4EBP1 (Ser64) (Fig. 5.2). This demonstrates that cAMP can inhibit mTORC1 independently of TSC1/2 and potentially reveals a novel and additional mechanism by which cAMP inhibits mTORC1. Moreover, no change in the phosphorylation state of PRAS40 was detected indicating that the suppression of mTORC1 activity is likely independent of PRAS40 in these cells (Fig. 5.2).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TSC2+/+MEFs
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P-S6K1 T389
S6K1
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rpS6
P-4EBP1 S64
4EBP1
P-PKB S473
P-PRAS40 T246
P-Erk1/2

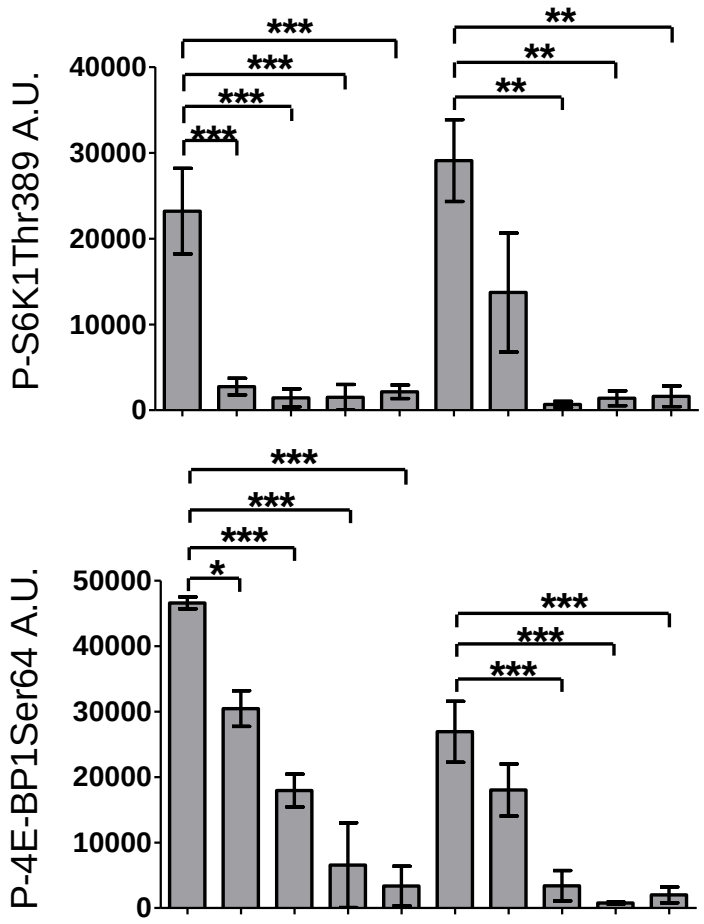
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Figure 5.2 cAMP inhibits the activation of mTORC1 independently of TSC2. $TSC2^{-/-}$ and $TSC2^{+/+}$ MEFs were serum starved for 16 h, and then incubated in KRB buffer supplemented with 0.5X amino acids for 30 min, pre-treated with forskolin (10 μM) and IBMX (1 mM) for 30 min, before stimulated with insulin (100 nM) for a further period of 30 min. Cell were lysed and proteins were resolved by SDS-PAGE and immunoblotted for indicated phospho (P)-S6K1 Thr389 (T389), P-rpS6 Ser240/Ser244 (S240/244), P-4EBP1 Ser64 (S64), P-PKB Ser473 (S473), P-PRAS40 Thr246 (T246), P-Erk1/2, as well as total S6K1, rpS6 and 4EBP1.. P-S6K1 T389 and P-4EBP1 S64 levels were quantified by densitometry analysis and are presented as arbitrary units (A.U.). Results were shown as means ± SE; $n = 3$. *P = 0.05-0.01, **P = 0.01-0.001, ***$P < 0.001$ using Dunnett's test following one-way ANOVA. Immunoblots are representative of three independent experiments.

5.2.3 AMPK is not involved in the inhibition of mTORC1 by cAMP

Glucagon, a GsPCR agonist which augments $[cAMP]_i$, can inhibit mTORC1 and this has been shown to correlate with the phosphorylation of AMPK (Kimball, Siegfried and Jefferson. 2004, Baum, Kimball and Jefferson. 2009). Interestingly, AMPK can also block the activity of mTORC1 independently of TSC1/2 via the phosphorylation of RAPTOR (Gwinn et al. 2008). To investigate whether the inhibitory effect of cAMP could be mediated by AMPK; $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were infected with increasing concentrations of adenovirus expressing dominant-negative AMPK (Ad DN-AMPK) (Fig. 5.3). Firstly, I sought to find out the amount of virus that should be used for an effective block to AMPK activation. Cells were treated with AICAR (5-Aminoimidazole-4-carboxamide ribotide) to evoke the phosphorylation and activation of AMPK and serum treatment was used as a negative control. As shown in Fig. 5.3A, 20 μl of the virus in

$TSC2^{+/+}$ or 10 μl of the virus in $TSC2^{-/-}$ MEFs were sufficient to effectively inhibit AICAR induced AMPK phosphorylation. These amounts of viruses were applied in the subsequent experiment (Fig. 5.3B). As anticipated, the over-expression of DN-AMPK abolished AICAR induced phosphorylation of AMPK, yet cAMP was still able to suppress insulin-mediated mTORC1 activation in both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells, as determined by the phosphorylation status of S6K1 and 4EBP1 (Fig 5.3B). In addition, I was unable to detect any increase in AMPK phosphorylation in cells treated with forskolin and IBMX. Therefore, I conclude that an elevation in $[cAMP]_i$ can inhibit mTORC1 independently of AMPK activity.

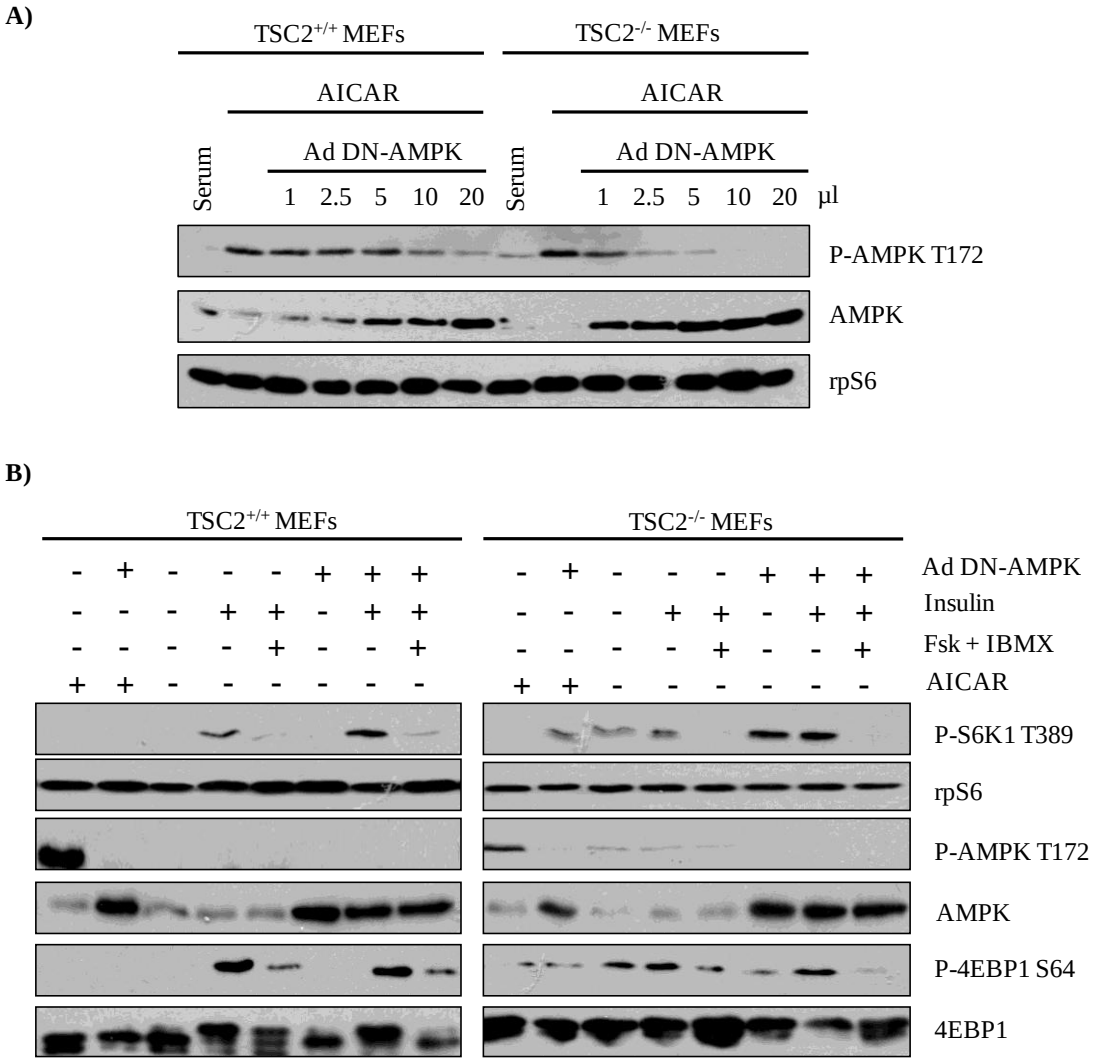

Figure 5.3 AMPK is not involved in the inhibition of mTORC1 by cAMP. **A)** Titration of the Ad DN-AMPK. $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were infected with indicated amount of adenovirus over-expressing dominant negative AMPK (Ad DN-AMPK), 32 h post infection, cells were serum starved and then incubated in KRB buffer supplemented with 0.5X amino acids for 30 min, before treated with AICAR (1 mM) or serum (10%) for 1 h. **B)** $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were infected with Ad DN-AMPK virus (20 µl for $TSC2^{+/+}$ MEFs and 10 µl virus for $TSC2^{-/-}$ MEFs), 32 h post-infection, cells were serum starved for 16 h, and then incubated in KRB buffer supplemented with 0.5X amino acids for 30 min, before treated with forskolin (10 µM) and IBMX (1 mM) for another period of 30 min, cells were then treated with AICAR (1 mM) or insulin (100 nM) for a final period of 30 min. Cell lysates were run on a SDS-PAGE gel followed

by western blotting of indicated phospho (P)-S6K1 Thr389 (T389), P-AMPK Thr172 (T172), P-4EBP1 Ser64 (S64), as well as AMPK, rpS6 and 4EBP1. Immunoblots in A) are from one experiment and immunoblots from B) are representative of two independent experiments.

5.2.4 cAMP inhibits amino acid signalling to mTORC1

Amino acids alone are able to stimulate mTORC1 via a mechanism that bypasses TSC1/2 (Smith et al. 2005). To investigate whether cAMP can suppress mTORC1 activity driven by amino acids, $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were incubated in the absence or presence of forskolin and IBMX prior to activation of mTORC1 by the addition of amino acids (Fig. 5.4A). As anticipated, the activity of mTORC1 was augmented in response to amino acids in both $TSC2^{+/+}$ and $TSC2^{-/-}$ cells as demonstrated by the phosphorylation of S6K1 (Thr389) and 4EBP1 (Ser64) and a decrease in the electrophoretic mobility of S6K1 and 4EBP1 (Fig. 5.4A). PKB, PRAS40 and ERK were not phosphorylated upon amino acids treatment. Interestingly, amino acid stimulated mTORC1 activation was attenuated in cells treated with forskolin/IBMX indicating that cAMP inhibits mTORC1 independently of TSC1/2 (Fig. 5.4A).

The over-expression of Rheb can overcome the effect of amino acids depletion and constitutively activate mTORC1 (Nobukuni et al. 2005, Smith et al. 2005). Therefore, to provide additional evidence that the effect of cAMP on mTORC1 is independent on Rheb, $TSC2^{-/-}$ MEFs (Fig. 5.4B) and HEK293 cells (Fig. 5.4C) were co-transfected with FLAG tagged Rheb (FLAG-Rheb) and HA tagged S6K1 (HA-S6K1). As anticipated, Rheb over-expression caused the constitutive activation of mTORC1, even in the absence

of amino acids, as determined by the phosphorylation of S6K1 on Thr389 (Fig. 5.4B and C). Importantly, in Rheb over-expressing cells, the phosphorylation of S6K1 was attenuated in the presence of forskolin/IBMX in both $TSC2^{-/-}$ MEFs and HEK293s (Fig. 5.4B and C). These results provide evidence that the inhibitory effect of cAMP is downstream of Rheb.

Rag GTPases are crucial for amino acids signalling to mTORC1 (Sancak et al. 2008, Kim et al. 2008). In order to study whether cAMP is exerting its inhibitory effect through Rag GTPases, active Rag GTPases [$RagB^{GTP}$Q99L and $RagC^{GDP}$S75L, as reported previously in (Sancak et al. 2008)] and HA-S6K1 were co-transfected in HEK293 cells, cells were incubated in KRB in the absence of amino acids. As amino acids play a permissive role in insulin signalling to mTORC1 (Avruch et al. 2009), insulin alone was unable to induce the phosphorylation of S6K1 (Fig. 5.4D). In contrast, the over-expression of active Rag GTPases evoked the phosphorylation of S6K1, which was potentiated by the addition of insulin. Treatment of cells with forskolin/IBMX was still able to inhibit mTORC1 in the presence of active Rag GTPases (Fig. 5.4D). These results indicate that inhibition of mTORC1 by cAMP is downstream of Rag GTPases.

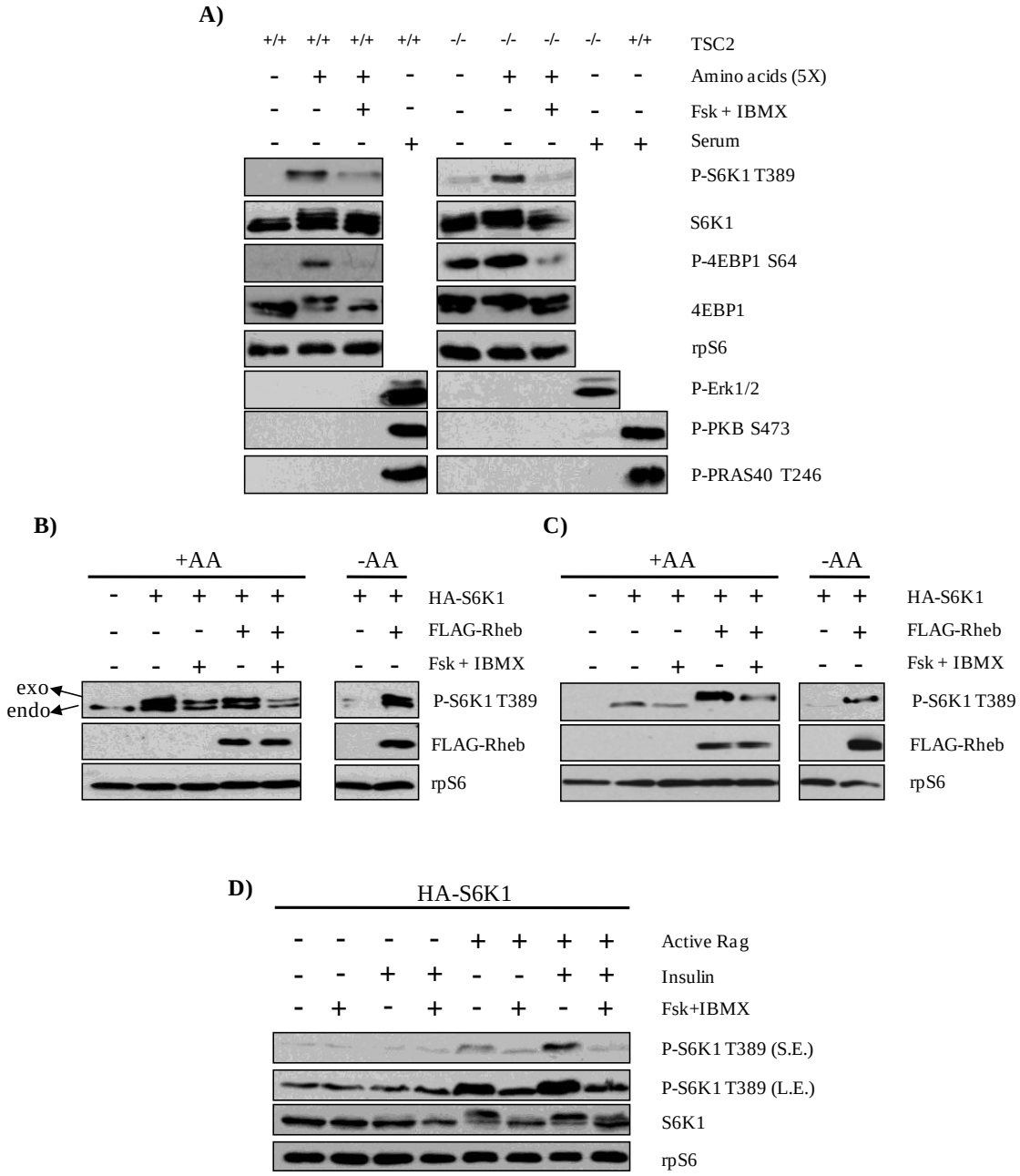

Figure 5.4 cAMP suppresses amino acids signalling to mTORC1. **A)** $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were serum-starved in DMEM for 16 h before preincubation in KRB buffer supplemented with 20 mM glucose but in the absence of amino acids. Forskolin (10 µM) and IBMX (1 mM) were added 30 min post-preincubation, and then after another period of 30 min, cells were treated with amino acids (5X) or serum (10%). Cells were lysed 30 min post-amino acids treatment. **B)** $TSC2^{-/-}$ MEFs and **C)** HEK293 cells were

co-transfected with FLAG-Rheb and HA-S6K1 for 48 h prior to the experiment. At 16 h before the experiment, cells were serum-starved in DMEM, and then preincubated in KRB buffer supplemented with 20 mM glucose and with (+AA) or without (-AA) amino acids (5X) but with 20 mM glucose. At 30 min post-preincubation, cells were treated with forskolin (10 μM) and IBMX (1 mM) for 1 h before lysis. exo: exogenous S6K1, endo: endogenous S6K1. **D)** HEK293 cells were co-transfected with $RagB^{GTP}Q99L$, $RagC^{GDP}S75L$ (constitutively active rag) and HA tagged S6K1 (HA-S6K1). At 32 h post-transfection, cells were serum-starved in DMEM for 16 h before preincubation in KRB supplemented with 20 mM glucose but in the absence of amino acids for 30 min, followed by the addition of forskolin (10 μM) plus IBMX (1 mM) for 30 min, and then further treated with insulin (100 nM) for another period of 30 min before lysis. Lysates were separated on SDS-PAGE and Western blotted using antisera against phosphorylated (P)-S6K1 Thr389 (T389), P-4EBP1 Ser64 (S64), P-PKB Ser473 (S473), P-PRAS40 Thr246 (T246), P-Erk1/2, as well as exogenous Rheb (FLAG-Rheb), 4EBP1, rpS6 and S6K1. S.E.: short exposure; L.E.: long exposure. All results are representative of three independent experiments.

5.2.5 cAMP inhibits mTORC1 via PKA

PKA is a major effector of cAMP (Pearce, Komander and Alessi. 2010). To determine whether the inhibitory effect of cAMP on mTORC1 is mediated through PKA, $TSC2^{-/-}$ MEFs (Fig. 5.5A) and HEK293 cells (Fig. 5.5B) were pretreated with the PKA selective inhibitor H89 prior to forskolin and IBMX treatment. As anticipated, amino acids stimulated the phosphorylation of S6K1 on Thr389 and this was inhibited by increased $[cAMP]_i$. As expected, H89 dose-dependently inhibited the phosphorylation of rpS6 in Ser236/236 which is mediated by PKA (Moore et al. 2009). Importantly this correlated with the recovery of S6K1 phosphorylation on Thr389 (Fig. 5.5), indicating that the inhibitory effect of cAMP on mTORC1 is likely mediated by PKA.

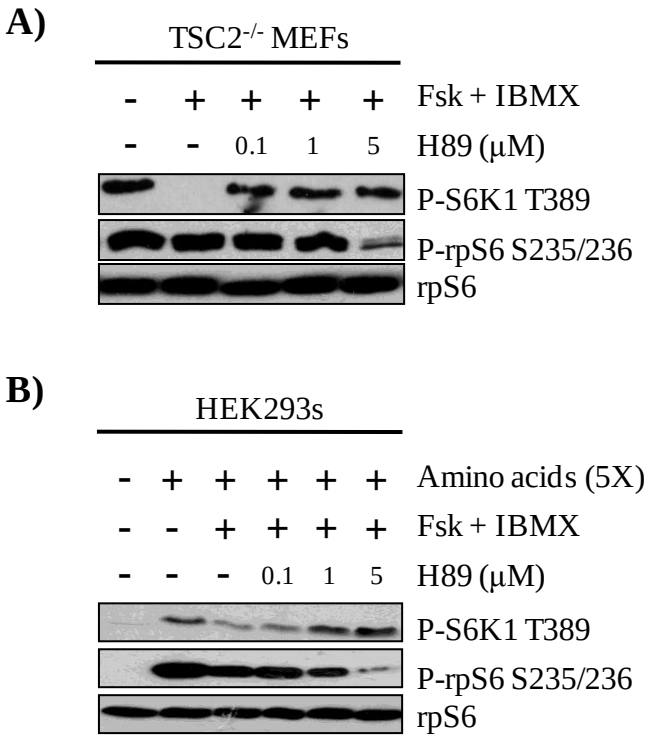

Figure 5.5 The inhibition of mTORC1 is PKA dependent. **A)** TSC2$^{-/-}$ MEFs and **B)** HEK293 cells were serum-starved in DMEM for 16 h. For A), cells were incubated in KRB supplemented with 0.5X amino acids. For B), cells were incubated in KRB supplemented with 20 mM glucose but in the absence of amino acids. For both A) and B), after 30 min, cells were treated with increasing concentrations of H89, a PKA inhibitor, for 30 min, followed by treatment with forskolin (10 μM) and IBMX (1 mM) for 30 min, before stimulation with amino acids (5X) for a further period of 30 min (for B). Proteins were resolved on SDS-PAGE and Western blotted using antibodies against phosphorylated (P)-S6K1 Thr389 (T389), P-rpS6 Ser235/236 (S235/236), and as a loading control, rpS6. All results are representative of three independent experiments.

5.2.6 Increased $[cAMP]_i$ causes dissociation of mTOR complexes

The results so far indicate that cAMP inhibits mTORC1 via the activation of PKA and independently of the classical regulatory inputs upstream of mTORC1. One possibility is that an elevation in $[cAMP]_i$ causes the PKA-dependent dissociation of mTORC1. Therefore, I investigated whether the integrity of mTOR complexes is disturbed upon increases in $[cAMP]_i$. For this purpose, HEK293 cells transfected with myc-tagged mTOR (myc-mTOR) were incubated with forskolin and IBMX prior to stimulation with serum, myc-mTOR and associated proteins were then immunoprecipitated from these cells. In serum repleted cells mTORC1 was active, as determined by the phosphorylation status of S6K1. In addition, mLST8 and the mTORC1 specific components RAPTOR and PRAS40, and the mTORC2 specific component RICTOR were co-immunoprecipitated with myc-mTOR, indicating the integrity of mTORC1 and mTORC2 under these conditions. As expected, the preincubation of cells with forskolin/IBMX inhibited serum-stimulated mTORC1 activity, as determined by the phosphorylation status of S6K1. Moreover, preincubation of cells with forskolin/IBMX caused the dissociation of the mTORC1 specific component RAPTOR and PRAS40. In addition, forskolin/IBMX caused the dissociation of the mTORC2 specific component RICTOR from myc-mTOR. However, the association of mLST8 to myc-mTOR was unaffected (Fig. 5.6). In conclusion, elevations in $[cAMP]_i$ lead to the dissociation of mTORC1 and mTORC2.

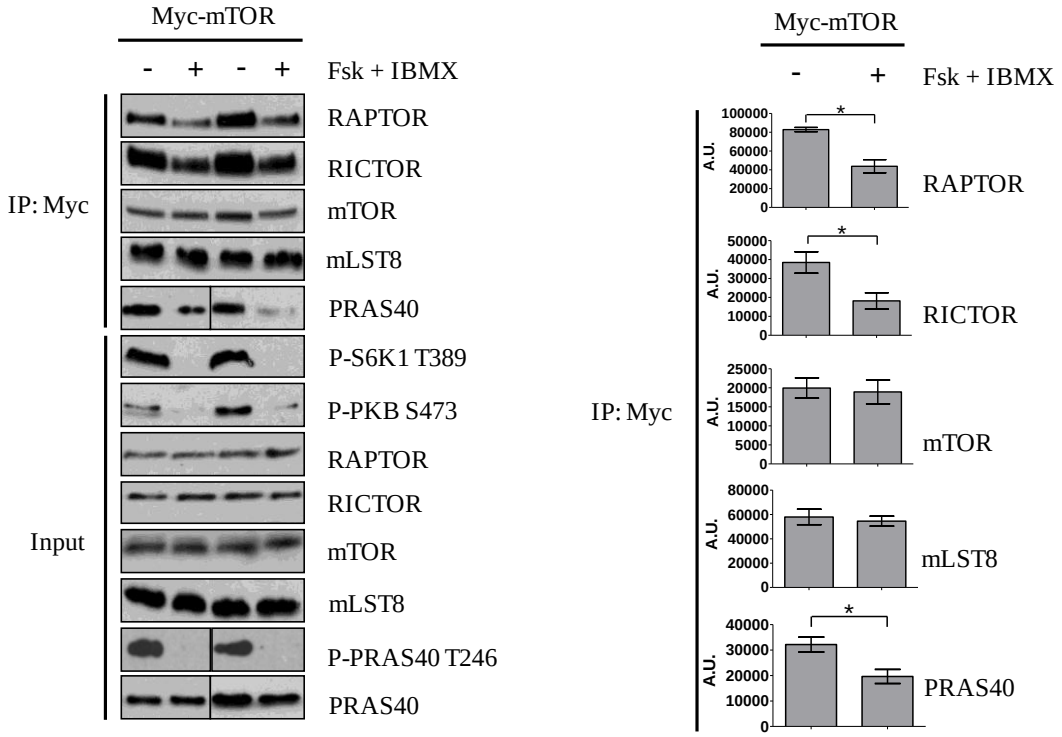

Figure 5.6 cAMP leads to dissociation of the mTOR complexes. HEK293 cells were transfected with myc-tagged mTOR (myc-mTOR). At 32 h post-transfection, cells were serum-starved in DMEM for 16 h before preincubation in Dulbecco's-PBS for 30 min, and then stimulation with DMEM supplemented with 10% serum in the presence or absence of forskolin (10 μM) and IBMX (1 mM) for 1 h. Cell lysates were immunoprecipitated with anti-myc antibodies and immunoblotted as indicated. Levels of RAPTOR, RICTOR, mTOR, mLST8 and PRAS40 from immunoprecipitation were quantified by densitometric analysis. Proteins were resolved on SDS-PAGE and Western blotted using antibodies against RAPTOR, RICTOR, mTOR, mLST8, PRAS40, as well as phosphorylated (P)-S6K1 Thr389 (T389), P-PKB Ser473 (S473) and P-PRAS40 Thr246 (T246). Results are means ± SE. * $P < 0.05$ by Student's paired *t*-test, $n = 3$ where the analysis was performed on the raw absorbance data. Immunoblots are representative of three independent experiments.

5.2.7 Increased $[cAMP]_i$ inhibits mTOR autophosphorylation

To further investigate the molecular mechanism by which cAMP inhibits mTORC1, I monitored the auto-phosphorylation of mTOR on Ser2481, which has been shown to be a biomarker for intrinsic mTORC-specific catalytic activity (Soliman et al. 2010). In $TSC2^{-/-}$ MEFs or in insulin-stimulated or FLAG-Rheb transfected HEK293 cells, forskolin/IBMX treatment led to a reduction in the phosphorylation of mTOR on Ser2481 (Fig. 5.7). Interestingly, the phosphorylation of mTOR on Ser2448, which has been reported to be mediated by S6K1 (Holz and Blenis. 2005, Chiang and Abraham. 2005), was unaffected by cAMP, whereas rapamycin treatment led to a reduction in the phosphorylation of mTOR on both sites (Ser2481 and Ser2448). However, S6K1 phosphorylation is as reduced in forskolin/IBMX as much as rapamycin treated cells. Therefore, this phosphorylation event is occurring independently to S6K1 (Fig. 5.7).

Taken together, these results suggest that cAMP inhibits mTORC1 via the perturbation of mTOR complex assembly and the reduction of mTOR catalytic activity.

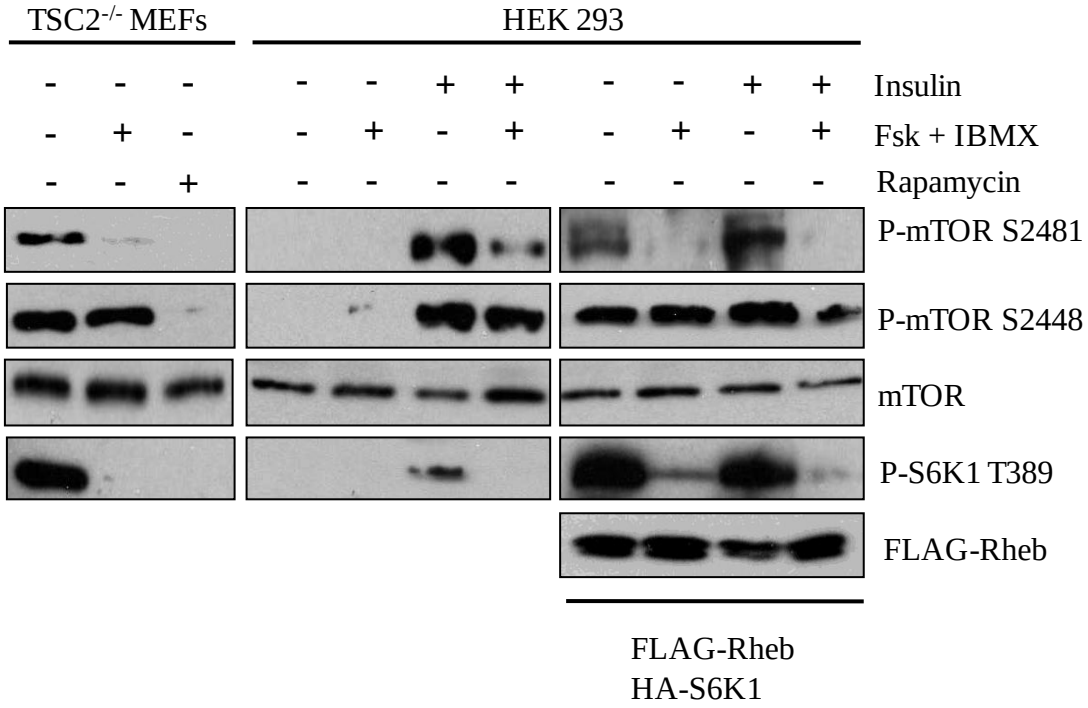

Figure 5.7 cAMP inhibits the catalytic activity of mTOR. TSC2$^{-/-}$ MEFs and HEK293 cells were serum-starved in DMEM for 16 h before preincubation in KRB for 30 min, then incubated in KRB with forskolin (10 μM) plus IBMX (1 mM) for 30 min, before further treatment with insulin (100 nM) for another period of 30 min. HEK293 cells were also co-transfected with FLAG-Rheb and HA-S6K 48 h prior to the experiment. Cell lysates were separated on SDS-PAGE and subjected to immunoblotting with antisera against phosphorylated (P)-mTOR Ser2481 (S2481), Ser2448 (S2448), S6K1 Thr389 (T389), and as loading controls, mTOR and exogenous Rheb (FLAG-Rheb). All immunoblots are representative of three independent experiments.

5.2.8 cAMP inhibits mTORC2

As Forskolin/IBMX leads to the dissociation of mTORC2 and a decrease in mTOR activity it seems likely that elevations in $[cAMP]_i$ may also inhibit mTORC2. mTORC2 phosphorylates the turn motif on PKB, PKCα and PKCβII, resulting in their stabilisation

(Ikenoue et al. 2008, Facchinetti et al. 2008). mTORC2 is also responsible for the phosphorylation of PKB on Ser473 (Sarbassov et al. 2005). Therefore, to determine whether mTORC2 is also inhibited by $[cAMP]_i$, $TSC2^{+/+}$ MEFs were treated with forskolin and IBMX for up to 24 h and the phosphorylation and expression of PKB, PKCα and PKCβII were determined. Incubation of cells with Forskolin/IBMX caused a time-dependent reduction in turn motif phosphorylation on PKB, PKCα and PKCβII, and this paralleled a decrease in the protein levels of PKB and PKCα (Fig. 5.8). These results provide evidence that elevation of $[cAMP]_i$ not only inhibits mTORC1 (Fig. 5.1-5.7) but also inhibits mTORC2 (Fig. 5.8).

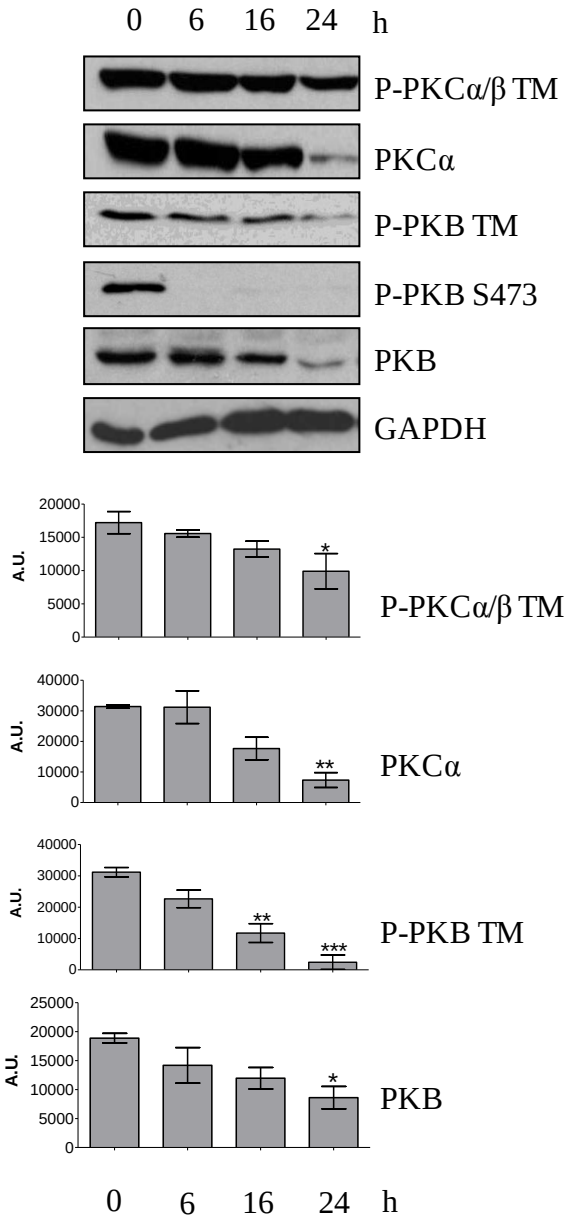

Figure 5.8 cAMP inhibits mTORC2. $TSC2^{+/+}$ MEFs were maintained in growth media, treated with forskolin (10 μM) and IBMX (1 mM) for 6, 16 or 24 h. Cell lysates were resolved by SDS-PAGE, followed by Western blotting of phosphorylated (P)-PKCα/βII turn motif (TM), P-PKB TM, P-PKB Ser473 (S473), total PKCα and total PKB. GAPDH was used as a loading control. P-PKCα/βII TM, PKCα, P-PKB TM and PKB levels were quantified by densitometric analysis. Results are means ± SE. **P* = 0.05-0.01, ***P* = 0.01-0.001, ****P* < 0.001 versus control (untreated) using one-way ANOVA followed by Dunnett's

test, $n = 3$ where the analysis was performed on the raw absorbance data. Immunoblots are representative of three independent experiments.

5.2.9 cAMP negatively regulates protein synthesis in MEFs

As mTOR is implicated in the regulation of anabolic processes like mRNA translation (see section 1.4.1), I sought to find out if increases in $[cAMP]_i$ affected protein synthesis in $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs. Rates of total protein synthesis were determined by monitoring the incorporation of [^{35}S]methionine into newly synthesized proteins. As shown in Fig. 5.9, in $TSC2^{+/+}$ cells, forskolin and IBMX significantly reduced protein synthesis in the absence or presence of insulin stimulation. Protein synthesis was doubled in $TSC2^{-/-}$ control cells compared to $TSC2^{+/+}$ cells, and was substantially decreased by the effect of cAMP elevation. Insulin alone also leads to a reduction of [^{35}S]methionine incorporation in $TSC2^{-/-}$, probably due to the activation of negative feedback mechanisms (i.e. S6K1 to IRS or mTORC1 to Grb10, see section 1.3.1 for details) as a result of over-stimulation of mTORC1. Surprisingly, rapamycin treatment had no effect on insulin induced mRNA translation, this is in agreement with previous observations that many mTORC1 functions, such as cap-dependent translation, are rapamycin resistant [reviewed in (Thoreen and Sabatini. 2009, Huo, Iadevaia and Proud. 2011)]. Taken together, these results suggested that total protein synthesis can be attenuated by increases in $[cAMP]_i$ independently of TSC2.

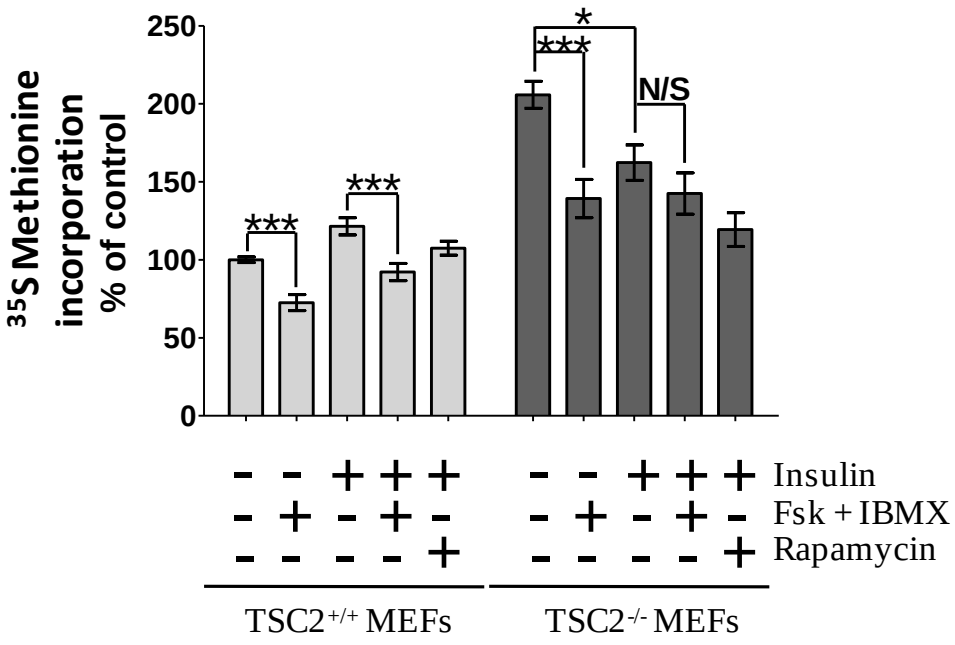

Figure 5.9 cAMP negatively regulates protein synthesis in MEFs. $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were preincubated in KRB for 30 min, and treated with forskolin (10 µM) and IBMX (1 mM) for another period of 30 min. Cells were then treated with insulin and [^{35}S]-methionine for a further period of 1 h, before lysed in ice cold lysis buffer. 20 µg of proteins were spotted onto Whatman papers, washed by boiling them in 5% trichloroacetic acid containing 0.1 g/L L-methionine. Papers were then dried out at 80 °C and the radioactivity was determined by scintillation counting. Results shown are representative of three independent experiments, two of which performed in duplicates. Results are means ± SE; $n = 5$. *P = 0.05-0.01, ***$P < 0.001$, N/S = not significant using one-way ANOVA followed by Bonferroni adjustment.

5.2.10 cAMP negatively regulates cell proliferation in MEFs

Activation of the mTOR pathway positively controls cell cycle progression (see section 1.4.3). In order to determine whether increases in $[cAMP]_i$ affects proliferation, $TSC2^{+/+}$ MEFs were serum starved for 48 h to synchronize them into G_1, and then released by the

addition of serum in the absence or presence of forskolin/IBMX and/or rapamycin. As shown in Fig. 5.10A, cells cycle was progressed from G_1 to S/G_2 phase upon serum repletion from 16 to 24 h, which is considerably blocked by either increasing $[cAMP]_i$ or rapamycin pre-treatment. cAMP and rapamycin also have an additive effect on the inhibition of cell cycle progression, because the percentage of cells found in S phase is approximately 10% lower than forskolin/IBMX or rapamycin treated ones (Fig. 5.10A). On the other hand, because the deletion of TSC2 in cells confers constitutive mTORC1 activation (Zhang et al. 2003), serum starvation was unable to cause cell cycle arrest into G_1 in $TSC2^{-/-}$ MEFs (Fig. 5.10B), therefore, these cells were incubated with forskolin/IBMX or rapamycin for 24 h directly in growing media, and this also led to a decrease in the percentage of S phase cells. Therefore, it can be concluded from these results that cAMP is able to prevent G_1 to S cell cycle progression in a TSC2 independent manner.

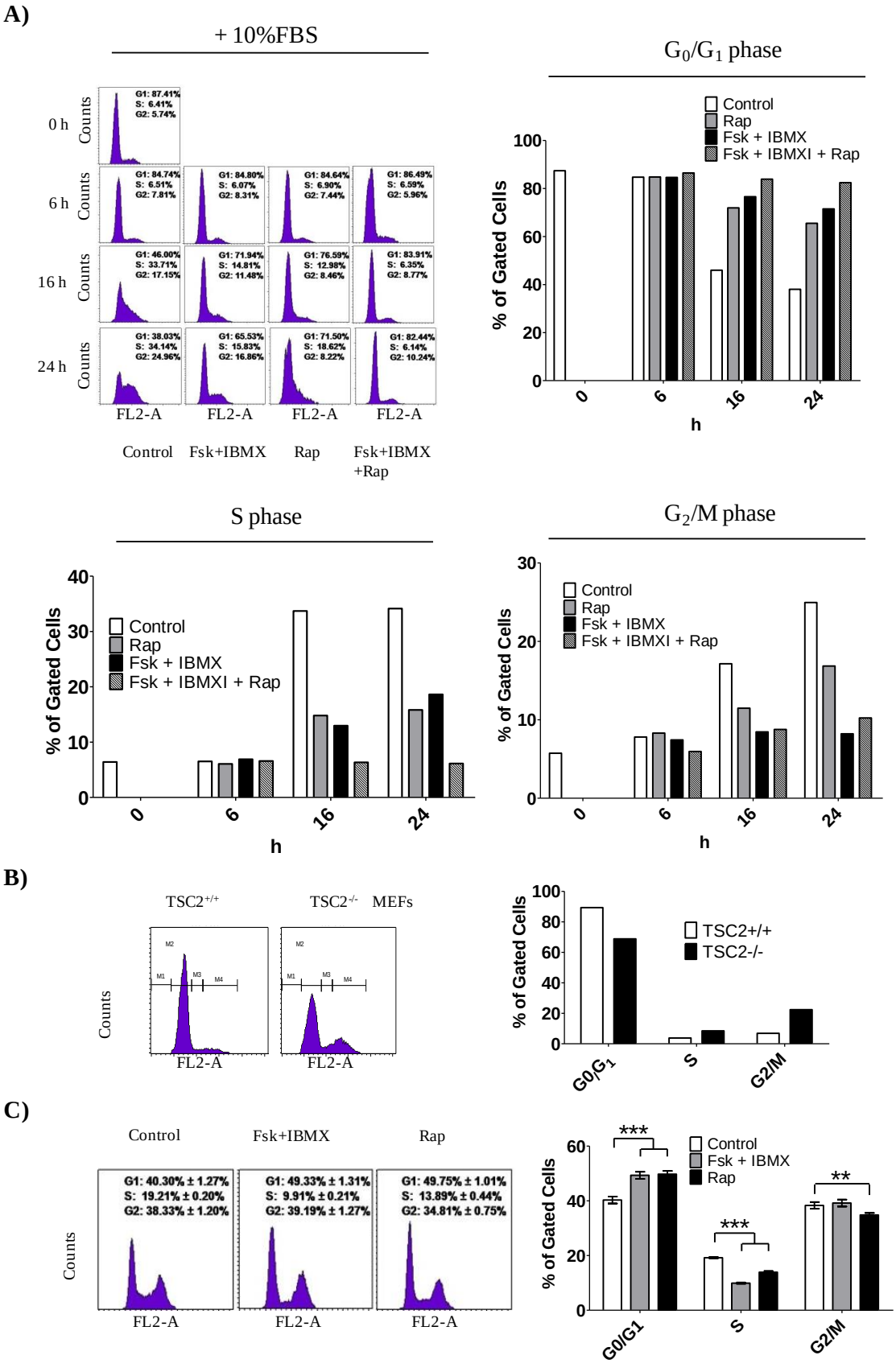
A)
+ 10%FBS
0 h
6 h
16 h
24 h
Counts
G1: 87.41%
S: 6.41%
G2: 5.74%
G1: 84.74%
S: 6.51%
G2: 7.81%
G1: 84.80%
S: 6.07%
G2: 8.31%
G1: 84.64%
S: 6.90%
G2: 7.44%
G1: 86.49%
S: 6.59%
G2: 5.96%
G1: 46.00%
S: 33.71%
G2: 17.15%
G1: 71.94%
S: 14.81%
G2: 11.48%
G1: 76.59%
S: 12.98%
G2: 8.46%
G1: 83.91%
S: 6.35%
G2: 8.77%
G1: 38.03%
S: 34.14%
G2: 24.96%
G1: 65.53%
S: 15.83%
G2: 16.86%
G1: 71.50%
S: 18.62%
G2: 8.22%
G1: 82.44%
S: 6.14%
G2: 10.24%
FL2-A
Control
Fsk+IBMX
Rap
Fsk+IBMX +Rap
G0/G1 phase
% of Gated Cells
Control
Rap
Fsk + IBMX
Fsk + IBMXI + Rap
h
S phase
G2/M phase
B)
TSC2+/+
TSC2-/-
MEFs
M1
M2
M3
M4
TSC2+/+
TSC2-/-
G0/G1
S
G2/M
C)
Control
Fsk+IBMX
Rap
G1: 40.30% ± 1.27%
S: 19.21% ± 0.20%
G2: 38.33% ± 1.20%
G1: 49.33% ± 1.31%
S: 9.91% ± 0.21%
G2: 39.19% ± 1.27%
G1: 49.75% ± 1.01%
S: 13.89% ± 0.44%
G2: 34.81% ± 0.75%
***
**
G0/G1
S
G2/M

Figure 5.10 cAMP negatively regulates cell cycle progression in MEFs. **A)** $TSC2^{+/+}$ MEFs only or **B)** $TSC2^{+/+}$ and $TSC2^{-/-}$ MEFs were cultured on 4 cm plates (5 x 10^5 cells per plate) and maintained in normally growing media (DMEM + 10% FCS) for 48 h, before being grown in low serum media (DMEM + 0.2% FCS) for 48 h, then cells were re-stimulated with normally growing media in the absence or presence of forskolin (10 μM) and IBMX (1 mM), or/and rapamycin (200 nM) for 6, 16 or 24 h, cell cycle distribution was determined by flow cytometry. % of gated cells in each of the cell phases $n = 1$. **C)** $TSC2^{-/-}$ MEFs were cultured on 4 cm plates (5 x 10^5 cells per plate) and maintained in normally growing media for 24 h, before treated with forskolin (10 μM) and IBMX (1 mM), or rapamycin (200 nM) for another 24 h, cell cycle distribution was determined by flow cytometry. $n = 3$. $^{**}P = 0.01\text{-}0.001$, $^{***}P < 0.001$, obtained using two-way ANOVA followed by Bonferroni adjustment.

5.3 Discussion

cAMP and mTOR signalling pathways coordinate to regulate fundamental cellular processes including metabolism, protein synthesis, proliferation and cell survival (Lawrence et al. 1997, Li et al. 2000). Therefore, it is of immense interest to understand the signalling events which integrate these two signalling pathways. In this chapter, I provide evidence that cAMP is able to inhibit the activation of mTORC1 and mTORC2 and this is likely mediated through PKA-dependent disruption of the mTOR complexes 1 and 2 and the inhibition of mTOR catalytic activity. I also show that cAMP suppresses protein synthesis and prevents cell cycle progression independently of the mTORC1 upstream regulator TSC2.

Hormones and growth factors can activate mTORC1 via the PKB- and/or ERK/RSK-dependent phosphorylation of TSC2 and the inactivation of the TSC1/2 complex (Sengupta, Peterson and Sabatini. 2010). However, in some cell types, cAMP inhibits these signalling pathways (Kimball, Siegfried and Jefferson. 2004, Baum, Kimball and Jefferson. 2009, Kim et al. 2001, Brennesvik et al. 2005, Filippa et al. 1999, Liu, Xie and Lou. 2005, Hong et al. 2008b, Dumaz and Marais. 2005, Hurley et al. 2006, Djouder et al. 2010) and hence inhibit hormone and growth factors activation of mTORC1. However, I demonstrate that cAMP can inhibit mTORC1 independently of these signalling pathways and independently of TSC1/2. Therefore, this work reveals an alternative and/or additional mechanism by which cAMP can inhibit mTORC1 in mammalian cells.

mTORC1 can also be regulated by the intracellular localization of Rheb independently of TSC1/2 (Sancak et al. 2008, Sancak et al. 2010). For example, amino acids promote the co-localisation of mTOR with Rheb through a Rag GTPase dependent mechanism, which alone can activate mTORC1 (Sancak et al. 2008, Kim et al. 2008). Interestingly, the over-expression of Rheb can overcome the inhibitory effect of amino acids withdrawal and this is thought to be mediated by the inappropriate co-localisation of Rheb with mTORC1 (Sancak et al. 2008, Sancak et al. 2010). It is unlikely that the inhibitory effect of cAMP on mTORC1 is caused by an alteration of the intracellular localization of Rheb as I show that forskolin/IBMX is able to reduce mTORC1 activity under conditions where Rheb or active Rag GTPases are over-expressed (Fig. 5.4). However, it cannot be excluded the possibility that cAMP perturbs Rheb-GTP loading independently of TSC1/2, perhaps through impinging on its guanine nucleotide exchange factor.

Although the molecular mechanism by which cAMP inhibits mTOR is not fully understood, it is dependent on the activation of PKA (Fig. 5.5). One possibility is that PKA directly phosphorylates mTOR and inhibits mTOR kinase activity. Indeed, cAMP inhibits mTOR intrinsic catalytic activity as determined by the auto-phosphorylation state of mTOR on Ser2481 (Fig. 5.7) and, interestingly, PKA has been reported to be associated with mTOR (Mavrakis et al. 2006). However, I and others have been unable to significantly phosphorylate mTOR *in vitro* with recombinant catalytic subunit of PKA [Xie, J. and Herbert, T.P., unpublished data, and (Scott and Lawrence. 1998)]. On the other hand, it has been reported that glucagon, which elevates $[cAMP]_i$, stimulates an increase in the phosphorylation of mTORC1 on Ser2448 in hepatocytes and this correlates with decreased mTORC1 activity (Mothe-Satney et al. 2004). However, this is unlikely to be the mechanism of inhibition as the mutation of this site has no effect on mTORC1 kinase activity (Sekulic et al. 2000, Mothe-Satney et al. 2004). Moreover, I could not detect any changes in the phosphorylation of mTOR on Ser2448 in response to elevated $[cAMP]_i$ in either HEK293 cells or MEFs (Fig. 5.7).

Importantly, I also showed that increased $[cAMP]_i$ led to the dissociation of both mTORC1 and 2 (Fig. 5.6), which is known to inhibit both mTORC1 and mTORC2 activity (Oshiro et al. 2004, Sarbassov et al. 2006, Kim et al. 2002). For example, upon rapamycin treatment, mTORC1 dimerization is compromised and the complex is disassembled in a time-dependent manner (Yip et al. 2010). However, whether complex

dissociation follows inactivation of mTOR or that mTOR inactivation follows the dissociation of the complex is unclear.

The phosphorylation of PRAS40 at Thr246 by PKB has been reported to promote mTORC1 activation through the dissociation of PRAS40 from mTOR (Sancak et al. 2007, Wang et al. 2007, Vander Haar et al. 2007). Surprisingly, the binding of PRAS40 to mTOR was reduced even though PRAS40 phosphorylation on Thr246 was ablated in response to cAMP (Fig. 5.7A). This raises doubt as to whether Thr246 can be used as an indicator of PRAS40 binding to mTORC1. As PRAS40 binds to RAPTOR within the complex (Yip et al. 2010, Sancak et al. 2007, Thedieck et al. 2007), the dissociation of PRAS40 from mTOR upon forskolin/IBMX treatment is likely caused by the dissociation of RAPTOR.

During cancer development, the mTOR pathway is often abnormally up-regulated, which favours cancer cell survival, growth, replication, angiogenesis and metastasis [reviewed in (Zoncu, Efeyan and Sabatini. 2011)]. Therefore, the inhibition of mTOR is a potential treatment for certain forms of cancer [reviewed in (Sparks and Guertin. 2010, Guertin and Sabatini. 2009, Menon and Manning. 2008)]. Similarly, cAMP negatively regulates protein synthesis, cell cycle progression and cell motility in cancer cells, most likely through the down-regulation of signalling pathways that control these processes, such as PI3K-PKB pathways, MAPK pathways and AMPK pathways, therefore the augmentation of $[cAMP]_i$ is a promising future cancer treatment (Kim et al. 2002, Rocha et al. 2008, Kim and Lerner. 1998, Murata et al. 2000, Lerner and Epstein. 2006). Here I have shown

that cAMP inhibits protein synthesis and cell cycle progression in cells where mTORC1 is constitutively activated as a result of TSC2 deletion. It can be tempting to speculate that at least part of these catabolic effects of cAMP is mediated through the inhibition of mTOR. However, cAMP can also target a number of cell cycle regulators such as $p21^{Cip1/Waf1}$, $p27^{Kip1}$, Rb (retinoblastoma protein) (Chen et al. 2002, Naderi et al. 2005) and CDK4 (cyclin D dependent kinase 4) (Rocha et al. 2008), although some of these proteins can also be regulated by mTORC1 [reviewed in (Wang and Proud. 2009)]. Therefore, it is difficult to differentiate mTOR dependent and independent effects of cAMP on the control of protein synthesis and proliferation.

In conclusion, I have shown that elevation of $[cAMP]_i$ suppresses mTORC1/2 by promoting mTOR complex disassembly and inhibiting mTOR's intrinsic catalytic activity, cAMP is also suppressive to protein synthesis and proliferation independently of TSC2, which lies upstream of mTORC1. These observations provide new insights into the crosstalk between cAMP and mTOR, which may also contribute to the design of novel mTOR inhibitors for future strategies in the fight against cancer.

Chapter 6. Final Discussion

6.1 Overview

mTOR is a master regulator of many anabolic and catabolic processes of the cell. The understanding of how mTOR is regulated and how it controls these cellular functions has great implications in the design of future strategies against human diseases like cancer, diabetes, cardiovascular diseases, neurodegenerative diseases and aging. In this thesis, I have investigated the role of mTOR in the control of pancreatic β-cell mass and have found that GLP-1 or exendin-4-induced β-cell proliferation is mediated through the IGF1R-PKB-mTOR pathway, although expressing hypophosphorylated 4EBP1 in MIN6 cells impairs cell viability, it is unlikely that the proliferative effect of GLP-1 is mediated through 4EBP1 because GLP-1 does not affect 4EBP1-eIF4E binding. Conversely, inhibition of mTOR using rapamycin decreases β-cell viability through the inhibition of mTORC2 and the subsequent suppression of PKB activity. In contrast to the anabolic effect of $[cAMP]_i$ elevating agents on pancreatic β-cells, increases in $[cAMP]_i$ are catabolic in some cell types such as cancer cell lines HEK293 cells and MEFs. I have demonstrated that cAMP is inhibitory to mTORC1 and 2 in these cells through the suppression of mTOR catalytic activity and the dissociation of mTOR complexes. These data further unravelled the role of mTOR and cAMP in the regulation of β-cell and cancer cell functions, and contributed to our understanding of the importance of mTOR in diabetes and cancer.

6.2 mTOR and pancreatic β-cells

Age-related body weight gain and the loss of insulin sensitivity (insulin resistance) leads to the expansion of pancreatic β-cell mass, and consequently augments insulin secretion in order to meet the increasing insulin demand [reviewed in (Bouwens and Rooman. 2005, Lingohr, Buettner and Rhodes. 2002)]. Maladaptation of β-cell mass and function to compensate for the insulin resistance results in the pathogenesis of T2DM (Lingohr, Buettner and Rhodes. 2002). Many growth factors and hormones stimulate protein synthesis and β-cell mass expansion in a rapamycin-sensitive manner, implying a critical role of mTOR in the regulation of β-cell mass and function (Gomez et al. 2004, Kwon et al. 2004, Bartolome, Guillen and Benito. 2010, Xu et al. 1998b, Xu et al. 1998a).

In human islets, β-cell proliferation rate decreases in a progressive fashion after gestational period and reaches less than 0.1% after 6-month postnatally (Kassem et al. 2000). During gestation and pancreatectomy in rodents, β-cell proliferation plays a critical role in maintaining normal β-cell mass (Kassem et al. 2000, Dor et al. 2004). Moreover, GLP-1, a gut hormone that increases insulin secretion in response to oral glucose intake (McIntyre, Holdsworth and Turner. 1965), stimulates β-cell proliferation *in vivo* (Xu et al. 1999). Therefore, considerable efforts have been focused on determining the molecular mechanisms upon which GLP-1 drives β-cell replication (Buteau et al. 1999, Buteau et al. 2003, Cornu et al. 2010, Park et al. 2006, Wang et al. 2004, Buteau, Spatz and Accili. 2006, Buteau et al. 2001, Liu and Habener. 2008, Li et al. 2005, Bastien-Dionne et al. 2011). I have revealed that GLP-1 and exendin-4 increases β-cell replication through the IGF1R-PKB-mTOR pathway. GLP-1-mediated PKB-mTOR

activation is through the stimulation of PI3K (El El-Sayed NM, Herbert TP, unpublished data) and presumably involving IRS2. On the other hand, the inhibition of FoxO, a downstream target of mTORC2 (Guertin et al. 2006), contributes to the proliferative effect of GLP-1 (Buteau, Spatz and Accili. 2006), and suppression of FoxO activity is mediated through the activation of mTORC2 and PKB in β-cells (Gu et al. 2011), and βRicKO mice represented defects in β-cell replication. Therefore, it is mostly likely that the mTORC2-PKB-FoxO pathway also mediates the positive effect of GLP-1 on β-cell proliferation.

Interestingly, although hypophosphorylated 4EBP1 led to a dramatic increase in cell death in MIN6 cells, GLP-1 has no effect on 4EBP1-eIF4E binding yet it strongly induces the phosphorylation of rpS6 on Ser240/Ser244 which is controlled by S6K1 (Fig. 3.9), because rapamycin inhibits β-cell replication exerted by GLP-1, it is most likely that S6K1 rather than 4EBP1 is responsible for the proliferative effect of GLP-1. S6K1 is essential for the maintenance of β-cell size (Pende et al. 2000), yet the effect of S6K1 deletion in mice on β-cell replication has not been reported. At least four S6K downstream targets have been shown to be implicated in the induction of proliferation by S6K, including CCTβ (Abe et al. 2009), ERα (Yamnik et al. 2009), Mad1 (Zhu, Blenis and Yuan. 2008) and GSK3 (Zhang et al. 2006), it is therefore plausible that GLP-1 enhances β-cell proliferation through the activation of S6K and subsequently one or some of its downstream targets.

[^{3}H]thymidine incorporation into the DNA in INS1E cells treated with AKTi or rapamycin were dramatically reduced to approximately 20-40% of basal levels (Fig. 3.2A), implying that the inhibition of PKB and mTOR may severely impair cell viability in β-cells. Indeed, previous studies have indicated that rapamycin causes cell apoptosis *in vitro* in rodent and human islets (Bell et al. 2003, Fabian et al. 1993, Zhang et al. 2006), an increase in β-cell apoptosis has also been observed in rapamycin *in vivo* treated animals (Fraenkel et al. 2008). Therefore, in the forth chapter of my thesis, I carried out experimentation to study the molecular mechanism by which rapamycin exerts its detrimental effect on islets of Langerhans. My data have shown that prolonged rapamycin treatement causes the inhibition of mTORC2 and subsequent impairment of PKB activity, which is the major cause of rapamycin islet toxicity. This observation provides a mechanistic insight into rapamycin islet toxicity and further explains why patients who received islet transplantation using rapamycin as an immunosuppressant failed to maintain insulin independency (Ryan et al. 2005). Importantly, the inhibition of mTORC2 is also responsible for the development of insulin resistance in rapamycin treated animals (Lamming et al. 2012). Therefore, mTORC2 plays an important role in the maintenance of glucose homeostasis.

6.3 mTOR and cAMP

cAMP is produced in response to a wide range of hormones, neurotransmitters and other chemical agents, and regulates many aspects of the cell (Cheng et al. 2008). In the third chapter of my thesis, I have shown that cAMP has anabolic effect on pancreatic β-cells. In contrast, in other cell types cAMP can antagonize (Bolster et al. 2002, Kimball,

Siegfried and Jefferson. 2004, Baum, Kimball and Jefferson. 2009, Kim et al. 2001) the effect of insulin, and leads to the inactivation of various anabolic signalling pathways including the mTOR pathway. These observations give rise to the intriguing question of how mTOR is regulated by cAMP. Here I have demonstrated that increased $[cAMP]_i$ leads to the dissociation of the mTOR complexes (Fig. 5.7) and impairs the catalytic activity of mTOR (Fig. 5.8), it is therefore plausible that a downstream effector of cAMP may directly associate with one of the mTORC component, which ultimately causes its inactivation. Indeed, the regulatory subunit Iα of PKA (RIα), which is one of the cAMP downstream target (Cheng et al. 2008), has been found to directly bind to mTOR, and the lack of RIα causes autophagic deficiency and the development of primary pigmented nodular adrenocortical disease (Mavrakis et al. 2006), characterized by the accumulation of macromelanosomes in skin and multiple neoplasia (Shenoy, Carpenter and Carney. 1984), implying a role of cAMP in the prevention of cancer development.

The cell-dependent effect of cAMP can be the result of many factors, such as duration and intensity of the signal and the abundancy of signalling molecules. For example, it is evidenced that cAMP activates MAPK pathway through the stimulation of B-Raf yet it can also inhibit the pathway through the suppression of C-Raf. Therefore, the abundancy of B-Raf and C-Raf may determine whether cAMP is stimulatory or suppressive to MAPK pathway (Dumaz and Marais. 2005).

Tuberous sclerosis is a genetic disease characterized by the presence of non-malignant tumours in vital organs such as brain, lungs and heart, it is caused by the mutation and

subsequent loss of function of either the *TSC1* or *TSC2* gene (Huang and Manning. 2008). Results from the fifth chapter have indicated that increases in $[cAMP]_i$ can inhibit protein synthesis (Fig. 5.9) and cell proliferation (Fig. 5.10) in TSC2 null cells, this provides a rationale of using cAMP elevating agents to treat tuberous sclerosis. Indeed, some studies have suggested the use of PDE inhibitors as a potential treatment for chemoresistent cancers, such as colon cancer (Murata et al. 2000, McEwan et al. 2007) and leukemia (Lerner and Epstein. 2006, Lerner, Kim and Lee. 2000). Therefore, cAMP elevating drugs may be used as an alternative approach to inhibit cancer cell proliferation. However, on a cautionary note, giving the cell type specific nature of the cAMP signalling, these drugs may cause the opposite effect on some of the non-cancer cells.

In the physiological context, depicting the relationship between cAMP and mTOR also help us to understand how hormones that increase $[cAMP]_i$ and hormones that activate mTOR interact with each other simultaneously to determine cell fate. For instance, adrenaline potentiates insulin-induced mTORC1 activation in skeletal muscle cells via EPAC (Brennesvik et al. 2005), whereas glucagon suppresses insulin-induced mTORC1 activation in liver cells in a PKA and AMPK dependent manner (Kimball, Siegfried and Jefferson. 2004, Baum, Kimball and Jefferson. 2009), in these studies both adrenaline and glucagon lead to increases in $[cAMP]_i$. Results obtained in this thesis are based on cancer cell lines, whether it also occurs in primary cells under physiological conditions awaits further study.

6.4 Future studies

In the third chapter I have provided evidence that GLP-1 induces β-cell proliferation through the activation of mTOR. However, it remains to be investigated which downstream target of mTOR is responsible for this effect. One potential candidate is 4EBP, which has been shown to regulate replication in mammalian cells (Fingar et al. 2004, Dowling et al. 2010). Exogenously expressing non-phosphorylable 4EBP1 in MIN6 cells impairs cell survival (Fig. 3.7), therefore, it could be of interest to develop 4EBP1 (β-cell specific) knock-in mice to study whether this happens *in vivo*. Moreover, GLP-1, which induces β-cell proliferation, strongly activates S6K and rpS6 in β-cells (Fig. 3.9), yet it is still unknown which S6K1 downstream target mediates this effect. Therefore, further study is required to determine whether CCTβ (Abe et al. 2009), ERα (Yamnik et al. 2009), Mad1 (Zhu, Blenis and Yuan. 2008) or GSK3 (Zhang et al. 2006), which are downstream targets mediating the proliferative effect of S6K in other cell types, play roles in GLP-1 induced β-cell replication.

Islet transplantation provides a potential cure for T1DM. However, insulin independency is difficult to maintain over time after transplantation because all tested immunosuppressants, including glucocorticoids, calcineurin inhibitors (Liu and Herold. 2000, Winter and Schatz. 2003, Burke, Ciancio and Sollinger. 2004), mycophenalate mofetil (Johnson et al. 2009) and rapamycin analogues [(Bell et al. 2003, Fabian et al. 1993, Zhang et al. 2006) and this thesis] have detrimental effect on islet engraftment and function. Nonetheless, the suitability of many alternative immunosuppressants remained to be studied. For example, interferons, TNFα-binding proteins and antibodies against

lymphocyte and thymocyte can potentially be used for islet transplantation. Ideally, the effect of these drugs on mTORC2 and PKB activity should be investigated before being used.

I have demonstrated that cAMP inhibits mTORC1 and 2 through the suppression of mTOR catalytic activity and complex dissociation. Although PKA is unlikely to directly phosphorylate mTOR [Xie, J. and Herbert, T.P., unpublished data, and (Scott and Lawrence. 1998)], it is still plausible that cAMP affect mTORC activity through altering the phosphorylation of mTOR or other mTOR components, and this can be further investigated using mass spectrometry, in the event of discovering novel phosphorylation sites, it can be tested whether they contribute to the regulation of cellular processes by cAMP and mTOR.

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Appendices

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