The role of glutamine in the regulation of mTOR signalling in pancreatic β-cells

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I. Abstract

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The mammalian target of rapamycin (mTOR), a serine/threonine kinase, is a master regulator of cellular growth and proliferation. It integrates several signals and inputs, such as nutrient and energy levels, stress, growth factors and amino acids, to regulate cell growth. The mTOR signalling pathway is dysregulated in several disease states, such as cancer and type 2 diabetes. In type 2 diabetes, an increase in demand for insulin is met by an increase in pancreatic β-cell function and mass in a mechanism termed "β-cell compensation". There is evidence to suggest that this is mediated, at least in part, by the mTOR pathway. However, hyper-activation of mTOR signalling, can lead to its inhibition by a negative feedback loop, and this has been implicated in a reduction in β-cell mass and function which coupled with insulin resistance, could lead to type 2 diabetes. In this work, I investigated the role of L-glutamine in mTOR signalling in β-cells. I show that L-glutamine activates mTORC1. L-glutamine deprivation results in rapid mTORC1 inhibition, the activation of the MAPK pathway and activation of AMPK. In the absence of glutamine, its metabolites glutamate and α-ketoglutarate restore signalling to mTOR and reverses AMPK activation; however, inhibition of glutamine metabolism does not inhibit mTOR activity. Glutamine transporters SNAT 2 and SNAT 3 are the main transporters in INS1e cells, and inhibition of glutamine transport does not inhibit signalling to mTOR. Glutamine withdrawal for 4 hours inhibits signalling to mTORC2 in INS1e, HEK293 and HepG2 cells, but not SH-SY5Y cells; thus, glutamine is required for insulin-stimulated phosphorylation of pPKB Ser 473, a site phosphorylated by mTORC2, but not for PI3K-dependent phosphorylation of pPKB Thr 308. This work provides further insights into how glutamine regulates insulin-dependent signalling to mTORC1 and mTORC2 in pancreatic β-cells.

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V. Abbreviations

%	Percentage
α	Alpha
β	Beta
[ ]	concentration
˚C	degrees Celsius
Ci	Curie
µg	microgram
µl	microlitre
µM	micromolar
^{3}H	tritium
4EBP1	eIF4e-binding protein 1
aa	amino acid
AU	arbitrary Units
ATP	adenosine 5’-triphosphate
ADP	adenosine 5’-diphosphate
AMP	adenosine 5’-monophosphate
AMPK	5’-AMP-activated protein kinase
ANOVA	analysis of variance
AICAR	5-aminoimidazole-4-carboxamide ribotide
APS	ammonium persulphate
Arf1	ADP-ribosylation factor 1
ASK	apoptosis-signal regulating kinase
Asp	aspartate
Asn	asparagine
Ala	alanine
BSA	bovine serum albumin
cPKC	conventional PKC
ddH_2O	double distilled water
DEPTOR	DEP domain-containing mTOR interacting protein
DMEM	Dulbecco’s modified Eagle’s medium

DMSO	dimethyl Sulphoxide
DPM	disintegrations per minute
DPBS	Dulbecco's phosphate-buffered saline
e.g.	*exemplī grātiā*
EBSS	Earle's balanced salt solution
ECL	enhanced chemiluminescence
EDTA	ethylenediaminetetraacetic acid
eEF2	eukaryotic elongation factor 2
eEF2K	eEF2 kinase
EGTA	ethyleneglycol-bis(β-aminoethyl ether)-N,N,N',N'-tetraacetic acid
EGF	epidermal growth factor
et al.	*et alii* (and others)
ERK	extracellular signal-regulated kinase
ERK1/2	extracellular signal-regulated kinase 1 and 2
FAT	FRAP, ATM, TRAP
FATC	C-terminal FAT
FBS	fetal bovine serum
FKBP12	FK506-binding protein of 12 kDa
FKBP38	FK506-binding protein of 38 kDa
FOXO1	forkhead box protein O1
FRB	FKBP12-rapamycin binding
g	gram
GAP	GTPase-activating protein
GEF	guanine nucleotide exchange factor
Gln	glutamine
Glu	glutamate
Gly	glycine
GPCR	G-protein coupled receptor
Grb10	growth factor bound-receptor protein 10
GSK3β	glycogen synthase kinase 3β
HEAT	Huntington, EF3, a subunit of PP2A, TOR1
HEK293	human embryonic kidney 293

HEPES	4-(2-hydroxylethyl)-1-piperazineethanesulphonic acid
HM	hydrophobic motif
His	L-histidine
HPLC	High Pressure Liquid Chromatography
i.e.	*id est* (it is)
IMS	industrial methylated spirit
INS1e	rat insulinoma cell line 1E
IR	insulin receptor
IRS	insulin receptor substrate
IP	immunoprecipitation
JNK	Jun N-terminal kinase
L-DON	6-diazo-5-oxo-L-norleucine
LKB1	liver kinase B1
Lys	lysine
Leu	leucine
M	Molar
mM	milliMolar
MAPK	mitogen-activated protein kinase
MAP4K3	mitogen-activated protein kinase kinase kinase kinase 3
MEF	mouse embryonic fibroblast
MEK	MAPK/ERK kinase
mTOR	mammalian target of rapamycin
MeAIB	α-(methylamino)isobutyric acid
mLST8	mammalian ortholog of lethal with sec thirteen
mSIN1	mammalian stress activated protein kinase interacting protein 1
MWt	Molecular Weight
n	Number
NEB	New England Biolabs
ng	nanogram
nM	nanomolar
PBS	phosphate buffered saline
PI3K	phosphoinositide 3-kinase

PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PDK1	phosphoinositide-dependent kinase-1
PDCD4	programmed cell death protein 4
PH	pleckstrin homology
PSN	penicillin sulphate, streptomycin, neomycin
PMA	Phorbol 12-myristate 13-acetate
PI3K	phosphatidylinositol 3-kinase
PIKKs	PI3K-related kinases
PI	phosphoinositides
PIP_2	phosphatidylinositol (4,5)-bisphosphate
PIP_3	phosphatidylinositol (3,4,5)-trisphosphate
PKA	protein kinase A
PKB	protein kinase B
PKC	protein kinase C
PP2A	protein phosphatase 2A
PRAS40	pro-rich Akt substrate of 40 kDa
PROTOR1/2	protein observed with RICTOR1/2
PTEN	phosphatase and tensin homolog
pmol	picomol
PVDF	polyvinylidene fluorine
RAPTOR	regulatory-associated protein of mTOR
Ras	rat sarcoma
Rb	retinoblastoma protein
REDD1	regulated in development and DNA damage response 1
Rheb	Ras homolog enriched in brain
RICTOR	rapamycin-insensitive companion of TOR
RP	ribosomal protein
RPMI	Roswell Park Memorial Institute
rpS6	ribosomal protein S6
S6K1	p70 rpS6 kinase 1
SDS	sodium dodecyl sulphate

SGK1	serum- and glucocorticoid-induced protein kinase
SH2	Src-homolgy-2
siRNA	small interfering RNA
SNAT	sodium-coupled neutral amino acid transporters
SKAR	S6K1 Aly/REF-like target
SLC	solute carrier family
T1D	type 1 diabetes
T2D	type 2 diabetes
TCA	tricarboxylic acid cycle
TSC	tuberous sclerosis
RT	room temperature
S.E.M.	Standard Error of Mean
Ser	serine
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Thr	threonine
TM	turn motif
TOR	target of rapamycin
TOS	TOR signalling
Tti1	Tel2 interacting protein 1
Tyr	tyrosine
ULK1	UNC-51-like kinase
TEMED	N,N,N',N'-tetramethylethylethylenediamine
vATPase	vacuolar H^+-ATPase
WB	western blotting
WT	wild-type

Chapter 1. Introduction

1.1 Introduction to mTOR

It is critical for an organism to finely balance energy consumption with energy production, and there exist several mechanisms by which cells can integrate extracellular nutrient and energy availability with signalling pathways to regulate cell growth, proliferation, function and death. Careful balancing of catabolic and anabolic processes allows the cell to regulate its growth. In environmental conditions of low nutrient and energy levels (i.e. a fasting state), cells are able to maintain sufficient metabolites via autophagy; conversely, in the presence of high energy and nutrient levels, cells sense and activate signalling pathways in order to utilise the abundance of nutrients for growth (Zoncu, Efeyan and Sabatini, 2011).

The Mammalian Target of Rapamycin (mTOR, also known as Mechanistic Target of Rapamycin) pathway is one such signalling network highly capable of regulating cellular metabolic homeostasis. Activated by growth factors, glucose, nutrients and stressors (Cornu, Albert and Hall, 2013), mTOR signalling acts to regulate processes such as protein synthesis, lipid biosynthesis, ribosome biogenesis, metabolism and autophagy (Kalaitzidis *et al.*, 2017). See **Figure 1.1**.

First discovered and identified in yeast in the early 1990s, the target of the immunosuppressant rapamycin (TOR) is heavily involved in the regulation and control of cellular growth, proliferation and viability (Aylett *et al.*, 2016). As such, functional abnormalities in mTOR signalling are heavily implicated in several disease states, including type 2 diabetes and cancer (Zoncu, Efeyan and Sabatini, 2011).

As an evolutionarily conserved 289-kDa Serine/Threonine kinase belonging to the phosphoinositide-3-kinase (PI3K)-related kinases (PIKK) family (Sengupta, Peterson and Sabatini, 2010) (Laplante and Sabatini, 2012), mTOR exists in two biochemically and functionally distinct multi-component complexes – mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2) – characterised by their unique components alongside the difference in their sensitivity to rapamycin. mTORC1 is acutely sensitive to rapamycin, whilst mTORC2 is often called the "rapamycin-insensitive complex" (Guertin and Sabatini, 2007). As an inhibitor of mTOR, Rapamycin binds to FK506-binding protein of 12kDa (FKBP12), which in turn binds to the FKBP12-rapamycin binding (FRB) domain of mTOR in mTORC1 but not mTORC2, thereby resulting in the inhibition of mTORC1 activity. Whilst mTORC1 is rapidly inhibited by rapamycin, the activity of mTORC2 is not affected by short-term treatment with rapamycin (Guertin and Sabatini, 2007).

mTOR belongs to the PIKK (phosphatidylinositol kinase-related kinase) family, and is composed of the following: N-terminal HEAT (Huntingtin, Elongation factor 3, A subunit of protein phosphatase 2A, and TOR1) repeats, a FAT domain, a FRB domain, the kinase domain and a C-terminal domain termed

FATC that is critical for mTOR function (Takahashi *et al.*, 2000) (Bosotti, Isacchi and Sonnhammer, 2000) (Dames *et al.*, 2005). See ***Figure 1.2***

Figure 1.1 has been omitted for copyright reasons. Please refer to Laplante and Sabatini (2012), or hard copy version of this thesis for the image.

Figure 1.1. Regulation and function of mTORC1 and mTORC2.

Existing in two complexes, both mTORC1 and mTORC2 are differentially regulated and have different functional roles. mTORC1 reacts to changes in oxygen levels, is activated by amino acids signalling, inhibited by stress signals, responds to changes in energy levels, and is activated by growth factor signalling. mTORC1 is acutely sensitive to Rapamycin. Activation of mTORC1 signalling results in an increase in macromolecule biosynthesis, decreased autophagy, increased growth and metabolism and promotion of cell cycle progression. mTORC2 responds to growth factor signalling, and is insensitive to short-term Rapamycin treatment. Activation of mTORC2 promotes cell survival and regulates cytoskeletal organisation. Taken from (Laplante and Sabatini, 2012).

Common to both mTOR complexes 1 & 2 are the catalytic unit mTOR, mLST8 (mammalian lethal with SEC13 protein)/GβL (G-protein β-protein subunit-like) and DEPTOR (DEP domain-containing mTOR-interacting protein). DEPTOR, an mTOR interacting protein, is a negative regulator of mTOR activity; depletion of DEPTOR has been shown to activate both mTORC1 and mTORC2 resulting in

an increase in cell size and protection from apoptosis. At the transcriptional level, mTOR negatively regulates DEPTOR expression (Peterson *et al.*, 2009). Activation of mTOR signalling via external stimuli leads to a positive feedback loop whereby mTOR phosphorylates DEPTOR at several sites, allowing for the release of mTOR from DEPTOR inhibition (Gao *et al.*, 2011).

mLST8/GβL, another common component of both mTOR complexes, plays an unclear role in regulating mTORC1 and mTORC2 activities; whilst it may stabilise the mTORC1 kinase activation loop by binding to mTORC1's catalytic domain (Saxton and Sabatini, 2017), its deletion has no effect on mTORC1. On the other hand, mLST8/GβL may be required in mTORC2 for complex integrity (Guertin *et al.*, 2006).

Several proteins are specific to either mTORC1 or to mTORC2. RAPTOR (regulatory-associated protein of mTOR), a defining component of mTORC1, acts as a scaffolding protein allowing the recruitment of downstream targets via their TOR signalling (TOS) motifs, thus is essential for mTORC1 integrity (Nojima *et al.*, 2003). Another protein unique to mTORC1 is PRAS40 (Proline-rich Akt substrate of 40 kDa), which acts as a second mTOR inhibitor alongside DEPTOR, specifically as an insulin-regulated inhibitor of mTORC1 (Sancak *et al.*, 2007).

On the other hand, the defining components of mTORC2 are RICTOR (rapamycin independent companion of mTOR), mSIN1 (mammalian stress activated protein kinase interacting protein 1) and Protor1/2 (protein observed with RICTOR 1/2), the former two being critical components that mediate the

assembly of mTORC2 and the phosphorylation of its downstream targets (Frias *et al.*, 2006).

Figure 1.2 has been omitted for copyright reasons. Please refer to Saxton and Sabatini (2017), or hard copy version of this thesis for the image.

Figure 1.2. mTOR Complexes 1 and 2.

Schematic representation of mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2).

a) mTOR Complex 1 subunits and their respective binding sites on mTOR. mTORC1 includes mTOR, Raptor, mLST8, DEPTOR and PRAS40.
b) A 5,9- Å cyro-EM structure of mTORC1 in complex with the FKBP12-rapamycin complex without DEPTOR and PRAS40.
c) mTOR Complex 2 subunits and their respective binding sites on mTOR.
mTORC2 includes mTOR, Rictor, mLST8, DEPTOR, Protor1/2 and mSin1.

mTORC1 & C2*: mammalian target of rapamycin complex 1 & complex 2;* ***Raptor****: regulatory-associated protein of mTOR;* ***PRAS40****: Proline-rich Akt substrate of 40 kDa;* ***FKBP12-rapa****: FK506-binding protein of 12kDa, Rapamycin complex;* ***mLST8****: mammalian lethal with SEC13 protein;* ***HEAT****: Huntingtin, Elongation factor 3, A subunit of PP2A-TOR1 repeats;* ***FAT****: FRAP-ATM-TRRP;* ***FRB****: FKB12-rapamycin binding domain;* ***FATC****: FAT-carboxy terminal domain;* ***DEPTOR****: DEP domain-containing mTOR-interacting protein;* ***Protor1/2****: protein observed with RICTOR 1/2;* ***mSIN1****: mammalian stress activated protein kinase interacting protein 1.* Taken from (Saxton and Sabatini, 2017).

1.2 mTOR Complex 1

1.2.1 Upstream regulation of mTORC1

A plethora of extracellular signals are integrated by mTORC1 to regulate growth and function. Activation of mTORC1 occurs via the interaction with the small GTPase (guanosine 5'-triphosphatase) Rheb (Ras homolog enriched in brain), when Rheb is in its GTP-loaded state (Saucedo *et al.*, 2003). These signals include growth factors, nutrients, hormones and energy status – each activating signalling pathways that converge on to TSC1/2 (Tuberous Sclerosis Complex 1 and 2), a GTPase-activating protein (GAP) which when phosphorylated regulates its only physiological substrate, the GTPase Rheb – a potent activator of mTORC1 (Inoki *et al.*, 2003) (Zhang *et al.*, 2003). An obligate requirement for the activation of mTORC1 by these extracellular signals, is the presence of active amino acid signalling, to be discussed further in section 1.2.2 (Nicklin *et al.*, 2009a).

TSC1-TSC2 complex is a heterodimer consisting of TSC1, TSC2 as well as TBC1D7 (TBC1 Domain Family Member 7) (Dibble *et al.*, 2012). As a GAP, TSC1/2 causes dephosphorylation of GTP-bound Rheb, resulting in GDP-bound Rheb, which leads to mTORC1 inactivation (Inoki *et al.*, 2002); conversely, once inactivated, TSC1/2 would allow the GTP-loading of Rheb. Thus, TSC1/2 acts as an upstream negative regulator of GTPase Rheb, and by consequence mTORC1. Therefore, loss of TSC1/2 leads to continuous activation of mTORC1 (Zhang *et al.*, 2003). See ***Figure 1.3*****a**.

Figure 1.3 has been omitted for copyright reasons. Please refer to Huang and Manning (2008), or hard copy version of this thesis for the image.

Figure 1.3. Regulation of mTORC1 by TSC1/2 and Rheb.

a) Under poor growth conditions, an activated TSC1/2 complex promotes GDP-Rheb formation via its GAP activity. Under stimulatory conditions, TSC1/2 is inactivated, thereby promoting GTP-loading of Rheb, which can bind to and activate mTORC1 activity. A GEF for Rheb is currently unknown.

b) FKBP38 association with mTORC1 inhibits mTORC1 signalling. Under stimulatory conditions, an inhibited TSC1/2 complex allows the accumulation of GTP-Rheb, which may sequester the mTOR inhibitor FKBP38 and thus allowing the activation of mTORC1 signalling.

***TSC1/2**: Tumour suppressor complex 1/2; **Rheb**: Ras homolog in brain; **GTP/GDP**: guanosine triphosphate/guanosine diphosphate; **FKBP38**: FK506-binding protein 38. Taken from (Huang and Manning, 2008).*

TSC1/2 is also able to regulate mTOR by integrating signals from several signalling pathways, each with distinct TSC phosphorylation events, such as the PI3K-PKB (phosphoinositide 3-kinase/Protein Kinase B) pathway, ERK1/2 (extracellular-signal-regulated kinase 1 and 2) pathway and the AMPK (AMP-dependent protein kinase) pathway.

Inhibition of TSC1/2 allows the association of the lysosome-located GTP-Rheb with mTORC1 and thus activation of mTORC1; most likely via interactions

with the kinase domains of mTOR, mLST8 and RAPTOR (Dibble and Cantley, 2015). On the other hand, Rheb can activate mTORC1 via interactions with mTOR's inhibitor FKBP38, a member of the FK506-binding protein family, that acts in a similar fashion to the FKBP12-rapamycin complex (FRB); the sequestering of FKBP38 by GTP-Rheb stops its association with mTOR therefore promoting mTORC1 activity (Bai *et al.*, 2007)(Duan *et al.*, 2015). See ***Figure 1.3*****b**.

1.2.1.1 Growth factor regulation of mTORC1

One extracellular stimuli that affects TSC1/2, and thus mTOR complexes 1 and 2, is the hormone insulin, acting via the insulin-PI3K signalling pathway upon binding to the insulin receptor (IR). The IR protein consists of both extracellular and transmembrane subunits: two extracellular α subunits, and two transmembrane β subunits, held together by disulphide bonds. Importantly, the cytoplasmic portion of the β subunits possesses intrinsic tyrosine kinase activity (Belfiore *et al.*, 2009). Upon binding of insulin to the α subunits of the IR, a conformational change bringing together the two β subunits occurs, resulting in autophosphorylation events and kinase activity towards insulin substrate-1 (IRS1). IRS1, acting as an adaptor protein to potentiate downstream insulin signalling, is then able to activate PI3K signalling (Vanhaesebroeck, Stephens and Hawkins, 2012).

1.2.1.2 PI3K signalling

The family of PI3Ks are lipid kinases that phosphorylate phosphatidylinositols (PtdIns), and act as major downstream effectors of both receptor tyrosine kinases (RTKs) and G-protein coupled receptors (GPCRs). Divided into three classes, the most commonly studied are the class I PI3Ks, of which class I_A PI3Ks are activated by RTKs. See **Figure 1.4**.

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Figure 1.4. Signalling by class I PI3K isoforms.

Upon activation of RTKs by growth factors, or GPCRs by chemokines, class I_A PI3Ks are recruited to the plasma membrane where they phosphorylate PIP_2 to generate PIP_3. This recruitment of PI3K happens via interactions between the p85 regulatory subunit with adapter proteins (such as IRS1) or interactions with GPCR $G_{\beta\gamma}$ subunit, allowing the p110 catalytic subunit to generate PIP_3. PTEN, a lipid phosphatase, acts to inhibit PI3K signalling by dephosphorylating PIP_3 back to PIP_2. The second messenger, PIP_3, is then able to activate downstream targets that regulate translation, growth, metabolism and survival, amongst others.

***RTK**: receptor tyrosine kinase; **GPCR:** G-protein coupled receptor; **PTEN**: phosphatase and tensin homolog; **PIP_2**: phosphatidylinositol (3,4,5) triphosphate; **PIP_3**: phosphatidylinositol (4,5) triphosphate. Adapted from (Thorpe, Yuzugullu and Zhao, 2015).*

Composed of two subunits – p85 regulatory protein and a p110 catalytic subunit – these PI3Ks are recruited by direct interaction of the Src Homology domain of the p85 subunit (p85-SH) with IRS. This results in the activation of the p110 catalytic subunit, which phosphorylates its substrate phosphatidylinositol (4,5)-bisphosphate (PIP_2) to generate phosphatidylinositol (3,4,5) trisphosphate (PIP_3) (Liu *et al.*, 2009). The levels of PIP_3 are tightly regulated, with the tumour suppressor PTEN (Phosphatase and tension homolog) possessing a lipid phosphatase activity able to reduce PIP_3 levels by converting them back to PIP_2 (Oudit *et al.*, 2004). In fact, of all mutations that affect tumour suppressors, PTEN mutations are one of the most common (Fruman and Rommel, 2014). Overstimulation of the downstream mTOR complexes 1 and 2 can also result in a negative feedback loop whereby overexpression of PTEN leads to the inhibition of PI3K-PKB-mTOR signalling by PTEN activity (Das *et al.*, 2012).

The second messengers, PIP_2 and PIP_3, act to recruit downstream effector proteins to the plasma membrane. Such proteins include PDK1 (Phosphoinositide dependent protein kinase 1) and PKB as their pleckstrin homology (PH) domains can bind to PIP_3 in the plasma membrane. Their recruitment initiates a signalling cascade that acts via PKB to phosphorylate and inactivate TSC1/2, leading to mTORC1 activation (Vanhaesebroeck, Stephens and Hawkins, 2012). See **Figure 1.5**.

Figure 1.5 has been omitted for copyright reasons. Please refer to Vanhaesebroeck, Stephens and Hawkins (2012), or hard copy version of this thesis for the image.

Figure 1.5. PI3K-PKB signalling

Activation of PI3K signalling results in the generation of phosphatidylinositol-3,4,5-triphosphate (PIP_3), which binds to the pleckstrin homology domain of Protein Kinase B (PKB; Akt). PKB is then translocated to the plasma membrane, where it is phosphorylated on Thr308 by phosphoinositide-dependent kinase 1 (PDK1) and by mTORC2 on Ser473, achieving full activation. PKB can then phosphorylate and regulate a multitude of downstream targets, including TSC2-mTORC1 and FOXO proteins. These downstream targets influence cellular growth, proliferation, survival, metabolism and autophagy.

***PI3K**: phosphoinositide 3-kinases; **PTEN**: phosphatase and tensin homologue; **TSC2**: tuberous sclerosis 2; **BAD**: BCL-2 antagonist of cell death; **BIM**: BCL-2-interacting mediator of cell death; **FOXO**: fork head box O; **GSK3**: glycogen synthase kinase 3; **eNOS**: epithelial nitric oxide synthase; **AS160**: AKT substrate of 160 kDa; **GS**: glycogen synthase; **S6K**: S6 kinase; **rS6**: ribosomal S6 protein; **4EBP1**: eIF4E-binding protein 1; **eIF4E**: eukaryotic translation-initiation factor 2.* Taken from (Vanhaesebroeck, Stephens and Hawkins, 2012).

PKB signalling

Protein Kinase B (PKB) is a serine/threonine kinase that is involved in the regulation of cellular growth and proliferation. It belongs to a family comprised of three members: PKBα, PKBβ and PKBγ, of which only PKBα is relevant to this thesis. Two phosphorylation events need to occur to fully activate PKB; its N-terminal PH domain binding to PIP_3 recruits it to the plasma membrane and changes the conformation of PKB as to allow the phosphorylation events to occur (Fayard *et al.*, 2005). The first event occurs with PDK1 phosphorylating PKB in its activation loop, on Thr308 (Alessi *et al.*, 1997). The second event occurs through mTORC2 recruitment to the plasma membrane – again via binding of PIP_3 to the PH domain of a critical component of mTORC2, mSIN1 – where it can phosphorylate PKB on Ser473 in its C-terminal hydrophobic motif (HM), thereby stabilising its active conformation state (Yang *et al.*, 2002) (Yang *et al.*, 2015). Once fully activated, PKB can translocate and phosphorylate several downstream targets, including: Fork-head transcription factors (FOXO) (Tran *et al.*, 2003), Glycogen synthase kinase (GSK) (Elghazi *et al.*, 2007) and TSC2. Phosphorylation on TSC2 by PKB occurs on several residues, including: Ser939, Ser981, Ser1132 and Thr1462 (Cai *et al.*, 2008), resulting in the inactivation of TSC1/2 (Inoki *et al.*, 2002) and thus the promotion of downstream mTORC1 activity (Manning *et al.*, 2002).

PKB activity is transiently regulated; alongside PTEN and negative feedback loops initiated by mTORC1, PKB phosphatases directly dephosphorylate and inactivate PKB, either via the actions of PP2A (protein phosphatase 2A) towards Thr308 (Kuo *et al.*, 2008), or via PHLPP (PH domain leucine-rich repeat protein phosphatase) towards Ser473 (Gao, Furnari and Newton, 2005).

Alongside the PI3K-PKB-mTORC1 signalling pathway, growth factors are also capable of regulating mTORC1 activity through phosphorylation of Ser540 and Ser664 on TSC2 by ERK1/2 (extracellular signal-regulated kinase 1 and 2); stimulation of ERK1/2 and its downstream target p90 ribosomal S6 kinases (RSKs) results in the inhibition of TSC1/2 and thus activation of downstream mTORC1 signalling (Ma *et al.*, 2005). ERK1/2 is also able to directly regulate mTORC1 activity through phosphorylation on mTORC1's defining component, RAPTOR, on three residues: Ser8, Ser696 and Ser863 (Carriere *et al.*, 2011).

Alongside growth factors, the cellular energy status also regulates cellular function and proliferation. mTORC1 has been shown to respond to several stressful stimuli, including DNA damage, hypoxia, glucose deprivation and changes in cellular ATP (adenosine triphosphate) levels (Saxton and Sabatini, 2017).

One metabolic regulator that responds to stressful stimuli is AMPK (adenosine 5' monophosphate-activated kinase); a heterotrimeric kinase composed a catalytic subunit (α), and two regulatory subunits (β and γ) (Gwinn *et al.*, 2008).

Rising 5'-AMP levels initiate a signalling cascade through binding of AMP to the γ-subunit of AMPK, resulting in decreased dephosphorylation activity and concurrently causing an increased phosphorylation of Thr172 on the α subunit of AMPK, by an upstream serine/threonine kinase, LKB1 (Hardie, 2005) (Hardie, 2007). Upon activation of AMPK, it directly phosphorylates and activates TSC2 on Thr1271 and Ser1387 (Inoki, Zhu and Guan, 2003). However, AMPK can also regulate mTORC1 activity directly, as evident in cells deficient of TSC2 which retain

their ability to respond to extracellular energy stressors; this is mediated by AMPK phosphorylating RAPTOR on several residues (Gwinn *et al.*, 2008).

Independently of AMPK, a decrease in oxygen levels has been shown to inhibit mTORC1, mainly by increased activity of REDD1 (Regulated in DNA damage and development 1) towards TSC1/2, activating it (Brugarolas *et al.*, 2004).

1.2.1.3 MAPK signalling

Cellular stress may also regulate cellular homeostasis through MAPK (mitogen-activated protein kinase) signalling through ERK1/2, p38 or JNK (Jun N-terminal kinase). p38 and JNK respond primarily to stress, however they are known to also be activated by other stimuli (Zuluaga *et al.*, 2007). The crosstalk between both the p38 and jnk MAPK pathways is important to coordinate an appropriate response. Their importance in regulating cellular homeostasis and response to extracellular stimuli is highlighted by their deregulation in disease states, including cancer (Koul, Pal and Koul, 2013).

Upon activation of p38 proteins by upstream kinases MKK3 and MKK6 (dual specificity mitogen-activated protein kinase kinase 3, 6), they translocate from the cytosol to the nucleus where they are able to phosphorylate their substrates – including the transcription factors p53, ATF2 (activating transcription factor 2), and kinases such as MK2 (MAPK-activated kinase 2), MSK1 (mitogen- and stress-activated protein kinase 1) (Wagner and Nebreda, 2009). JNK, once activated by upstream kinases MKK4 and MKK7, targets its major substrate, the transcription factor AP1. Dependent on the stimulus JNK activation leads to varying outcomes

ranging from inducing apoptosis to increased survival (Wagner and Nebreda, 2009). See **Figure 1.6**.

Figure 1.6 has been omitted for copyright reasons. Please refer to Wagner and Nebreda (2009), or hard copy version of this thesis for the image.

Figure 1.6. Mitogen-activated protein kinase signalling pathways.

Growth factors, environmental stresses and inflammatory cytokines activates the Mitogen-activated protein kinase (MAPK) signalling pathway, which signal downstream to the MAPKs JNK and p38, through MAP2Ks. The upstream MAP2Ks MKK7/MKK4 and MKK3/MKK6 activate JNK and p38, respectively. Downstream targets of JNK and p38 include transcription factors, collectively eliciting biological responses.

***LZK**: leucine-zipper kinase; **MLK**: mixed-lineage kinase; **TAO**: thousand-and-one amino acid kinase; **ASK**: apoptosis signal-regulating kinase 1; **DLK1**: dual leucine zipper-bearing kinase 1; **ZAK**: leucine-zipper and sterile-α motif kinase; **TAK1**: transforming growth factor β-activated kinase 1; **MNK1**: MAP kinase-interacting serine/threonine kinase 1; **MEF2**: myocyte-specific enhancer factor; **ATF2:** activating transcription factor 2; **CREB**: cAMP-responsive element binding protein.* Taken from (Wagner and Nebreda, 2009).

1.2.2 Amino acid regulation of mTORC1

As mentioned previously, the presence of active amino acid signalling is an obligate requirement for full mTORC1 activation – even in the presence of sufficient growth factor, nutrient and energy levels, the activity of mTORC1 is dependent on the presence of branch chain amino acids (BCAA). BCAA input into mTORC1 signalling occurs independently of the TSC-Rheb arm (Roccio, Bos and Zwartkruis, 2006). Other amino acids have also been shown to modulate mTORC1 activity including Glutamine and Arginine (Nicklin *et al.*, 2009a) (Jewell *et al.*, 2015).

Amino acid signalling is initiated in the lysosomal lumen and stimulates the translocation of mTORC1 to the lysosomal surface in close proximity to its activator GTP-Rheb (Zoncu *et al.*, 2011). It has been proposed that a lysosomal amino acid transporter, SLC38A9 (solute carrier family 38 member 9), can sense and signal intraluminal amino acid levels, particularly those of arginine, leucine and glutamine (Rebsamen *et al.*, 2015)(Rebsamen and Superti-Furga, 2016) (Wang *et al.*, 2015).

The translocation of mTORC1 to the lysosome is dependent on the small Rag GTPases. These Rags are obligate heterodimers composed of Rag_A or Rag_B coupled with Rag_C or Rag_D (Kim *et al.*, 2008); amino acid sufficiency promotes the conversion of $Rag_{A/B}$ to their GTP-bound state, and $Rag_{C/D}$ to their GDP-bound state, thus allowing them to recruit mTORC1 to the lysosomal surface through interactions with RAPTOR (Jewell *et al.*, 2015). Upon translocation of mTORC1 to the lysosomal surface, it is free to interact with its activator, GTP-Rheb. (Saxton and Sabatini, 2017). See **Figure 1.7.**

Figure 1.7 has been omitted for copyright reasons. Please refer to Bar-Peled et al. (2012) or hard copy version of this thesis for the image.

Figure 1.7. mTORC1 activation by amino acids.

During amino acid sufficiency, the accumulation of amino acids in the lysosomal lumen activates a v-ATPase-dependent signal that activates the GEF activity of the Ragulator towards Rag_A. Once Rag_A is GTP-loaded, the activated Ragulator-Rag recruits mTORC1 to the lysosomal surface where it may interact with and activated by Rheb (Rheb is not shown).

v-ATPase*: vacuolar H^+-adenosine triphosphatase;* ***GEF****: guanine nucleotide exchange factor;* ***GTP/GDP****: guanosine triphosphate/guanosine diphosphate.* Taken from (Bar-Peled *et al.*, 2012).

These Rag heterodimers are tethered to the lysosome via their association with a pentameric Rag-interacting complex, termed Ragulator. The Ragulator complex is composed of p14, p18, MP1, HBXIP and C7orf59, all of which are necessary for the lysosomal localisation of both Rag and mTORC1 (Bar-Peled *et al.*, 2012). Ragulator interacts with a lysosomal v-ATPase (vacuolar H^+-adenosine triphosphatase) which acts as Ragulator's positive regulator, promoting the guanine-nucleotide exchange factor (GEF) activity of Ragulator under active amino acid signalling (Zoncu *et al.*, 2011).

As a regulator, v-ATPase controls mTORC1 signalling; in parallel to stimulating Ragulator's GEF activity through conformational changes in conditions of amino acid sufficiency (Yoon and Choi, 2016). v-ATPase can also negatively regulate mTORC1 activity through interactions with SPAR (small regulatory polypeptide of amino acid response), which inhibits the lysosomal translocation of mTORC1 through an unknown mechanism (Matsumoto *et al.*, 2017).

v-ATPase can also inhibit a GATOR1/2 complex that has GAP activity towards Rag_A and Rag_B (Bar-Peled *et al.*, 2013). Rag_C and Rag_D on the other hand, are regulated by tumour suppressor folliculin (FCLN) which through binding with FNIP1 (folliculin-interacting protein 1), has GAP activity towards Rag_C and Rag_D, thereby maintaining an activated Rag heterodimer, and promoting RAPTOR-mTORC1 translocation to the lysosomal surface (Petit, Roczniak-Ferguson and Ferguson, 2013) (Tsun *et al.*, 2013).

The GTPase-activating protein GATOR 1 (named after its GAP Activity TOwards Rags) consists of three proteins: DEPDC5 (DEP domain-containing 5),

Nprl2 and Nprl3 (Nitrogen permease regulator 2-like protein, 3-like protein) (Bar-Peled *et al.*, 2013). GATOR1 is tethered to the lysosomal surface, by a recently identified complex, KICSTOR – named after the complexes' components: KPTN, ITFG2, C12orf66 and SZT2, -containing regulator of mTORC1 (Wolfson *et al.*, 2017).

Upstream of GATOR1 lies GATOR2, a positive regulator of mTORC1 signalling due to its inhibitory effect on its downstream target, GATOR1. Existing as a pentameric complex which contains: Mios, WDR24, WDR59, Seh1L and Sec13 (Bar-Peled *et al.*, 2013), WDR24 has been shown to be critical in GATOR1 regulating TORC1 activity and lysosomal function (Cai *et al.*, 2016).

GATOR2 sensing of cytosolic amino acid levels, particularly leucine and arginine, and therefore its inhibition of downstream GATOR1, promotes downstream GTP-loading of $Rag_{A/B}$. This mechanism of action depends on the actions of two upstream GATOR2-interacting proteins, that act as either leucine or arginine sensors (Kim *et al.*, 2015).

As a primary sensor of leucine levels (and methionine to a lesser extent), Sestrin2 belongs to a protein family made up of Sestrin 1, Sestrin 2 and Sestrin3. Sestrin2 is upstream of mTORC1, and in conditions of leucine starvation, it acts to inhibit GATOR2 (Wolfson *et al.*, 2016). This inhibition relieves GATOR1 inhibition by GATOR2, therefore promoting the GAP activity of GATOR1. As a result, GDP-loading of $Rag_{A/B}$ leads to inhibition of mTORC1 signalling. Leucine-Sestrin2 interaction therefore, directly regulates Sestrin2-GATOR2 interaction and mTORC1 activation (Saxton *et al.*, 2015). See **Figure 1.8**.

In a mechanism, alike that by mediated by leucine, arginine acts to regulate mTORC1 activation through the recently identified arginine sensor, CASTOR1

(cellular arginine sensor for mTORC1). CASTOR1, in the presence of arginine, is dissociated from its inhibitory interaction with GATOR2 and thus allows the activation of downstream mTORC1 signalling (Chantranupong *et al.*, 2016), see **Figure 1.9**.

Whilst the essential amino acids (EAA) leucine and arginine, alongside Glutamine, are known to activate mTORC1 signalling (Wang *et al.*, 1998) (Kimball *et al.*, 1999), the exact mechanism was unknown. Whereas EAA such as Leucine activate mTORC1 signalling through modulation of the small Rags as discussed earlier, the mechanism by which the non-essential amino acid Glutamine regulates mTORC1 activity appears to be multifaceted – as discussed further below – and only recently was shown to modulate mTORC1 activity independent of the Rags (Jewell *et al.*, 2015).

Figure 1.8 has been omitted for copyright reasons. Please refer to Bar-Peled et al (2013), or hard copy version of this thesis for the image.

Figure 1.8. The GATOR1/2 complex.

GATOR1 possesses GAP activity towards $Rag_{A,}$ promoting GDP-loading of Rag_A and inhibition of mTORC1 signalling. It is inhibited by GATOR2, a negative regulator of GATOR1. GATOR1 is composed of DEPDC5, Nprl2 and Nprl3. GATOR2 consists of Mios, Seh1L, WDR24, WDR59, Sec13.

DEPDC5*: DEP domain-containing 5;* ***Nprl2****: Nitrogen permease regulator 2-like protein;* ***Nprl3****: Nitrogen permease regulator 3-like protein;* ***Mios****: WD repeat-containing protein mio;* ***Seh1L****: nucleoporin SEH1;* ***WDR24****: WD repeat-containing protein 24;* ***WDR59****: WD repeat-containing protein 59;* ***Sec13****: protein SEC13 homolog.* Taken from (Bar-Peled *et al.*, 2013).

Figure 1.9 has been omitted for copyright reasons. Please refer to Saxton and Sabatini (2017), or hard copy version of this thesis for the image.

Figure 1.9. Amino acid sensing and signalling pathway upstream of mTORC1.

Recruitment of mTORC1 to the lysosomal surface where it may interact with GTP-Rheb, is mediated through v-ATPase-Ragulator-Rag interactions. Amino acid sensing in the lysosomal lumen activates the Ragulator complex through the SLC38A9 transporter. Upon activation of Ragulator, its GEF activity towards Rag_A results in GTP-loaded Rag_A and leading to the translocation of mTORC1. This mechanism of activation is also regulated through the activity of a GAP towards Rag_A, GATOR1, and its inhibitor GATOR2.

In the presence of sufficient concentrations of Leucine and Arginine, the inhibitory effects of Sestrin1 and CASTOR1 towards GATOR2 are impeded. GATOR2 is now able to inhibit GATOR1, and thus its GAP activity towards the Rag GTPases, promoting mTORC1 translocation and activation. Conversely, in the absence of amino acid sufficiency, the GEF activity of Ragulator is inhibited, whilst the GAP activity of GATOR1 is activated. This results in GDP-loaded Rag_A and thus the inability to recruit mTORC1.

SLC38A9*: Member 9 of the solute carrier family 38;* ***v-ATPase****: vacuolar H^+-adenosine triphosphatase;* ***KICSTOR****: KPTN, ITFG2, C12orf66 and SZT2, -containing regulator of mTORC1;* ***FLCN****: folliculin,* ***FNIP2****: folliculin-interacting protein 1*. Taken from (Saxton and Sabatini, 2017).

1.2.2.1 Glutamine: Metabolism and Transport

One of twenty different amino acids, glutamine is one of the most abundant and versatile amino acids in plasma, accounting for approximately 20% of plasma amino acids, and plays a significant role in cellular proliferation (Reitzer, Wice and Kennell, 1979)(Bergström *et al.*, 1974). It is a non-essential amino acid, although it is a conditionally essential amino acid as often its demand exceeds its supply (Fuchs and Bode, 2006). It plays a multitude of key roles in cellular homeostasis and growth, acting as a precursor for glucose, nucleotide, protein and amino sugar synthesis (Neu, Shenoy and Chakrabarti, 1996) and is heavily implicated in mTORC1 signalling (Cohen and Hall, 2009). As a non-essential amino acid (NEAA), it can be synthesised through the metabolism of other amino acids; likewise, it can be utilised to synthesise other NEAAs (Hosios *et al.*, 2016).

Intracellular concentrations of glutamine range between 2 mM to 20 mM, with the extracellular concentrations being much lower, averaging 0.7 mM (Newsholme *et al.*, 2003). Glutamine is utilised as an important fuel for cellular growth in many differing tissue types, and is involved in many critical cellular processes. Various biological molecules – such as the antioxidant glutathione, the neurotransmitters glutamate and GABA (γ-amino butyric acid) (Bhutia and Ganapathy, 2016)

In rapidly proliferating cells, such as with cancer, glutamine “addiction” – or dependency on glutamine to fuel this rapid growth – is evident (Wise and Thompson, 2010). In glutamine-addicted cancer cells, apoptosis is induced upon glutamine deprivation (Yuneva *et al.*, 2007).

One of glutamine's key roles arises from its property as a carbon and nitrogen donor. Its carbon can be incorporated into glucose and fatty acids for ATP production, whilst the nitrogen can be utilised for the synthesis of purines and pyrimidines (Bhutia and Ganapathy, 2016). See **Figure 1.10**.

Figure 1.10 has been omitted for copyright reasons. Please refer to Newsholme et al (2003), or hard copy version of this thesis for the image.

Figure 1.10. Overview of glutamine metabolism pathways in mammalian cells.

Glutamine is transported intracellularly through glutamine transporters, where it may be a precursor for the synthesis of nucleic acids, nucleotides and proteins. Through the actions of glutaminase enzyme, glutamate is produced. It may then be converted to GABA, glucose via gluconeogenesis, 2-oxoglutarate (also known as α-ketoglutarate) via glutaminolysis, amino acids via transaminases, orthenine, or the antioxidant glutathione.

***GABA**: γ-amino butyric acid; **NO**: nitric oxide; **iNOS**: inducible nitric oxide synthase.* Taken from (Newsholme *et al.*, 2003).

With transport of glutamine intracellularly by their transporters (to be discussed in section 1.4) glutamine is utilised in many metabolic pathways. One pathway relevant to this thesis, is glutaminolysis (see **Figure 1.11**).

The first step of glutaminolysis is the conversion of glutamine to glutamate through the action of the glutaminase (GLS) enzyme, reversed via the enzyme glutamine synthetase (GS). The activity of GLS is induced in correlation with tumour growth (Tennant *et al.*, 2009). Secondly, glutamate is deaminated again and converted to α-ketoglutarate (αKG; also known as 2-oxoglutarate), catalysed by glutamate dehydrogenase (GDH). GDH is inhibited by DON (6-diazo-5-oxo-L-norleucine), acivicin and azaserine (Durán and Hall, 2012), as well as by epigallocatechin gallate (EGCG) (Li *et al.*, 2006).

As a critical component of the tricarboxylic acid (TCA) cycle, αKG production allows cells to maintain the required energy levels (DeBerardinis *et al.*, 2007)(Still and Yuneva, 2017). Glutamate dehydrogenase (GDH) is negatively regulated by GTP, and positively regulated by leucine (Li *et al.*, 2012). Activation of GDH by leucine is mediated by the direct binding of leucine to, and thus activation of, GDH. This appears to be an important regulatory mechanism for insulin secretion in pancreatic β-cells (Carobbio *et al.*, 2009).

Figure 1.11 has been omitted for copyright reasons. Please refer to Hensley, Wasti and DeBerardinis (2013), or hard copy version of this thesis for the image.

Figure 1.11. Glutamine metabolism via glutaminolysis.

In mitochondria, glutamine is metabolised by the enzyme GLS, which is inhibited by compound 968 and BPTES. Glutamate is converted to α-KG via the enzyme GDH and/or transaminases (TA). GDH is inhibited by EGCG, DON, Acivicin and Azaserine (not shown). AOA inhibits transaminases. α-KG then enters the TCA cycle.

***GLS**: glutaminase; **968**: compound 968; **BPTES**: bis-2-(5-phenylacetamido-1,2,4-thiadiazol-2-yl)ethyl sulphide; **α-KG:** α-ketoglutarate; **AOA**: aminooxyacetic acid; **OAA**: oxaloacetate; **ME**: malic enzyme; **DON**: 6-diazo-5-oxo-L-norleucine. Taken from (Hensley, Wasti and DeBerardinis, 2013)*

Intracellular transport of glutamine is required for the import of leucine – inwards transport of leucine utilises a glutamine concentration gradient (Cohen and Hall, 2009). In fact, inhibition of bidirectional transport of glutamine or leucine inhibits mTORC1 signalling (Nicklin *et al.*, 2009a). On the other hand, transport of glutamine and leucine and the consequent induction of glutaminolysis and production of αKG, promotes the GTP-loading of the $Rag_{A/B}$ (Durán *et al.*, 2012); this coupled with leucine (and arginine) positively regulating the GATOR-Ragulator signalling, results in the activation of mTORC1 signalling.

Unlike leucine and arginine, glutamine was recently discovered to mediate mTORC1 activity independently of the v-ATPase-Ragulator-Rag complex (Jewell *et al.*, 2015; Shimobayashi and Hall, 2016). Although glutamine can mediate and influence mTORC1 activity through its activator leucine – by facilitating leucine transport – and by glutamine metabolism promoting GTP-loading of $Rag_{A/B}$ (Durán *et al.*, 2012), glutamine requires another GTPase, Arf1 (adenosine diphosphate ribosylation factor-1), to be sensed and influence mTORC1 activation (Jewell *et al.*, 2015); see **Figure 1.12**.

Figure 1.12 has been omitted for copyright reasons. Please refer to Jewell et al (2015), or hard copy version of this thesis for the image.

Figure 1.12. Proposed model of amino acid induced activation of mTORC1 signalling.

Growth factor signalling, acting through the TSC-Rheb arm of mTORC1 signalling, promotes activation of mTORC1 by GTP-loading of the lysosomal-bound GTPase Rheb. The amino acid leucine promotes activation of mTORC1 through the v-ATPase-Ragulator-Rags arm of mTORC1 signalling. Glutamine regulates mTORC1 activity by facilitating leucine transport, and through its metabolism. It may also act independently of the Rags, through another GTPase ARF1 in an unknown mechanism.

***Leu**: leucine; **Gln**: glutamine; **ARF1**: adenosine diphosphate ribosylation factor-1.* Adapted from (Jewell *et al.*, 2015).

1.2.3 Downstream of mTORC1

The mTORC1 pathways play a key role in the regulation of mammalian cell growth and proliferation. In favourable environmental conditions, an activated mTORC1 promotes protein synthesis and strongly inhibits autophagy (Noda and Ohsumi, 1998). Conversely, in response to stressful stimuli, mTORC1 is inhibited leading to increased autophagy, replenishing the cellular lysosomal population (Yu *et al.*, 2010).

Characteristically, alongside mTOR, mLST8/GβL and DEPTOR, mTORC1 also includes RAPTOR (regulatory associated protein of mTOR) and PRAS40 (proline-rich AKT substrate of 40 kDa). RAPTOR plays a critical role in mediating downstream signalling from mTROC1, acting as a scaffold that recruits to mTOR its downstream targets ribosomal S6 Kinases 1 and 2 (S6K1/2), and eIF4E-binding protein 1 (4E-BP1), two important components of the translational machinery. S6K1 is phosphorylated at Thr389, S6K2 at Thr388 and eIF4B is phosphorylated on Ser422 (Pearson *et al.*, 1995)(Shahbazian *et al.*, 2006).

4E-BP1 inhibits protein synthesis by preventing the assembly of the eIF4F complex (containing EIF4E, the RNA helicase eIF4A, and the scaffolding protein eIF4G) by binding and sequestering eIF4E; phosphorylation of 4E-BP1 by mTORC1 on several residues releases its association with eIF4E, allowing the formation of the eIF4F complex and therefore the promotion of mRNA translation (Gingras *et al.*, 1999)(Saxton and Sabatini, 2017) .

Activation of S6K1 by mTORC1 phosphorylation of Thr389 on its hydrophobic domain (HM), followed by phosphorylation by PDK1 (Saxton and Sabatini, 2017), leads in turn to phosphorylation of S6K1's downstream targets, including ribosomal S6 (rpS6), eukaryotic elongation factor 2 kinase (eEF2K) (Wang *et al.*, 2001) and eIF4B (Holz *et al.*, 2005) – thereby promoting mRNA translation; see **Figure 1.13**.

The activation of eIF4B by S6K1 – a positive regulator of the eIF4F complex (Holz *et al.*, 2005) – results in an enhancement of eIF4A activity, a RNA helicase involved in the unwinding of secondary structures in the 5' untranslated regions (UTRs) of many mRNAs (Zoncu, Efeyan and Sabatini, 2011). Conversely, programmed cell death protein 4 (PDCD4) inhibits protein translation by binding to and inhibiting the eIF4A helicase activity; phosphorylation of PDCD4 by S6K1 targets it for degradation, therefore relieving its inhibitory effect on eIF4A (Guertin and Sabatini, 2007). mTORC1, via S6K1, also potentiates the transcriptional activity of RNA polymerase I (RNAPI), through modulation of TIF-IA (transcription intermediary factor1-α) activity – a regulatory factor that responds to nutrients and growth-factors (Mayer *et al.*, 2004). Ribosomal S6 protein, a component of the 40S ribosome, is phosphorylated by S6K on Ser240/244 (Ferrari *et al.*, 1991) and is often used as a marker of mTORC1-S6K activity.

Figure 1.13 has been omitted for copyright reasons. Please refer to Laplante and Sabatini (2012), or hard copy version of this thesis for the image.

Figure 1.13. Downstream targets of mTORC1.

mTORC1-mediated activation of translation and ribosome/mRNA biogenesis is mediated through phosphorylation of its two best characterised substrates, S6K1 and 4E-BP1.

S6K1*: ribosomal protein S6 kinase 1;* ***eIF4B****: eukaryotic translation initiation factor 4B;* ***eIF4A****: eukaryotic translation initiation factor 4A;* ***S6****: ribosomal protein s6;* ***CBP80****: nuclear cap-binding protein subunit 1, 80kDa;* ***eEF2K****: eukaryotic elongation factor 2 kinase;* ***eEF2****: eukaryotic elongation factor 2;* ***SKAR****: S6K1 Aly/REF-like target;* ***TIF1A****: transcription initiation factor 1A;* ***Pol I****: polymerase I;* ***Pol III****: polymerase III;* ***PCDC4****: programmed cell death protein 4;* ***4E-BP1****: 4E-binding protein 1;* ***Maf1****: MAF1 homolog, negative regulator of RNA polymerase III.* Taken from (Laplante and Sabatini, 2012).

Activated S6K is also capable of initiating both a negative and a positive feedback loop. The phosphorylation of insulin receptor substrate-1 (IRS-1) by S6K1 on Ser302, as well as S6K1 leading to the repression of IRS-1 gene expression, acts to reduce insulin-induced PI3K signalling. Phosphorylation of mTOR at Ser-2448 results in stimulating mTOR activity (Harrington *et al.*, 2004).

PRAS40 is an inhibitory component of mTOR complex 1; an active upstream PKB signalling phosphorylates PRAS40 on residue Thr246, to relieve inhibition of mTOR (Haar *et al.*, 2007). Thus, a negative feedback loop to IRS-1 initiated by S6K, results in reduced PKB signalling and therefore an enhanced inhibition of mTORC1 by PRAS40 (Guertin and Sabatini, 2007); see **Figure 1.14**.

Figure 1.14 has been omitted for copyright reasons. Please refer to Kim, Cook and Chen (2017a), or hard copy version of this thesis for the image.

Figure 1.14. Overview of mTOR signalling pathway.

Environmental stimuli, including but not limited to growth factors, energy levels and amino acids, activate mTORC1 and mTORC2 through several pathways. Activation of receptor tyrosine kinases or GPCRs leads to the activation of PI3K and/or MAPK signalling pathways. An activated PI3K pathway promotes the accumulation of PIP3, allowing the recruitment of PKB/Akt and mTORC2 to the membrane. PKB/Akt is then phosphorylated on two residues, Thr308 by PDK1 and Ser473 by mTORC2. Fully activated PKB/Akt may now activate mTORC1 signalling by inhibiting TSC2, a GAP for the GTPase Rheb. PKB/Akt can also directly regulate mTORC1 activity by phosphorylating the mTORC1 inhibitory component, PRAS40. ERK and RSK, members of the MAPK signalling pathway, may also activate mTORC1 signalling by inhibiting TSC2 and PRAS40. Under high energy levels, AMPK – an activator of TSC2 – is inhibited, thus promoting mTORC1 activation. Lysosomal arginine and cytoplasmic leucine mediate mTORC1 localisation to the lysosome and therefore its activation, through the Ragulator-Rags complex. Glutamine can also promote mTORC1 localisation to the lysosome via the GTPase ARF1. mTORC1 can phosphorylate its downstream targets, including S6K and 4EBP1, affecting protein synthesis. S6K1 can initiate a negative feedback loop, via IRS1, to inhibit PI3K signalling upstream of mTORC1. mTORC2 downstream targets include PKB/Akt, PKC and SGK.

***RTK**: receptor tyrosine kinase; **GPCR**: G-protein coupled receptor; **PIP_3**: phosphatidylinositol (3,4,5)-triphosphate; **PI3K**: phosphoinositide 3-kinase; **PTEN**: phosphatase and tensin homolog; **PDK1**: phosphoinositide-dependent kinase-1; **AKT**: protein kinase B; **AMPK**: AMP-activated protein kinase; **TSC1/2**: tuberous sclerosis 1/2; **RHEB**: ras homolog enriched in brain; **S6K1**: ribosomal protein S6 kinase 1; **4EBP1**: 4E-binding protein 1; **MEK**: mitogen-activated protein kinase kinase, also known as MAP2K and MAPKK); **ERK**: extracellular signal-regulated kinases; **RSK**: ribosomal s6 kinase; **PKC**: protein kinase C; **SGK**: serum/glucocorticoid-regulated kinase; **IRS1**: insulin receptor substrate 1.* Taken from (Kim, Cook and Chen, 2017a).

1.3 mTOR Complex 2

1.3.1 Upstream regulation of mTORC2

Whilst mTORC1 is heavily studied, mTORC2 is less defined (Kim, Cook and Chen, 2017b), due to the lack of a specific inhibitor, and its insensitivity towards acute treatment with Rapamycin; although prolonged incubation with Rapamycin will lead towards diminished mTORC2 activity, at least in some cell types (Laplante and Sabatini, 2012). The two most probable causes of mTORC2's diminished activity in response to rapamycin, albeit linked, are as follows: firstly, the sequestering of unbound mTOR by Rapamycin and therefore the inability of nascent mTORC2 to incorporate free mTOR (Xie and Herbert, 2012), and secondly an inhibited mTORC1 results in reduced biogenesis of ribosomes, an activator of mTORC2 (Zinzalla *et al.*, 2011).

mTORC2 is insensitive to nutrients, but responds to growth factors through the PI3K pathway. As a mechanism to ensure the activation of mTORC2 only occurs in growing cells, mTORC2 associates with and is activated by the ribosome – an association promoted by insulin-mediated PI3K signalling, giving mTORC2 a signalling platform (Zinzalla *et al.*, 2011) (Laplante and Sabatini, 2012). mTORC2 is also able to promote the folding and stability of its substrates, PKB and PKC (protein kinase C), by phosphorylating nascent PKB and PKC on their TM at Thr450 (Oh *et al.*, 2010).

Alongside the common components of mTOR complexes – mTOR, DEPTOR, mLST8 – mTORC2 also incorporates RICTOR (rapamycin-insensitive companion of mTOR), mSIN1 (mammalian stress-activated MAPK-interacting protein 1) and Protor 1/2 (protein observed with RICTOR 1/2) (Kim *et al.*, 2002).

Although the function and regulation of mTORC2 is still being debated and studied, it's most likely that mTORC2's most important function is its critical interactions with PI3K signalling, mainly via mSIN1 (Saxton and Sabatini, 2017). Strong evidence points to the localisation of mTORC2 to the plasma membrane where it is recruited and activated by plasma membrane-produced PIP_3, upon activation of PI3K signalling by growth factors (Gan *et al.*, 2011). This interaction is mediated by the pleckstrin homology (PH) domain of mSIN1 (PH-mSIN1), an essential component of mTORC2; under unstimulated conditions, PH-mSIN1 binds to the mTOR kinase domain, thereby inhibiting mTORC2 activity. PIP_3 interaction with PH-mSIN1 releases PH-mSIN1 from its inhibitory interaction with the mTOR kinase domain, thus activating mTORC2 by allowing access to the kinase domain (Schroder *et al.*, 2007)(Liu *et al.*, 2015).

Further illustrating the critical importance of mSIN1, full activation of PKB appears to be dependent upon PKB-mSIN1 interaction. Partial activation of PKB on Thr308 by PDK1, initiates a phosphorylation cascade that phosphorylates mSIN1 on Thr81, resulting in increased mTORC2 activity, and culminating in the phosphorylation of PKB on Ser473 (Yang *et al.*, 2015). This demonstrates an indirect regulation of mTORC2 by mTORC1; firstly, by phosphorylating Grb10 (growth factor receptor-bound protein 10), mTORC1 negatively regulates receptor

signalling upstream of PKB (Yoon and Choi, 2016)(Yu *et al.*, 2011), and thus mTORC2. Secondly, via the negative feedback loop initiated by mTORC1-S6K1 on IRS-1, and therefore impinging on PI3K-mediated recruitment of mTORC2 (Harrington *et al.*, 2004). Likewise, PH-mSIN1 was shown to be able to interact with cytoplasmic phosphorylated Rb (retinoblastoma) proteins – which function as a tumour suppressor – which results in decreased PKB-mTORC2 interaction, therefore leading to reduced PI3K-PKB signalling (Zhang *et al.*, 2016)

However, other studies indicates that mTORC2 is also localised to the endoplasmic reticulum (ER) (Boulbés, Shaiken and Sarbassov, 2011), where it has been shown to interact with the ER proteins Hsp70 and Grp58 (Ramirez-Rangel *et al.*, 2011). As the mTORC2 substrate PKB can also be found on the ER, it is possible that activated PKB can translocate to the ER to be phosphorylated by activated mTORC2 (Betz and Hall, 2013).

1.3.2 Downstream of mTORC2

In addition to PKB, mTORC2 also targets many other AGC (protein kinase A/protein kinase G/protein kinase C) kinases such as SGK1 (serum- and glucocorticoid-induced protein kinase 1) and PKC (protein kinase C) on their TM and/or HMs, in a mSIN1-dependent manner (Cameron *et al.*, 2011); see **Figure 1.15**.

The PKC family is categorised by structure and regulation, and includes the conventional (PKCα, PKCβI/II and PKCγ), novel (PKCε, δ, θ, η) and atypical PKCs (PKCζ, τ, λ), many of which are downstream targets of mTORC2 (Xie *et al.*, 2017). They are serine/threonine kinases involved in many cellular processes including proliferation, apoptosis, survival, and differentiation (Griner and Kazanietz, 2007). Phosphorylation by mTORC2 on Thr450 of the turn motif of nascent PKB and cPKC (conventional PKCs) promotes stability and proper folding (Ikenoue *et al.*, 2008)(Oh *et al.*, 2010).

SGK1 functions as a regulator of cellular survival, and of ion transport – in a PI3K-dependent manner, it is activated by phosphorylation by PDK1 on Thr256 within its T-loop of the kinase domain, and on Ser422 in the HM (García-Martínez and Alessi, 2008). Interestingly, upon loss of mTORC2, SGK1 activity is abolished (Laplante and Sabatini, 2012).

Figure 1.15 has been omitted for copyright reasons. Please refer to Laplante and Sabatini (2012), or hard copy version of this thesis for the image.

Figure 1.15. Downstream outputs of mTORC2 pathway.

mTORC2 signalling regulates survival, metabolism and actin cytoskeleton dynamics through the regulation of its downstream targets including the AGC kinases (Akt, SGK1, PKCα) and the transcription factors FOXO.

SGK1*: serum/glucocorticoid- regulated kinase 1;* ***AKT****: protein kinase B;* ***FOXO****: forkhead box O;* ***PKCα****: protein kinase C-α.* Taken from (Laplante and Sabatini, 2012)

1.4 Amino acid transporters

The transport of amino acids is mediated by membrane transporters that are characterised by their differing substrate affinities, transport mechanisms, sensitivity towards pH changes and ion dependencies, amongst other factors (Scalise *et al.*, 2016). Thus far, fourteen transporters with an affinity for glutamine have been discovered – the redundancy reflecting the importance of glutamine transport – and two broad categories of transporters are relevant to this thesis. First, are the transporters that are Na^+-coupled, including System A and N transporters of the SLC38 family (solute carrier family 38), and System ASC of the SLC1 family (solute carrier family 1). Second, are the Na^+-independent tertiary transporters – the System L transporters of the SLC7 family (solute carrier family 7), named after their substrate leucine (Bhutia and Ganapathy, 2016).

1.4.1 SLC38 family

The SLC38 transporter family, subdivided into systems A and N, is composed of 11 ubiquitously expressed sodium-coupled neutral amino acid transporters (SNATs), with limited substrate profiles – they primarily transport small neutral amino acids such as glutamine, alanine, cysteine and serine (Bröer and Palacín, 2011). System A includes SLC38A (SNAT1) (Varoqui *et al.*, 2000), SLC38A2 (SNAT2) (M. Sugawara *et al.*, 2000a) and SLC38A4 (SNAT4) (Mitsuru Sugawara *et al.*, 2000b), whilst system N includes SLC38A3 (SNAT3, SN1) SLC38A5 (SNAT5, SN2), SLC38A7 (SNAT7) and SLC38A9. The remaining five orphan receptors functions and substrate specificities are unknown (Bröer, 2014b).

Members of the SLC38 family share similarities as well as differences. For example, both system A and system N transporters are pH sensitive – its strongest activation occurring between pH 6 and 8 (Bevington *et al.*, 2002)(Mackenzie and Erickson, 2004). However, they differ in their transport mechanisms and substrates. System A transporters utilise a Na^+ electrochemical gradient to transport its substrates, whilst system N transporters utilise a co-transport with Na^+ in antiport to H^+ (Bröer, 2014a).

α-methylaminoisobutyric acid (MeAIB), a non-metabolizable, N-methylated amino acid analogue, is a strong substrate and inhibitor for system A transporters (Tovar *et al.*, 2000) but not for system N transporters (Gu *et al.*, 2001), thus is the preferred substrate for functional assessments of system A, specifically SNAT2 (Desforges *et al.*, 2010).

1.4.1.1 System A transporters

System A transporters – A for "alanine-preferring" – are highly homologous and ubiquitously expressed in mammalian tissues, and except for SNAT4, all have an affinity towards transporting glutamine. They exhibit two phenomena that further characterises them; firstly, adaptive regulation of the transporters by the extracellular concentrations of its substrates (Ling *et al.*, 2001) . Secondly, inhibition of the transporters' activity as a result of rising intracellular amino acid concentrations (Bracy *et al.*, 1986).

SNAT1 – formerly known as ATA1, GlnT, SA2, SAT1 – is the first member of the SLC38 family to be identified, is highly expressed in the brain, and includes the following as its preferred substrates: glutamine, histidine, alanine,

asparagine, cysteine and serine (Mackenzie and Erickson, 2004). SNAT1 plays a critical role in arbitrating glutamine influx into neurons (Melone *et al.*, 2004).

SNAT2 is broadly expressed in the body, and is the most abundant system A transporter. It has the same substrate preference as SNAT1 does, with the addition of methionine, proline and glycine (Mackenzie and Erickson, 2004). SNAT2, alongside transporting glutamine, has a multitude of functions, including the regulation of gene expression, insulin secretion and nutrient signalling.

Alongside amino acid availability, insulin and growth factors are able to modulate SNAT2's activity in a PI3K-PKB-dependent manner (Hyde, Peyrollier and Hundal, 2002). Upon pharmacological inhibition of PKB, insulin-stimulated SNAT2 activity is dampened (Green *et al.*, 2008). This regulation of SNAT2 by insulin and growth factors implicates the transporter in mTOR signalling. In fact, a study in L6 myoblast cells have shown evidence that inhibition of SNAT2 by either MeAIB or siRNA (small-interfering RNA) against SNAT2 leads to impairment of signalling down to mTORC1 (Evans *et al.*, 2007). Coupling of SNAT2 with SLC7A5 (LAT1), an EEA (essential amino acid) transporter, allows the influx of leucine (an activator of mTORC1 signalling) at the expense of an efflux of glutamine, thus impacting mTOR signalling (Pinilla *et al.*, 2011).

Furthermore, stimulation by other growth factors and hormones, such as Interleukin-6 (IL-6) and tumour necrosis factor-α (TNFα) for example, act to stimulate System A transporter activity and increased expression of SNAT2 in trophoblast cells (Jones, Jansson and Powell, 2009). Similarly, SNAT2 can be up-

regulated upon treatment with EGF (epidermal growth factor) and glucagon in liver cells (McGivan and Pastor-Anglada, 1994).

SNAT2 has also been shown to possess a “transceptor” property, a property demonstrated in lower eukaryotes (Hundal and Taylor, 2009), whereby SNAT2 may act as both a transporter of amino acids, and as an amino acid receptor (Pinilla *et al.*, 2011). This transceptor property allows SNAT2 to regulate intracellular amino acid levels, and sense extracellular amino acid concentrations, and signal downstream accordingly. Thus, system A transporters are unique in their ability to be regulated by the extracellular and intracellular amino acid concentrations via adaptive regulation (Bröer, 2014b).

1.4.1.2 System N transporters

First described in 1980 (Kilberg, Handlogten and Christensen, 1980), and first isolated in 1999 (Chaudhry *et al.*, 1999), System N (SNAT3, SNAT5 and SNAT7) transporters are Na^+-dependent, pH sensitive and with a narrow substrate specificity only to glutamine, histidine, and asparagine – all of which contain Nitrogen in their side chain (Fuchs and Bode, 2006)(Nakanishi *et al.*, 2001).

System N includes two members of the SNAT family, SLC38a3 (SNAT3; SN1) and SLC38a5 (SNAT5; SN2). Transport of glutamine via system N involves the efflux of H^+ through the transporter – Na^+ alongside an amino acid are transported inwards, coupled with the efflux of H^+ (Fei *et al.*, 2000); however,

SN1 and SN2 exhibit some tolerance towards substituting Na^+ for Li^+ (Bhutia and Ganapathy, 2016); see **Figure 1.16**.

System N, quite surprisingly, has the ability to mediate both glutamine uptake and release (Bode, 2001). Unlike SNAT1 and SNAT2, SNAT3 expression in the brain is limited to astrocytes; it is also highly expressed in liver, kidney, skeletal muscle, pancreas and adipose tissues (Mackenzie and Erickson, 2004).

1.4.1 SLC1 family

System ASC transporters – named ASC for its preferred substrates *a*lanine, *s*erine and *c*ysteine – are Na^+-dependent transporters that belong to the SLC1 family (Christensen, Liang and Archer, 1967). Unlike system A transporters, system ASC transporters are not pH sensitive and are not adaptively regulated (Bussolati *et al.*, 1992).

Two isoforms exist, ASC1 and ASC2, of which only ASC2 is capable of transporting glutamine, and thus of relevance to this thesis. ASC2 is able to transport the following amino acids: glutamine, serine, threonine, cysteine, alanine and asparagine (Bode, 2001). Interestingly, unlike system A transporters, ASC2 is capable of mediating both influx and efflux of glutamine (Bröer *et al.*, 1999).

Figure 1.16 has been omitted for copyright reasons. Please refer to Scalise et al (2016), or hard copy version of this thesis for the image.

Figure 1.16. Transport mechanisms of the different glutamine transporters.

Membrane-bound glutamine transporters are shown above. Arrows indicate direction of glutamine (indicated in blue), other amino acids (indicated in grey) and ions (indicated in black).

***Gln**: glutamine; **Leu**: leucine; **Na^+:** sodium; **H^+**: hydrogen; **aa**: amino acid; **ex**: extracellular; **in**: intracellular.* Taken from (Scalise *et al.*, 2016)

1.5 Physiological roles of mTOR signalling in disease

Dysfunction of mTOR signalling is a major contributor to disease. As a signalling pathway that integrates many environmental cues and signals downstream accordingly, the regulation of mTOR signalling is crucial. Maintenance of metabolic homeostasis under conditions of increased or reduced energy levels, through anabolism and catabolism, requires sufficient regulation of mTOR signalling. Aberrations in mTOR signalling has been shown to play a role in aging, cancer, type 2 diabetes and neurodegeneration (Saxton and Sabatini, 2017).

mTOR signalling plays a vital role in mammalian aging. A decrease in mTOR expression was shown to correlate with an increased lifespan in mice (Wu *et al.*, 2013); in fact, the mTOR inhibitor rapamycin is shown to influence longevity (Robida-Stubbs *et al.*, 2012). Deletion of S6K1 – and an activation of AMPK – had resulted in an increase in mammalian life spans (Selman *et al.*, 2009). These effects are possibly mediated through a decrease in oxidative stress that may arise from decreased mRNA translation, and decreased metabolic by-products, and through an increase in autophagy which allows for the recycling of damaged proteins and organelles (Saxton and Sabatini, 2017).

Regulation of mTOR signalling is also heavily implicated in cancer; mutated oncogenic pathways, such as the PI3K-PKB and MAPK pathways, results in mTORC1 hyperactivation and unchecked growth (Zoncu, Efeyan and David M. Sabatini, 2011). Direct evidence linking mTOR with development of cancer arises from studies on negative mTOR regulators, such as TSC1/2 and PTEN; alongside the tumour suppressor p53, aberrations in PI3K-PKB signalling via PTEN (and mTOR signalling via TSC1/2), are amongst the most common mutations in cancers (Yuan and Cantley, 2008). Mutations in mTOR itself has also been reported in cancers (Grabiner *et al.*, 2014). Similarly, amino acid signalling to mTORC1 also has a role in tumour progression, through the tumour suppressor complex GATOR1 (Bar-Peled *et al.*, 2013).

A regulated mTOR signalling pathway has vital roles in neurological processes and neuron development, and thus implicated in neurodegenerative disorders such as Alzheimer's and Parkinson's disease (Lipton and Sahin, 2014). Deletions of Raptor and Rictor hindered neuronal development; on the other hand, hyperactive mTOR signalling – as seen in patients with TSC (tuberous sclerosis) – resulted in disorders such as epilepsy, which were reversed with treatment with mTOR inhibitor rapamycin (Zeng *et al.*, 2008).

1.5.1 Diabetes and β-cell compensation

Diabetes mellitus (DM) is a chronic metabolic disorder, characterised by chronic hyperglycaemia, as a consequence of impaired insulin secretion and/or peripheral insulin resistance. Its symptoms include increased thirst (polydipsia) and excessive passage of urine (polyuria). Persistence of hyperglycaemia would eventually lead to increased damage to tissues. DM is classified into type 1 diabetes (T1D), type 2 diabetes (T2D) and gestational diabetes (GD). T1D is caused by auto-immune destruction of insulin-producing pancreatic β-cells, and require constant and regular insulin treatment to survive. T2D on the other hand, is the most common form of DM – accounting for approximately 90% of diabetes cases globally – and is characterised by impaired insulin secretion coupled with insulin resistance. T2D patients require insulin treatments, careful control of circulating glucose levels as well as changes to lifestyle, to delay or prevent the development of DM.

T2D is heavily linked with obesity; increased weight-gain coupled with lifestyle choices such as lack of exercise, causes an increase in peripheral insulin resistance, and thus an increased demand for insulin (Lingohr, Buettner and Rhodes, 2002). Initially, β-cells can adapt to an increased demand for insulin secretion via increasing β-cell mass and function (and thus secretory capacity of insulin), in a process termed β-cell compensation. It is when this compensatory mechanism diminishes, or fails, that impaired glucose tolerance is developed, ultimately leading to T2D. A hallmark of T2D is pancreatic β-cell failure, arising from impaired insulin secretion due to reduced β-cell function and mass.

Augmentation of β-cell mass can occur through the generation of new β-cells, hyperplasia (increased rate of replication), hypertrophy (increased cell size) and/or decreased apoptosis (Ackermann and Gannon, 2007)(Bonner-Weir *et al.*, 2010).

As insulin resistance is primarily caused by over-nutrition and obesity, the mTOR signalling pathway plays a critical role in the regulation of β-cell mass and function (Alejandro *et al.*, 2017). Growth factors, hormones and nutrients were shown to regulate β-cell mass – thus implicating mTOR signalling – such as the stimulatory effects of glucose (in a mTORC1-dependent fashion) on β-cell mass, proliferation and viability (Paris *et al.*, 2003). In fact, mTOR inhibitor rapamycin was shown to result in loss of β-cell viability (Bell *et al.*, 2003). As a positive regulator of β-cell mass and function, chronic activation of mTORC1 causes a decrease in β-cell mass through a negative feedback mechanism. This can explain how obesity and over-nutrition may at first increase β-cell function and mass in an mTORC1-dependent mechanism, however would eventually lead to β-cell failure (Xie and Herbert, 2012). mTORC2 signalling is also reported to be critical in the maintenance of a balance between β-cell proliferation and cell size (Gu *et al.*, 2011).

1.6 Thesis aims

1. Investigate the molecular mechanism by which glutamine exerts its influence on mTOR activity in INS1e cell line.

2. To characterise the transport mechanism of glutamine in INS1e cell line and determine the role of amino acid transporters in the activation of mTORC1 and mTORC2.

3. Investigate the role of L-glutamine and its transporters in the regulation of mTORC2 in pancreatic β-cells.

Chapter 2. Materials and Methods

2.1 Reagents

All chemicals were purchased from Sigma Aldrich, Melford or Fisher Scientific, unless otherwise stated. Fetal bovine serum (FBS) was purchased from Invitrogen. Antibodies were obtained from Cell Signalling Technology. Tissue culture plates and flasks were obtained from Nunc, or VWR. Tissue culture pipettes were purchased from Greiner or Corning. Anti-mouse/anti-rabbit Ig HRP-linked antibodies were obtained from Cell Signaling Technology. Centrifuge tubes were purchased from VWR. 1.5 mL microtubes were purchased from Sarstedt. Rapamycin was purchased from Calbiochem. Torin was kindly provided by David Sabatini (Whitehead Institute for Biomedical Research, Cambridge, MA, USA). Trypsin/EDTA, penicillin/streptomycin/neomycin, L-glutamine and essential amino acids were purchased from Thermo Fisher Scientific. MeAIB, L-DON, acivicin, azaserine were purchased from Sigma Aldrich. ^{3}H-glutamine was purchased from Amersham.

2.2 Buffers

10X Tris-glycine buffer: 30 g Tris base, 144 g glycine.

10X PBS: 3 g KCl, 100 g NaCl, 14 g Na_2HPO_4, 3 g KH_2PO_4, pH 7.4, up to 1L with ddH_2O.

4X Laemmli sample buffer: 0.25 M Tris pH 6.8, 4% SDS, 40% glycerol, 10% β-mercaptoethanol, 20 µg/mL bromophenol blue.

SDS-PAGE running buffer: 1x Tris-glycine buffer, 0.1% (w/v) SDS.

Semi-dry transfer buffer: 1X Tris-glycine buffer, 0.01% (w/v) SDS, 20% (v/v) methanol.

PBST: 1x PBS, 0.1% (v/v) Tween-20.

2.3 Cell culture

2.3.1 Maintenance of cell lines

Rat insulinoma cell lines (INS1e cells) were used between passages 70 and 100 at approximately 90% confluence. Rat adrenal gland cell line (PC12 cells) and INS1e cells were cultured in Roswell Park Memorial Institute medium (RPMI 1640, Sigma Aldrich) supplemented with 11 mM glucose, 2 mM L-glutamine, 5% (v/v) heat-inactivated fetal bovine serum (FBS, Invitrogen), 1 mM sodium pyruvate, 10 mM HEPES, 50 μM β-mercaptoethanol, 100 μg/mL streptomycin, 100 units/mL penicillin and 100 units/mL neomycin (P/S/N).

Human embryonic kidney cell line (HEK293 cells), human liver cell line (HepG2 cells), human non-small cell lung cancer cell line (H1299 cells), human Osteosarcoma (U2OS cells), rat myocardium cell line (H9C2 cells) and human neuroblastoma cell line (SH-SY5Y cells) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 25 mM glucose, supplemented with 10% (v/v) FBS, 100 units/mL P/S/N.

2.3.2 Splitting of cell lines

Cells were maintained in a humidified incubator (95% air, 5% CO_2) at 37 ˚C. The medium was changed every 2 to 3 days; when approximately 90% confluent, media was removed and cells were washed with 1x phosphate buffered saline (PBS), followed by incubation with 1 mL 0.5% trypsin/EDTA (Gibco) for 3-5 minutes at 37˚C. Cells were then resuspended in their appropriate medium and plated out for maintenance, or as required for experimentation.

2.3.3 Cyroprotection of cell lines

Following trypsin treatment, cells were resuspended in appropriate growth medium, and centrifuged at 1100 rpm for 5 minutes. Cell pellets were resuspended in freezing-down buffer (composed of 10% sterile dimethyl sulphoxide (DMSO) and 90% FBS). Cells were then aliquoted in 1.5mL cryovials (StarLab), and stored in liquid nitrogen for long term storage. When required, frozen cell stocks were rapidly defrosted at 37 °C and added to the appropriate growth media. One day after incubation in a humidified incubator, the medium was changed to remove DMSO and unattached cells.

2.4 Treatment of cell lines

Detailed descriptions of treatments are provided in the appropriate figure legends. Generally, the majority of experimentation was conducted as follows: 16 hours prior to experimentation, cells were serum starved in CMRL-1066 medium (Sigma Aldrich) that contained low glucose (5.5 mM) and 0.25x L-glutamine (L-gln) over-night. Prior to experimentation the following day, CMRL-1066 medium was aspirated, and washed twice with EBSS medium (Sigma Aldrich). Experiments were then carried out with EBSS medium supplemented with 15 mM glucose, 1x essential amino acids (Sigma Aldrich), 1% P/S/N with 2 mM L-glutamine when appropriate. Insulin, PMA or EGF stimulation occurred in the final 30 minutes of incubation, or in the final 15 minutes as stated in figure legends. Inhibitors (rapamycin, torin, L-DON, acivicin, azaserine, MeAIB), cell-permeable esters (glutamate, α-ketoglutarate/2-oxoglutarate) and amino acids (D-threonine, L-histidine) where added to cells when and where stated in figure legends. Cells were incubated in a humidified incubator for the appropriate time.

2.4.1 Sample preparation

2.4.1.1 Cell lysis

After treatments, the cells were placed on ice and scrapped off the culture dishes with ice-cold lysis buffer, comprised of: 50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1 mM EDTA pH 8, 10 mM EGTA, 50 mM sodium flouride, 1 mM sodium orthovandatate, 1% Triton X-100, 10 mM β-glycerophosphate, and protease inhibitors (1 mM benzamidine, 0.2 mM PMSF, 1 µg/ml Pepstatin A, 1 µg/ml leupeptin). Lysates were centrifuged at 14,000 rpm at 4˚C for 10 minutes, followed by transferring the supernatent to a new microtube, and total protein content was determined using a Bradford Assay (BioRad).

2.4.1.2 Bradford Assay

Total protein concentration was analysed by Bradford Assay. A series of dilutions of Bovine Serum Albumin (BSA) ranging from 0.25 mg/mL to 2 mg/mL were prepared, and used to construct a standard curve plotting absorbance vs. protein concentrations. Bradford protein assay reagent (BioRad) was diluted 1:5 with ddH_2O, and 2 µL of each sample was incubated with 1 ml of Bradford protein assay reagent (BioRad) in cuvettes for 5 minutes at room temperature. Absorbance was then measured at 595nm, in a UV11-1 Biotech photometer (BioRad), and used to quantify protein content using the BSA standard curve. The samples were then normalised to the lowest protein concentration using lysis buffer.

2.5 SDS-Polyacrylamide Gel Electrophoresis

2.5.1 Preparation

Polyacrylamide gels were prepared with the BioRad SDS-PAGE system using the solutions listed in Table 2.1.

Table 2.1. Solutions and quantities used in BioRad SDS-PAGE gels.

Solution (in ml)	**7.5%**	**10%**	**12.5%**	**15%**	**17.5%**	**20%**	**Stacking**
40% acrylamide	1.95	2.55	3.15	3.75	4.35	4.85	1.24
2% Bis-acrylamide[1]	1.04	1.36	1.68	2	2.32	2.5	0.65
1.5M Tris[2]-HCl pH	2.5	2.5	2.5	2.5	2.5	2.5	-
1M Tris[2]-HCl pH	-	-	-	-	-	-	1.25
ddH_2O[3]	4.36	3.44	2.52	1.6	0.68	-	6.7
10% SDS[4]	0.15	0.15	0.15	0.15	0.15	0.15	0.1
TEMED[5]	0.005	0.005	0.005	0.005	0.005	0.005	0.001
10% APS[6]	0.05	0.05	0.05	0.05	0.05	0.05	0.075

[1]: N,N'-methylene bis-acrylamide
[2]: tris(hydroxymethyl)aminomethane
[3]: double distilled water
[4]: sodium dodecyl sulphate
[5]: N, N, N', N'-tetramethylethylenediamine
[6]: ammonium persulphate

2.5.2 Running a SDS-PAGE gel

Samples were diluted with 4x Laemmli sample buffer (62.5 mM Tris pH 6.8, 4% (w/v) SDS (sodium dodecyl sulphate), 40% (v/v) glycerol, 10% β-mercaptoethanol, 20 µg/mL bromophenol blue) to give a final concentration of 1x, and boiled at 100˚C for 3 minutes. The gel was assembled in the gel tank, and SDS-PAGE running buffer (1x Tris-glycine buffer, 0.1% (w/v) SDS) was added. Gels were run at 180 V for an appropriate time (60-120 minutes, dependent on gel and required protein separation).

2.5.3 Western Blotting

After seperation by gel electrophoresis, proteins were transferred onto Immobilon PVDF (polyvinylidene fluoride) filter membranes (Millipore) using a semi-dry transfer system (BioRad). The membranes were pre-soaked in methanol for 1 minute, and the gel was equilibriated in transfer buffer (1x tris-glycine buffer, 0.01% SDS, 20% (v/v) methanol) for 1 minute prior to transfer. A “sandwhich” was prepared as follows, from bottom to top: two 3MM papers (GE Healthcare), membrane, gel, two 3MM papers. Care was taken to remove air bubbles. Proteins were then transferred onto the PVDF membranes using semi-dry transfer system, for 30 minutes at 15 V. Transfer efficacy was determined by the transfer of prestained protein markers.

Following the transfer, the non-specific binding sites on the membranes were blocked with 5% skimmed milk in a phosphate-buffered saline (PBS) solution containing 0.1% Tween-20 for 60 minutes at room temperature on an orbital shaker. The membrane was then washed three times at room temperature with PBS-T (phosphate-buffered saline with 0.1% Tween-20), for a duration of 10 minutes each wash. The membranes were then incubated over night, at 4˚C, in an appropriate primary antibody in a PBS-T solution containing 5% BSA. The following day, the membranes were washed three times with PBS-T solution and incubated with the appropriate HRP-conjugated secondary antibody (horseradish

peroxidase) in 5% milk/PBS-T solution for 1 hour at room temperature. Following a final set of three washes in PBS-T for 10 minutes each, proteins were detected by enhanced chemiluminesence (ECL) reactions (ECL reagents obtained from Amersham Bioscience). Membranes were incubated in ECL reagent mixture for 1 minute, followed by exposure to X-ray film in the dark room. X-ray films were developed by a hyper-processor (Amersham Biosciences).

Table 2.2. List of Primary and Secondary Antibodies used in Western Blotting.

Antibody	**Source**	**Dilution**	**Supplier**
pPKB Ser473	Rabbit	1:1000	New England Biolabs
pPKB Thr308	Rabbit	1:500	New England Biolabs
PKB	Rabbit	1:1000	New England Biolabs
pS6K Thr389	Rabbit	1:1000	New England Biolabs
rpS6	Mouse	1:10000	New England Biolabs
pS6 Ser240/244	Rabbit	1:1000	New England Biolabs
p4E-BP1 Ser65	Rabbit	1:1000	New England Biolabs
4EBP1	Rabbit	1:1000	New England Biolabs
pERK1/2 Thr202/Tyr204	Rabbit	1:1000	New England Biolabs
pAMPK Thr172	Rabbit	1:1000	New England Biolabs
pmTOR Ser2481	Rabbit	1:1000	New England Biolabs
pPKC α/βII	Rabbit	1:1000	New England Biolabs
pp38 MAPK Thr180/182	Rabbit	1:1000	New England Biolabs
pJNK MAPK Tyr183/185	Rabbit	1:1000	New England Biolabs

2.6 HPLC

Following treatment of cells as per figure legend descriptions, INS1e cells in culture dishes were rapidly chilled on ice, followed by three washes with ice-cold phosphate-buffered saline (PBS) to remove extracellular amino acids. Cells were deproteinized by adding 150 µL of 0.3 M perchloric acid and scraped off culture dishes. The lysate was then transferred to microcentrifuge tubes and incubated on ice for 30 minutes, to allow maximal precipitation of protein. Precipitated proteins were then centrifuged at 140,000 rpm for 10 minutes, at 4˚C. Sedimented protein was retained for total protein assay, whilst the supernatent was filtered through a 0.45 µm microfilter. This was used for determination of amino acids on an Agilent 1100 high-performance liquid chromatograph (HPLC) with Zorbax Eclipse AAA columns, at 40 ˚C, with *o*-phtalaldehyde/3-mercaptopropionate/9-fluorenylmethylchloroformate precolumn derivatization, and ultraviolet and fluorimetric postcolumn detection (Evans *et al.*, 2007).

2.7 Transport Assay

Prior to treatment, serum-starved cells were washed twice with EBSS media. ^{3}H-glutamine plus unlabelled L-glutamine were added to EBSS medium to give a final radioisotope concentration of 0.98 µCi/mL and a final L-glutamine concentration of 10, 250 or 500 µM. 10 mM MeAIB, histidine and threonine were added to appropriate treatments. The culture with ^{3}H were then incubated at room temperature for 5 or 20 minutes. The cells were immediately placed on ice, the medium was aspirated off and the cells were rapidly washed 3x with ice-cold phosphate-buffered saline solution (PBS). 0.05 M NaOH was then added to the cells and the lysates were transferred to microcentrifuge tubes. The lysates were then incubated at 70 ˚C for 30 minutes. A fraction of the lysate was then transferred to a scintillation vial containing Ecoscint A scintillant and allowed to stand for at least 1 hour to allow chemiluminescence to decay before quench-correction scintillation counting using a scintillation counter. Another fraction was

used to quantify protein concentrations. ^{3}H-glutamine transport rate is expressed as DPM/mg protein/minutes (Cheng *et al.*, 2016).

2.8 Statistical analysis

Immunoblot intensities were quantified using the ImageJ software. Results are expressed as means ± SEM. Data were analysed by one-way analysis of variance (ANOVA) followed by Bonferroni correction for all pair-wise comparisons, using the GraphPad Prism 7.0 (GraphPad Software, San Diego, CA). A statistical test was only carried out when the experiments had at least n=3. Significance was assigned at $p<0.05$.

Chapter 3. Regulation of mTOR signalling by glutamine

3.1 Introduction

The deregulation of the mTOR pathway in disease states has given rise to extensive research into further understanding the dynamics of this signalling pathway. With the critical importance of amino acids in the regulation of mTOR signalling, one key area of research is the role of glutamine and essential amino acids (EEAs) in signalling to mTOR. Only recently has research furthered our understanding of how glutamine regulates mTOR signalling. Given the multitude of roles that the amino acid glutamine plays, acting as an energy source and as a vehicle by which EEAs mediate the activation of mTOR, and the role that its metabolites play in mTOR signalling, the first of the aims of my project was to investigate how glutamine acts to influence mTOR signalling in pancreatic β-cells.

A pancreatic β-cell line, the rat insulinoma cell line INS1e, was the primary cell line used for this research. First generated in 1992, this cell line displays characteristics of pancreatic β cells, being sensitive to physiological ranges of glucose, as well as to modulators of insulin secretion (Skelin, Rupnik and Cencic, 2010). Glucose-induced insulin secretion in human pancreatic cells, and their responses to amino acids exhibited similarities to those responses found in rat pancreatic islets (Merglen *et al.*, 2004).

Previous work from our laboratory had shown that glutamine withdrawal in pancreatic rat islets inhibits signalling to mTOR. Therefore, my first objective was to reproduce these results and, in parallel, to determine the concentrations of glutamine that may elicit an mTOR response, or inhibit it, in INS1e cells.

Glutamine may also act to regulate signalling pathways alongside mTOR. In intestinal cells for example, glutamine and its metabolism may activate MAPK signalling to induce proliferation (Rhoads *et al.*, 2000), whilst inhibiting apoptosis in an ERK-dependent manner (Larson *et al.*, 2007). Under stressful environmental conditions, glutamine can also induce autophagy and inhibit apoptosis, through its regulation of the mTOR and p38/JNK MAPK pathways (Sakiyama *et al.*, 2009). As cross-talk between the mTOR and MAPK pathways exist, this pathway integration allows for either the positive or negative regulation of mTORC1 and MAPK signalling (Mendoza, Er and Blenis, 2011): through regulation of the PI3K-mTORC1 arm by MAPK, mTORC1 can be cross-activated (Kodaki *et al.*, 1994); conversely, through cross-inhibition, these signalling pathways can negatively regulate the other (Zimmermann and Moelling, 1999). Further investigating the stress response upon glutamine withdrawal, the third question asked was whether glutamine withdrawal – in conditions of high glucose concentrations – would activate the AMPK signalling pathway, a potent inhibitor of mTOR signalling, possibly leading to the previously reported inhibition of mTORC1 signalling upon glutamine deprivation.

Finally, I investigated the role of glutamine metabolism in mediating mTOR signalling in INS1e cells. It had been reported that glutaminolysis activates mTORC1 signalling through the actions of α-ketoglutarate (Durán *et al.*, 2012). This finding contradicts another earlier study which had proposed that glutamine-dependent activation of mTOR signalling does not arise from its metabolism through glutaminolysis, but as a consequence of the coupling of SNAT2 glutamine transporter and LAT1 leucine transporter, in order to mediate leucine influx, a potent activator of mTORC1 (Nicklin *et al.*, 2009b).

3.1.1 Aims

Utilising INS1e cells, my aims were to: first, investigate the role of glutamine in mTOR signalling, and second, investigate how glutamine regulates mTOR signalling.

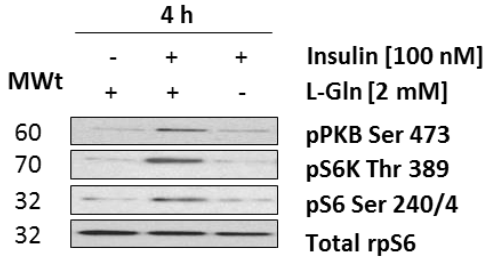
3.2 Results

3.2.1 Glutamine is required for signalling to mTORC1 in INS1e cells

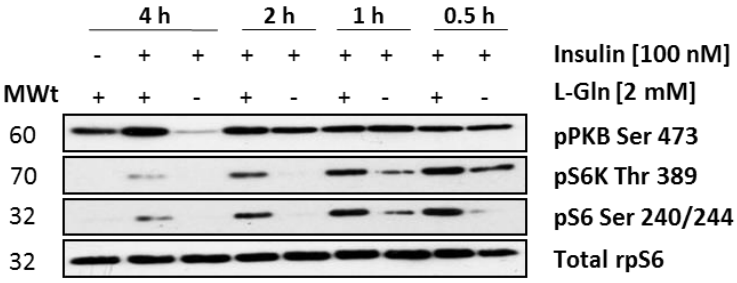
Previous studies in our laboratory had shown that in rat islets of Langerhans, glutamine is required for signalling to mTOR (Mustafa and Herbert, 2012). To investigate whether glutamine is required for signalling to mTOR in a clonal pancreatic β cell line. INS1e cells were serum-starved overnight prior to incubation in the presence of 20 mM glucose and essential amino acids, and incubated in the absence or presence of 2 mM glutamine for up to 4 h prior to treatment with insulin (100 nM), a known activator of mTORC1, for 30 minutes (**Figure 3.1**).

Withdrawal of glutamine for as little as 30 minutes inhibited insulin-mediated signalling to mTORC1 as determined by reduced phosphorylation of ribosomal S6 kinase (pS6K Thr389) and its target, ribosomal protein S6 (pS6 Ser240/244) (**Figure 3.1**)**.** Interestingly, after 4 h of glutamine starvation, inhibition of mTORC2 signalling – as indicated by the phosphorylation of its target PKB (pPKB Ser473) – was observed (**Figure 3.1**). **Figures 3.1 b) i), ii)** and **iii)** illustrate the significant dephosphorylation of pPKB Ser473, pS6K Thr389 and pS6 Ser240/244 upon glutamine withdrawal for 4 hours.

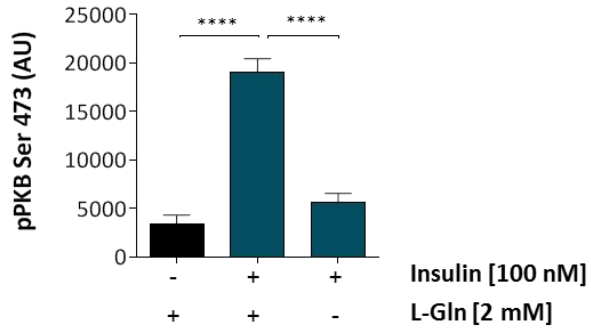
a)


4 h
- + +
MWt
+ + -
Insulin [100 nM]
L-Gln [2 mM]
60
70
32
32
pPKB Ser 473
pS6K Thr 389
pS6 Ser 240/4
Total rpS6

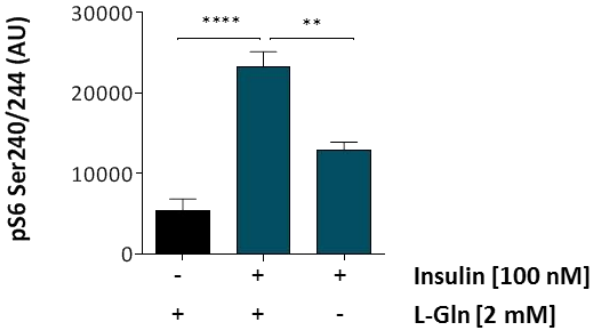
b)


4 h
2 h
1 h
0.5 h
- + + + + + + + +
MWt
+ + - + - + - + -
Insulin [100 nM]
L-Gln [2 mM]
60
70
32
32
pPKB Ser 473
pS6K Thr 389
pS6 Ser 240/244
Total rpS6

i)


pPKB Ser 473 (AU)
25000
20000
15000
10000
5000
0
****
****
- + +
+ + -
Insulin [100 nM]
L-Gln [2 mM]

ii)


pS6 Ser240/244 (AU)
30000
20000
10000
0
****
**
- + +
+ + -
Insulin [100 nM]
L-Gln [2 mM]

iii)

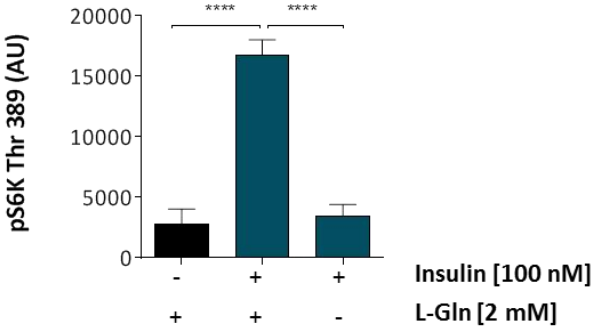

pS6K Thr 389 (AU)
20000
15000
10000
5000
0
****
****
- + +
+ + -
Insulin [100 nM]
L-Gln [2 mM]

Figure 3.1. Withdrawal of glutamine on insulin-mediated mTOR signalling for up to 4 hours.

Confluent INS1e cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with 2 mM of L-glutamine for 4 h as described in Chapter 2. Insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and lysates were immunoblotted for phospho-PKB (pPKB Ser 473) and phospho-S6 (pS6 Ser 240/244) and phospho-S6K (pS6k Thr 389). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative western blot of changes in phospho-PKB, phospho-S6 and phospho-S6K in response to insulin upon withdrawal of glutamine at 30 min, 1 h, 2 h and 4 h.

b) Representative western blot of changes in phospho-PKB, phospho-S6 and phospho-S6K in response to insulin upon withdrawal of L-glutamine for four hours. Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB (**i**), phospho-S6 (**ii**) and phospho-S6K (**iii**).

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

With intracellular concentrations of glutamine ranging from 2 mM to 20 mM (Newsholme *et al.*, 2003), the above data indicate that 2 mM L-glutamine supports insulin-dependent signalling to both mTOR complexes 1 and 2 in INS1e cells. This raised the question of how differing concentrations of glutamine might affect signalling to mTOR. As such, I next investigated the range of glutamine concentrations that may support this signalling. Serum-starved INS1e cells were incubated with varying concentrations of glutamine for 4 hours, with insulin stimulation for 30 minutes (as described in **Chapter 2.4**). As shown in **Figure 3.2**, even at the lowest concentration of L-glutamine used (0.25 mM), almost maximal phosphorylation of the target of mTORC1 (pS6 Ser240/244) and mTORC2 (pPKB Ser473) were observed.

HPLC analysis of selected amino acids were performed to assess how the various concentrations of glutamine affected their intracellular concentrations. Withdrawal of L-glutamine for 4 hours resulted in a significant fall in intracellular L-glutamine levels as expected, correlating to the concentrations used (**Figure 3.3 a**). Changes in extracellular concentration of glutamine correlated with intracellular changes in glutamine Intracellular concentrations of glutamate, leucine, alanine and aspartate were unaffected by the lowering of the glutamine concentration (**Figure 3.3 b-e**)**.**

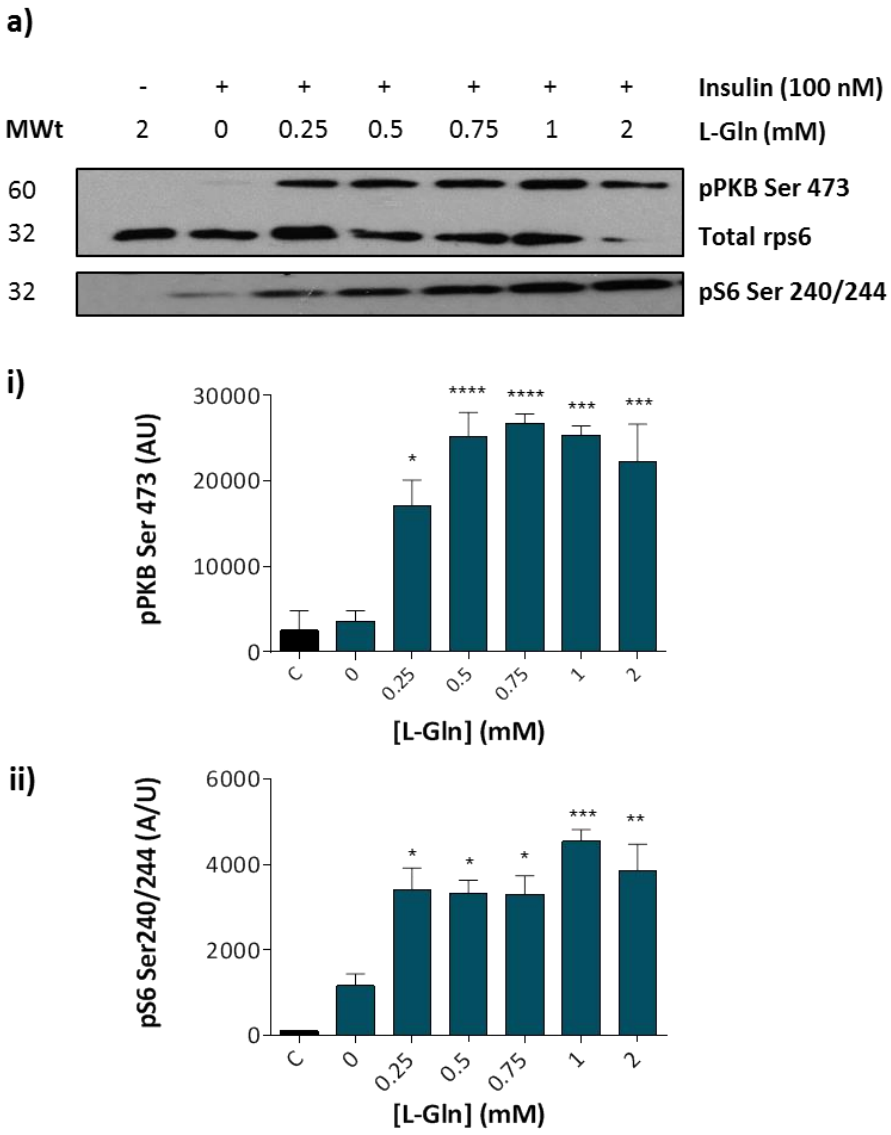


Figure 3.2. Varying concentrations of L-glutamine on insulin-mediated mTOR signalling.

Confluent INS1e cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with varying concentrations of L-glutamine (0-2 mM as indicated) for 4 h as described in **Chapter 2**. Insulin was added to all plates except the control (**C, no stimulation**) for the final 30 min of the incubation. Cell monolayers were then solubilised and lysates were immunoblotted for phospho-PKB (pPKB Ser 473) and phospho-S6 (pS6 Ser 240/244). Total rps6 used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin at different concentrations of L-glutamine. Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB (**i**) and phospho-S6 (**ii**).

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$, ****$p<0.0001$, calculated by Bonferroni's test following one-way ANOVA, relative to 0 mM L-Gln response. AU = arbitrary units.

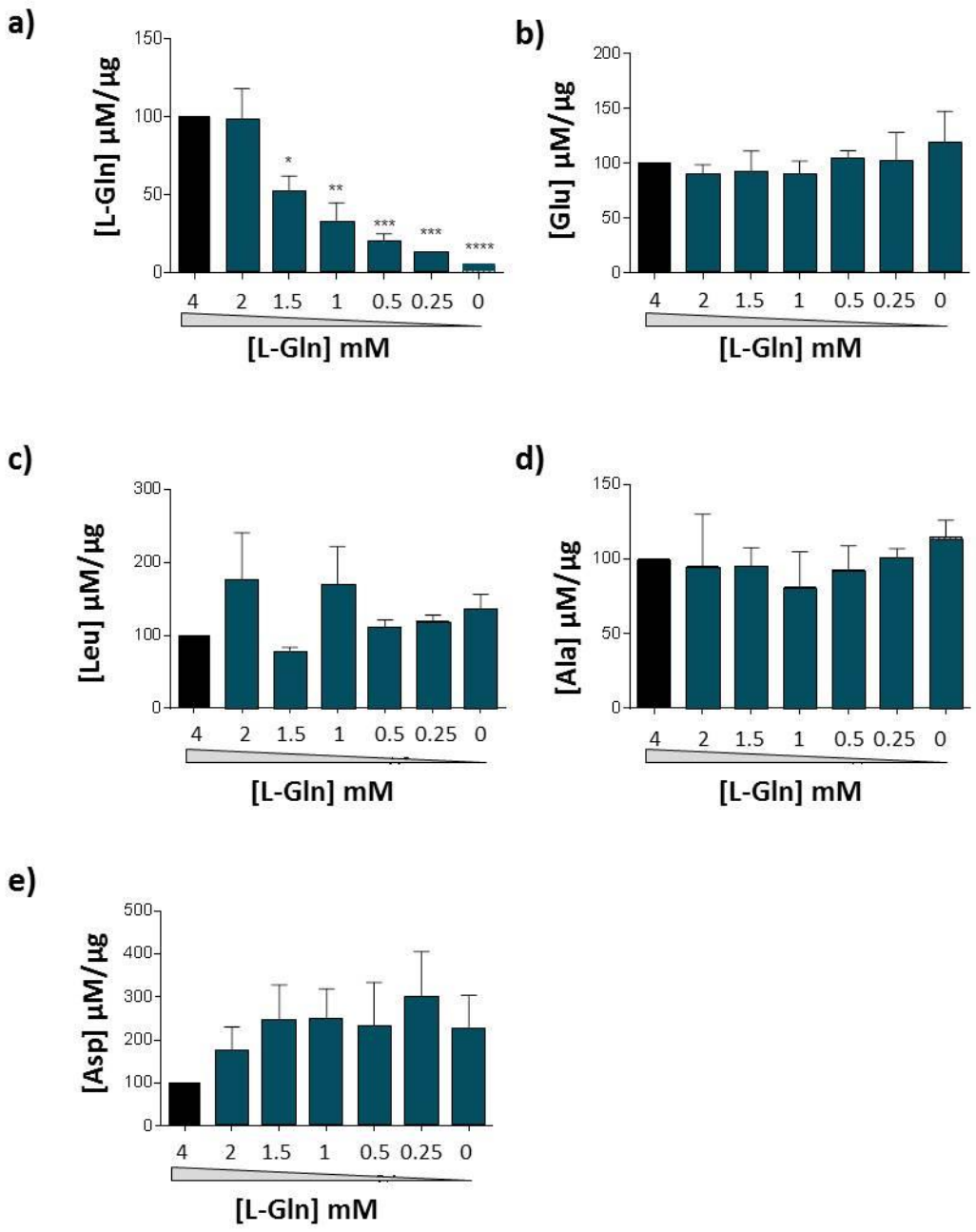


Figure 3.3. HPLC amino acid profile of INS1e cells upon treatment with varying concentrations of L-glutamine.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media with varying concentrations of L-glutamine (0 to 4 mM). Cell monolayers were then solubilised for HPLC analysis as described in **Chapter 2**. Selected amino acid profiles were analysed: **a)** Glutamine; **b)** Glutamate; **c)** Leucine; **d)** Alanine and **e)** Aspartate. The results are mean + S.E.M. of n=3 independent experiments. Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$ and *** $p<0.001$ calculated by Bonferroni's test following one-way ANOVA, relative to cells treated with 4 mM L-glutamine.

3.2.2 Withdrawal of glutamine activates the MAPK signalling pathway in INS1e cells

Given that withdrawal of glutamine inhibited insulin-dependent signalling to both mTOR complexes 1 and 2, I decided to investigate whether glutamine withdrawal causes a general inhibition of cellular signalling. Therefore, I next investigated whether insulin, phorbol ester (receptor independent activation of a signalling pathway) or EGF activated ERK phosphorylation was inhibited by glutamine withdrawal. Serum-starved INS1e cells were incubated in the presence or absence of 2 mM glutamine for 4 hours (as described in Chapter 2.4), prior to treatment with PMA, EGF or, as a control, insulin. As previously shown mTOR signalling was stimulated with insulin. The stimulation of mTOR and MAPK by insulin and PMA/EGF, respectively, occurred at either the final 30 minutes (**figure 3.4a** and **3.5a**) or 15 minutes (**figure 3.4b** and **3.5b**).

Withdrawal of glutamine activates MAPK signalling in INS1e cells. Upon PMA stimulation for 30 minutes (**figure 3.4a, ii**), and in the presence of glutamine, there is an increase in the phosphorylation levels of ERK1/2, albeit not statistically significant. This phosphorylation status of ERK1/2 (pERK1/2) is significantly further increased in the absence of glutamine. This observation is mimicked with 15 minutes of PMA stimulation (**figure 3.4b, ii**). Interestingly, in the experimental conditions lacking both glutamine and PMA stimulation, there is an apparent activation of MAPK signalling, as observed by the presence of phosphorylated ERK1/2, when compared to those conditions with no PMA stimulation in the presence of glutamine. In contrast, 30 minutes of PMA activation appeared to

inhibit signalling to pPKB Ser473 relative to the unstimulated cells, an inhibition occurring regardless of the presence of glutamine (**figure 3.4a, i**). Signalling to pPKB Ser473 appears to be unaffected by 15 minutes of PMA stimulation (**figure 3.4b, i**).

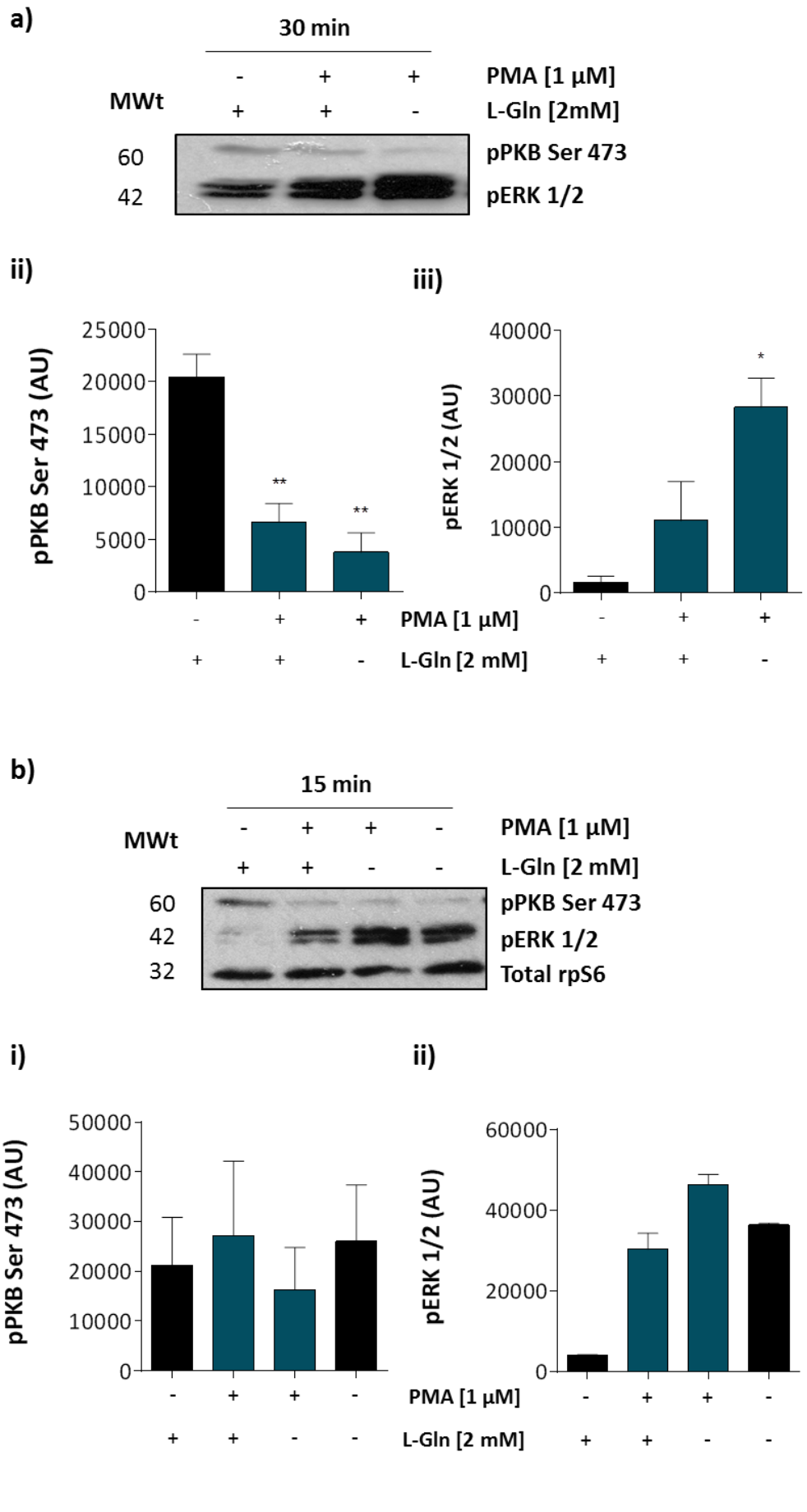

a)
30 min
MWt
PMA [1 µM]
L-Gln [2mM]
60
42
pPKB Ser 473
pERK 1/2
ii)
iii)
pPKB Ser 473 (AU)
pERK 1/2 (AU)
PMA [1 µM]
L-Gln [2 mM]
b)
15 min
MWt
PMA [1 µM]
L-Gln [2 mM]
60
42
32
pPKB Ser 473
pERK 1/2
Total rpS6
i)
ii)
pPKB Ser 473 (AU)
pERK 1/2 (AU)
PMA [1 µM]
L-Gln [2 mM]

Figure 3.4. Effects of L-glutamine withdrawal on PMA-induced MAPK and mTOR signalling.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with 2 mM L-glutamine for 4 h as described in **Chapter 2**. PMA (1 µM) was added to all plates except the control for the final 30 min **(a)** or 15 min **(b)** of the incubation. Cell monolayers were then solubilised and lysates were immunoblotted for phospho-PKB (pPKB Ser 473) and phospho-ERK 1/2 (pERK Thr 202/204). Total rps6 used as a loading control.

a) Representative blot of changes in phospho-PKB and phospho-ERK in response to 1 µM PMA for 30 min. Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB (**i**) and phospho-ERK 1/2 (**ii**).

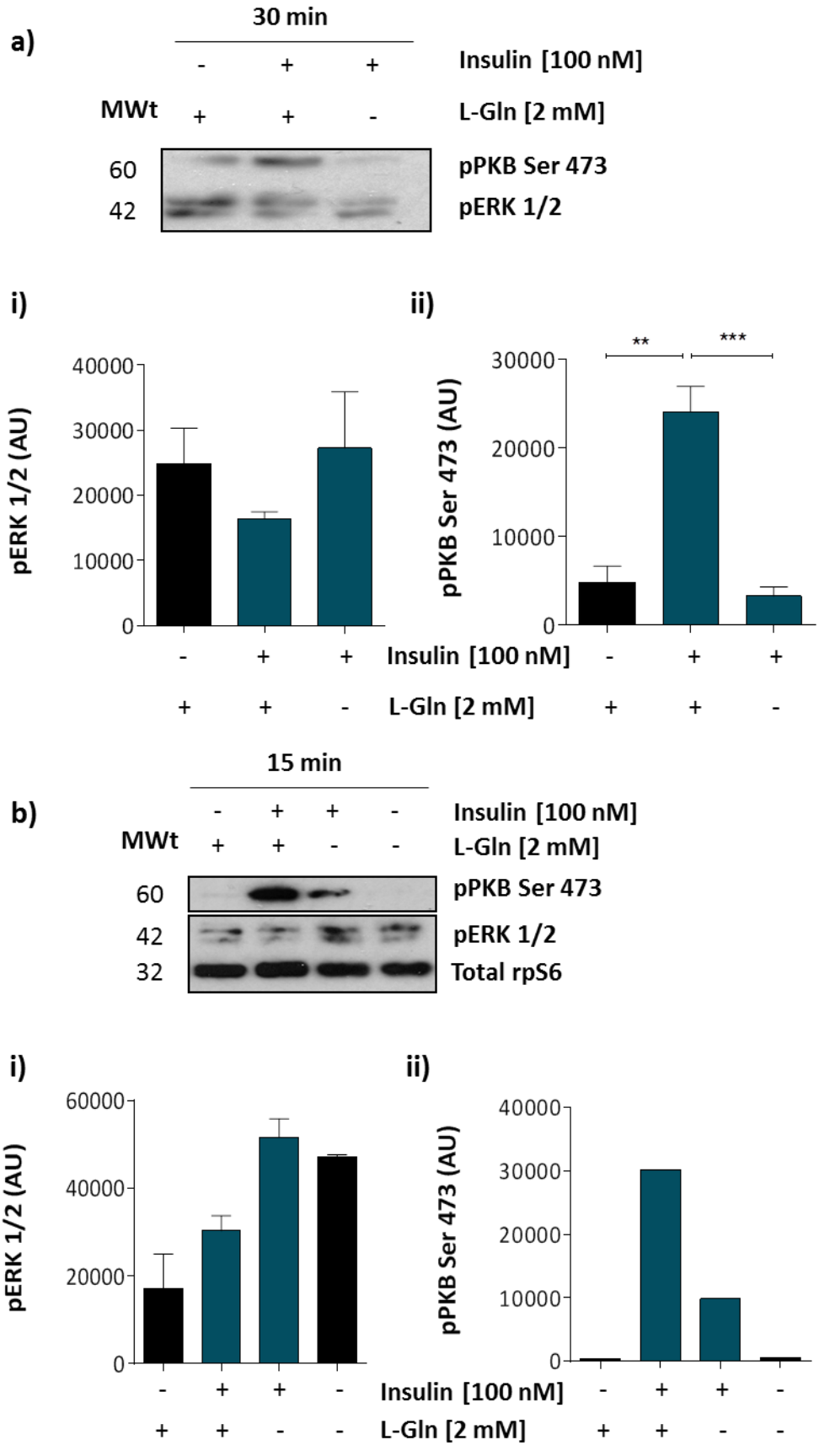
b) Representative blot of changes in phospho-PKB and phospho-ERK in response to 1 µM PMA for 15 min. Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB (**i**) and phospho-ERK 1/2 (**ii**).

The results are either representative or mean ± S.E.M. of n≥3 (**a**) and n=2 (**b**) independent experiments. Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, calculated by Bonferroni's test following one-way ANOVA, relative to unstimulated conditions (without PMA, black bars). AU = arbitrary units.

To ensure that any impact on MAPK signalling observed correlates with an inhibition of insulin-mediated mTOR signalling upon glutamine withdrawal, mTOR signalling was monitored as a control (**Figure 3**.**5**).

As expected, and observed previously, insulin stimulation for 30 minutes (**Figure 3.5a, ii**), and for 15 minutes (**Figure 3.5b, ii**), resulted in maximal phosphorylation of pPKB Ser473 in the presence of glutamine, and inhibition of signalling to pPKB Ser473 in the absence of glutamine. With regards to insulin-mediated MAPK signalling, 30 minutes of stimulation did not affect the phosphorylation status of pERK1/2 (**Figure 3.5a, i**); however, with insulin stimulation occurring for 15 minutes, pERK1/2 phosphorylation levels were as those observed under 30 minutes of PMA stimulation – an increased phosphorylation upon glutamine withdrawal, regardless of PMA stimulation (**Figure 3.5b, i**). EGF stimulation for 15 minutes did not affect the phosphorylation levels of pERK1/2 (**Figure 3.5c, i**), but led to a reduction in pPKB Ser473 phosphorylation levels upon glutamine withdrawal (**Figure 3.5c, ii**).

Collectively, these data illustrate the existence of an inverse relationship between mTOR and MAPK signalling pathways in INS1e cells; upon inhibition of mTOR signalling, there is an activation of MAPK signalling, or *vice versa*. The next question was whether the cross-talk between these two signalling pathways exist as a compensatory mechanism to regulate cellular homeostasis and autophagy/apoptosis, and/or as a consequence of ERK-dependent activation of stress pathways (Cowan and Storey, 2003)?


a)
30 min
- + +
Insulin [100 nM]
MWt + + -
L-Gln [2 mM]
60
42
pPKB Ser 473
pERK 1/2
i)
ii)
pERK 1/2 (AU)
40000
30000
20000
10000
0
pPKB Ser 473 (AU)
30000
20000
10000
0
**
***
- + + Insulin [100 nM] - + +
+ + - L-Gln [2 mM] + + -
b)
15 min
- + + - Insulin [100 nM]
MWt + + - - L-Gln [2 mM]
60
42
32
pPKB Ser 473
pERK 1/2
Total rpS6
i)
ii)
pERK 1/2 (AU)
60000
40000
20000
0
pPKB Ser 473 (AU)
40000
30000
20000
10000
0
- + + - Insulin [100 nM] - + + -
+ + - - L-Gln [2 mM] + + - -

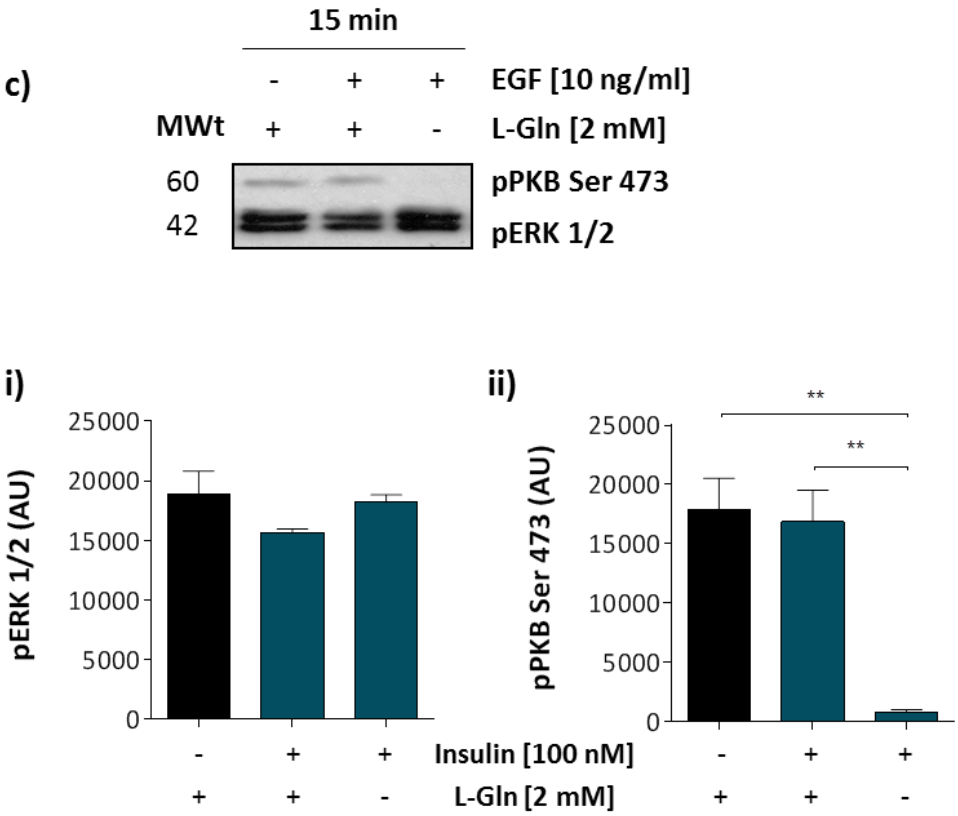


Figure 3.5. Effects of L-glutamine withdrawal on Insulin-induced and EGF-Induced MAPK and mTOR signalling.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with 2 mM L-glutamine for 4 h as **Chapter 2**. 100 nM Insulin or 10 ng/ml EGF were added to all plates except the control for the final 30 or 15 minutes of the incubation. Cell monolayers were then solubilised and lysates were immunoblotted for phospho-PKB (pPKB Ser 473) and phospho-ERK 1/2 (pERK Thr 202/204) with total rps6 used as a loading control.

a) Representative blot of changes in phospho-PKB and phospho-ERK in response to 100 nM Insulin for 30 minutes. Densitometric analysis was carried out to quantify relative protein levels of phospho-ERK Thr 202/204 (**i**) and phospho-PKB Ser 473 (**ii**) The results are n≥3.

b) Representative blot of changes in phospho-PKB and phospho-ERK in response to 100 nM Insulin for 15 min. Densitometric analysis was carried out to quantify relative protein levels of phospho-ERK Thr 202/204 (**i**) and phospho-PKB Ser 473 (**ii**). The results are n=1 experiments.

c) Representative blot of changes in phospho-PKB and phospho-ERK in response to 10 ng/ml EGF for 15 min. Densitometric analysis was carried out to quantify relative protein levels of phospho-ERK Thr 202/204 (**i**) and phospho-PKB Ser 473 (**ii**). The results are n=3.

* $p<0.05$, ** $p<0.01$ and *** $p<0.001$ by Bonferroni's test following one-way ANOVA. AU= Arbitrary Units.

3.2.3 L-glutamine withdrawal appears to induce cellular stress in INS1e cells

With the activation of ERK1/2 signalling upon glutamine deprivation, it was of interest to investigate if glutamine insufficiency gives rise to an increase in the activation of other MAPKs involved in stress pathways, as well as how glutamine regulates AMPK signalling to mTOR. Under conditions of glutamine withdrawal for 4 hours, and insulin stimulation for 30 minutes (as described in Chapter 2.4), phosphorylation levels of p38 and JNK – two stress activated MAPKs – alongside AMPK, were investigated.

In the absence of glutamine, there appears to be a non-significant increase in phosphorylation of both p38 MAPK (pp38 Thr180/182) (**Figure 3.6a**) and JNK (pJNK Thr 183/Tyr 185) (**Figure 3.6b**), compared to phosphorylation levels in the presence of glutamine. This observation was mirrored with regards to the phosphorylation levels of AMPK (pAMPK Thr 172), an energy sensor which plays an important inhibitory role on mTOR signalling when energy levels are low (**Figure 3.7**). Under unstimulated conditions, and in the presence of glutamine, high basal phosphorylation levels of AMPK Thr172 were observed (**Figure 3.7 a**). AMPK phosphorylation appears to be reduced upon insulin stimulation in the presence of glutamine, and induced significantly in the absence of glutamine (**Figure 3.7 a i** and **ii**). These results suggest an activation of the cellular stress machinery in response to L-glutamine deprivation in INS1e cells.

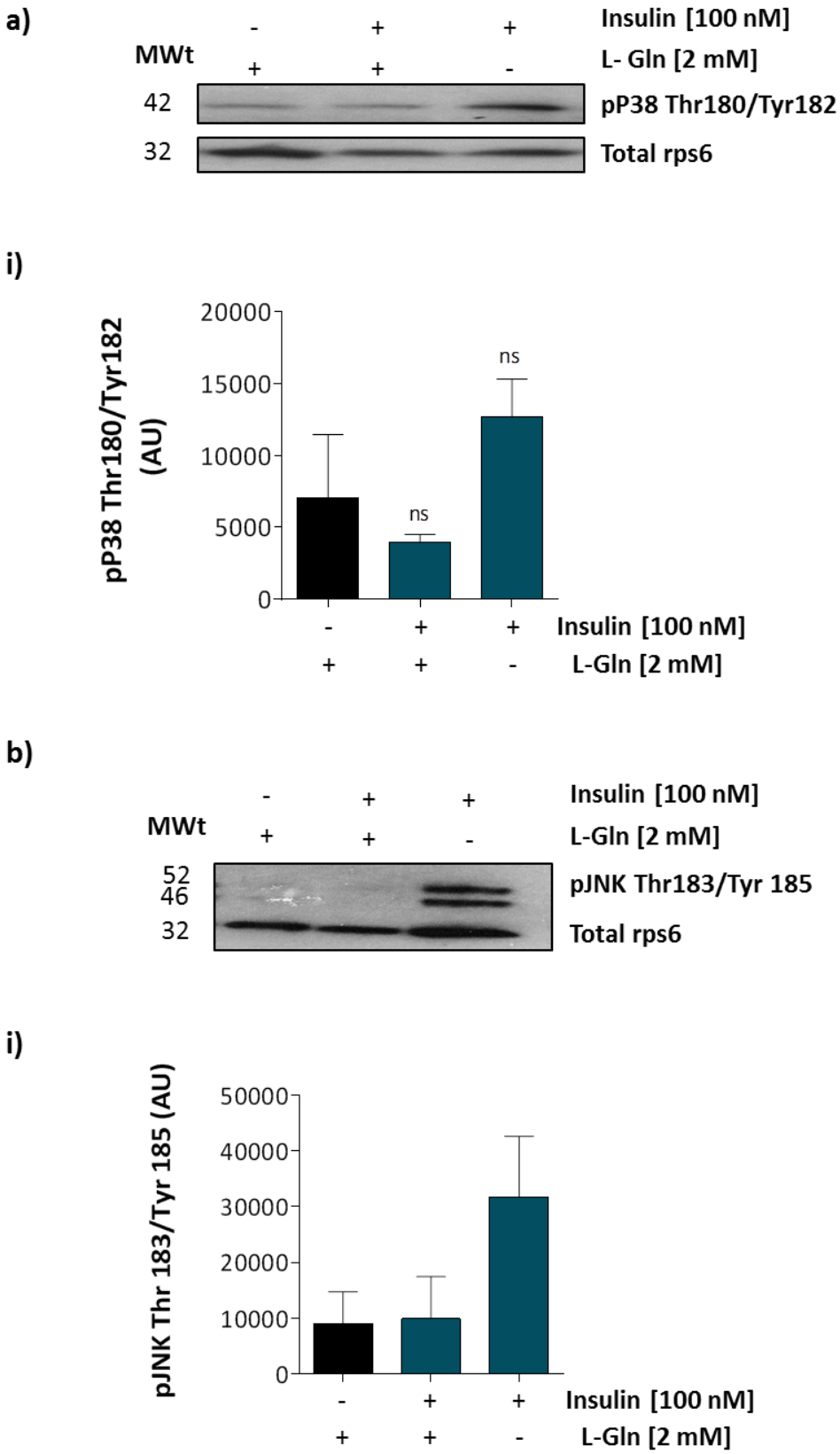


Figure 3.6. Effects of 4 hr L-Glutamine withdrawal on pP38 and pJNK in INS1-E cells.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media with/without 2 mM L-glutamine for 4 h as described in **Chapter 2**. Insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and lysates were immunoblotted for phospho-P38 (pP38 Thr190/Tyr182) and phospho-JNK (pJNK Thr183/Tyr185) with total rps6 as a loading control.

a) Representative blot of changes in phospho-P38 responses. Densitometric analysis was carried out to quantify relative protein levels of phospho-P38 (**i**). The results mean ± S.E.M. of n≥3.

b) Representative blot of changes in phospho-JNK responses. Densitometric analysis was carried out to quantify relative protein levels of phospho-JNK (**i**). The results mean ± S.E.M. of n=2.

ns = non-significant, calculated by Bonferroni's test following one-way ANOVA. AU= Arbitrary Units.

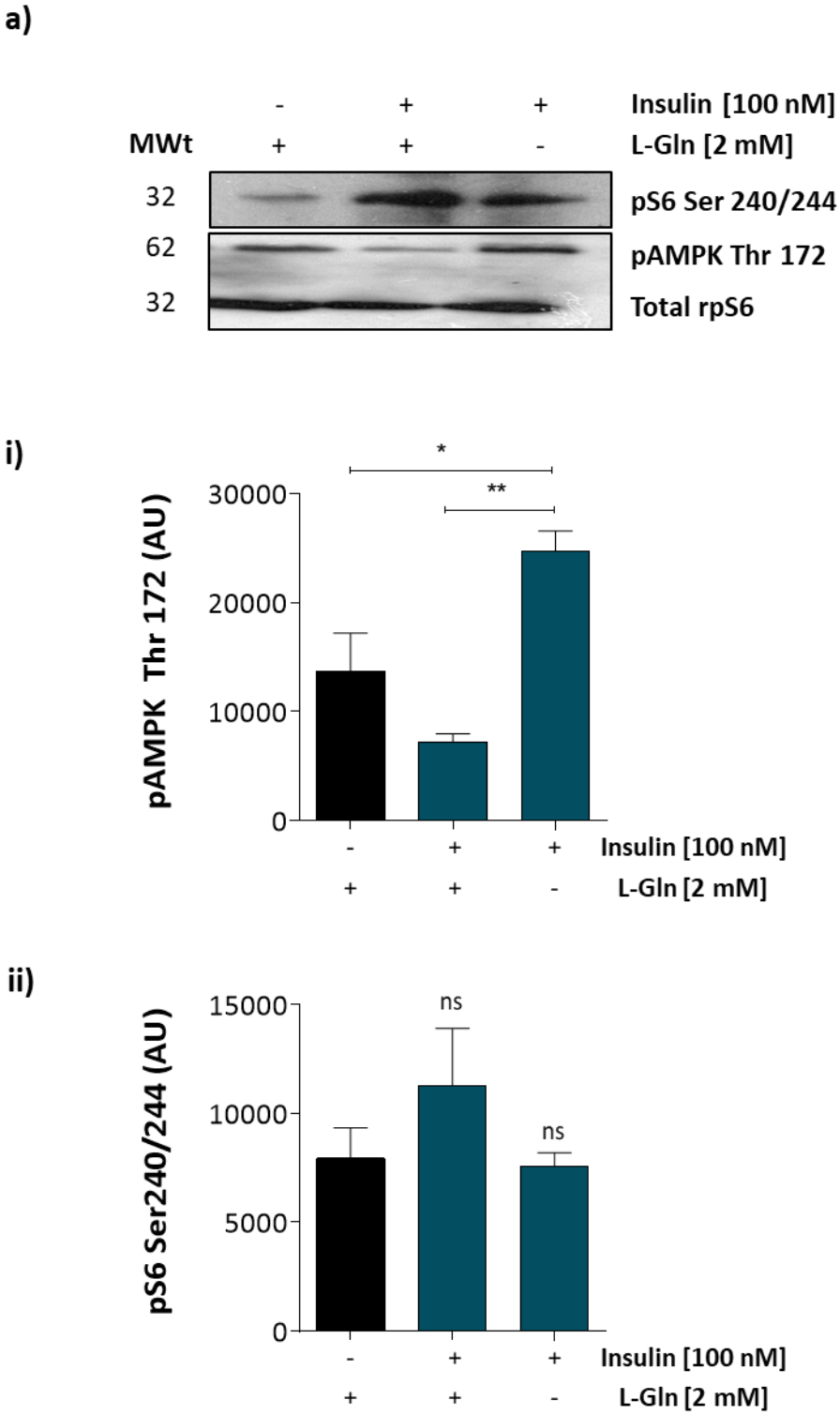


Figure 3.7. Effects of L-glutamine withdrawal on insulin-induced phospho-AMPK Thr172.

Confluent INS1e cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with 2 mM of L-glutamine for 4 h as described in Chapter 2. 100 uM Insulin was added to all plates except the control for the final 30 minutes of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-AMPK (pAMPK Thr172) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-AMPK and phospho-S6 in response to insulin upon withdrawal of glutamine for 4 hours.
i) Densitometric analysis of phospho-AMPK responses.
ii) Densitometric analysis of phospho-S6 responses.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

3.2.4 Glutamine metabolites restore signalling to mTOR in glutamine-deprived INS1e cells

The metabolism of glutamine, via glutaminolysis, is an important mediator of mTORC1 activity. Glutamine is metabolised by the glutaminase (GLS) enzyme to glutamate, which is further metabolised via the actions of the glutamate dehydrogenase (GDH) enzyme to produce α-ketoglutarate (also known as 2-oxoglutarate; αKG). Duran *et al*. (2012) has argued that glutaminolysis is a potent activator of mTORC1 signalling. Previous work in our laboratory had shown that in rat pancreatic islets, glutamine metabolites are not able to restore mTORC1 signalling in glutamine-deprived islets of Langerhans (Mustafa and Herbert, 2012).

With the understanding that glutamine is critical for the activation of mTOR signalling in insulin-stimulated INS1e cells, and that depriving cells of glutamine inhibits signalling to mTOR, whilst activating MAPK and AMPK signalling, my next research questions were firstly, to ask if the glutamine metabolites – glutamate and αKG – were able to restore mTORC1 (and mTORC2) activity in glutamine-deprived INS1e cells; and secondly, if they are able to influence mTORC1 activity, would inhibition of glutaminolysis with pharmacological inhibitors impede on such a restoration of mTOR activity?

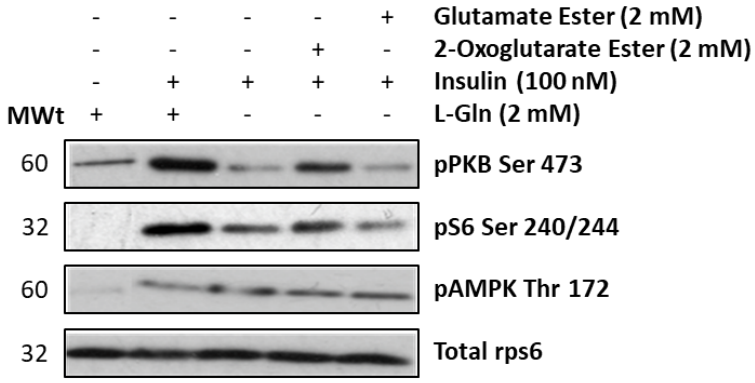
To assess whether glutamine metabolites would restore mTOR signalling in glutamine-deprived cells, serum-starved INS1E were treated with cell-permeable esters of either glutamate or αKG in the absence of glutamine, and mTOR

signalling was induced by the addition of insulin, as previously described Chapter 2.2.

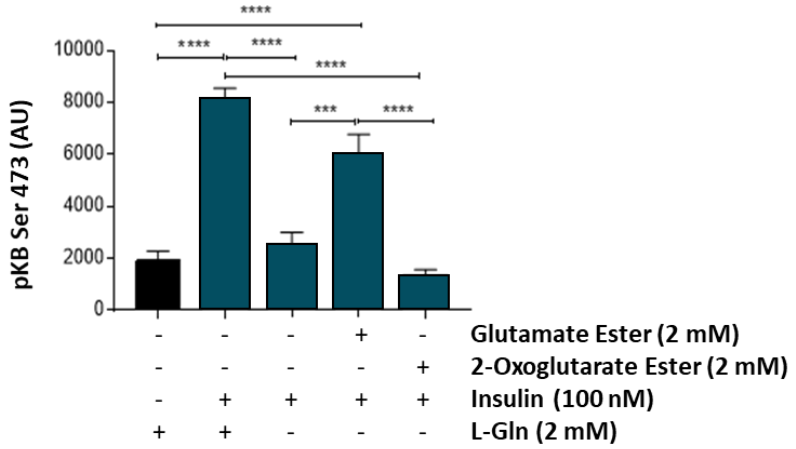
Interestingly, the cell-permeable ester of glutamate restores signalling to both mTORC1 and mTORC2 (**Figure 3.8a, i, ii**). The inhibition of insulin-stimulated mTOR signalling upon withdrawal of glutamine for 4 hours, was reversed upon the addition of an ester of glutamate, evident by phosphorylation levels of S6 at Ser240/244 for mTORC1 activity, and PKB at Ser473 for mTORC2 activity. This was not observed when an ester of αKG was added to INS1e cells deprived of glutamine; intriguingly, even though αKG failed to restore signalling to mTORC1 and mTORC2, both αKG and glutamate appear to reduce AMPK Thr172 phosphorylation levels to those seen in the presence of glutamine (**Figure 3.8a, iii**).

Due to the inability of αKG to restore signalling to mTORC1 and mTORC2, concerns regarding the cell permeability of this ester arose. The experiment was repeated with different ester of αKG, and this time both esters of glutamate and αKG rescued insulin-stimulated mTORC1 (pS6 Ser240/244) and mTORC2 (pPKB Ser473) activity in the absence of glutamine, relative to the withdrawal of glutamine (**Figure 3.9)**.

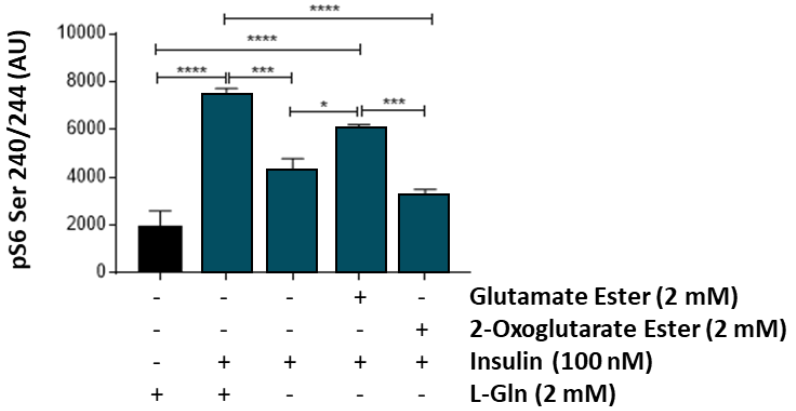
a)


- - - - + Glutamate Ester (2 mM)
- - - + - 2-Oxoglutarate Ester (2 mM)
- + + + + Insulin (100 nM)
MWt + + - - - L-Gln (2 mM)
60 pPKB Ser 473
32 pS6 Ser 240/244
60 pAMPK Thr 172
32 Total rps6

i)


pKB Ser 473 (AU)
10000
8000
6000
4000
2000
0
- - - + - Glutamate Ester (2 mM)
- - - - + 2-Oxoglutarate Ester (2 mM)
- + + + + Insulin (100 nM)
+ + - - - L-Gln (2 mM)

ii)


pS6 Ser 240/244 (AU)
10000
8000
6000
4000
2000
0
- - - + - Glutamate Ester (2 mM)
- - - - + 2-Oxoglutarate Ester (2 mM)
- + + + + Insulin (100 nM)
+ + - - - L-Gln (2 mM)

iii)

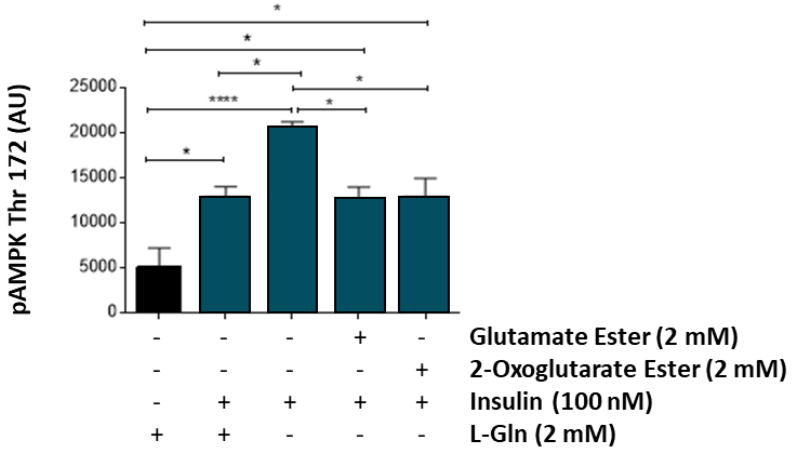

pAMPK Thr 172 (AU)
25000
20000
15000
10000
5000
0
- - - + - Glutamate Ester (2 mM)
- - - - + 2-Oxoglutarate Ester (2 mM)
- + + + + Insulin (100 nM)
+ + - - - L-Gln (2 mM)

Figure 3.8. Addition of glutamate and 2-oxoglutarate esters restores mTORC1 and mTORC2 signalling

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media in the presence or absence of 2 mM L-glutamine, 2 mM glutamate ester and 2 mM 2-oxoglutarate ester, where indicated, for 4 h as described in Chapter 2. 100 nM insulin was added to all plates except the control in the final 30 minutes of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473), phospho-AMPK (pAMPK Thr172) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB, phospho-AMPK and phospho-S6 in response 100nM Insulin.
i) ii) and iii) Densitometric analysis of phospho-PKB, phospho-S6 and phospho-AMPK responses.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

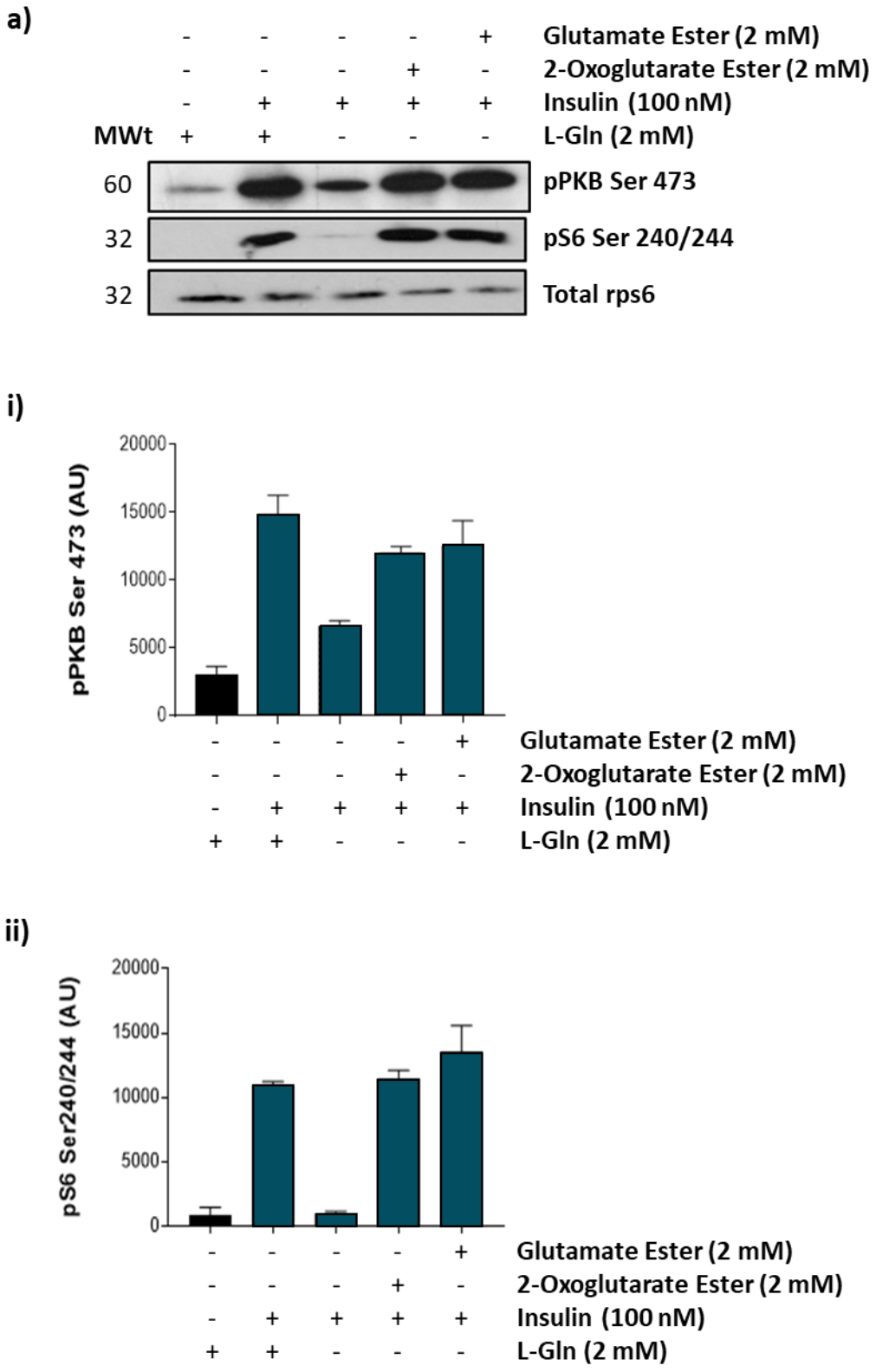


Figure 3.9. Addition of new esters of glutamate and 2-oxoglutarate restores mTORC1 and mTORC2 signalling.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media in the presence or absence of 2 mM L-glutamine, 2 mM glutamate ester and 2 mM 2-oxoglutarate ester for 4 h as described in Chapter 2. 100 nM Insulin was added to all plates except the control in the final 30 minutes of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control.

a) Representative blot of changes in phospho-PKB, and phospho-S6 in response 100uM Insulin.
i) and ii) Densitometric analysis of phospho-PKB and phospho-S6 responses.

The results are either representative or mean ± S.E.M. of n=2 independent experiments. AU = arbitrary units.

Collectively, the above observations paint glutamine as a critical signal for the activation of mTORC1 and mTORC2 signalling in INS1e cells, a role that depends – at least in part – on the functions of its metabolites glutamate and αKG. As my results corroborated Duran *et al.* findings (Durán *et al.*, 2012), my next question aimed to dissect these observations and ask if the process of glutaminolysis is the process by which glutamine (and its metabolites) exerts its influence on mTOR signalling in INS1e cells. Duran *et al.* proposes that one of many mechanisms by which leucine activates mTORC1 signalling is by promoting glutaminolysis, via modulating the activity of GDH, of which it is an allosteric activator. Does inhibition of glutaminolysis and glutamine metabolism impinge on signalling to mTORC1 and mTORC2?

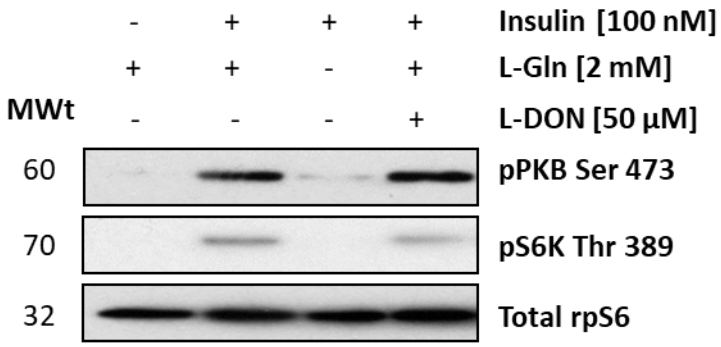
3.2.5 Inhibition of glutaminolysis does not inhibit mTOR signalling in INS1e cells

The two primary enzymes involved in glutaminolysis, glutaminase (GLS) and glutamate dehydrogenase (GDH), act to produce glutamate and α-ketoglutarate, respectively. They are inhibited by several pharmacological inhibitors, primarily L-DON (6-diazo-5-oxo-L-norleucine), acivicin and azaserine. The mechanism of action of these inhibitors stems from them being a glutamine analogue; their structural similarities allowing them to bind to and inhibit glutamine utilizing enzymes, thus interfering with glutamine and glutamate metabolic pathways. L-DON, for example, is a diazo compound that is a glutamine antagonist, and

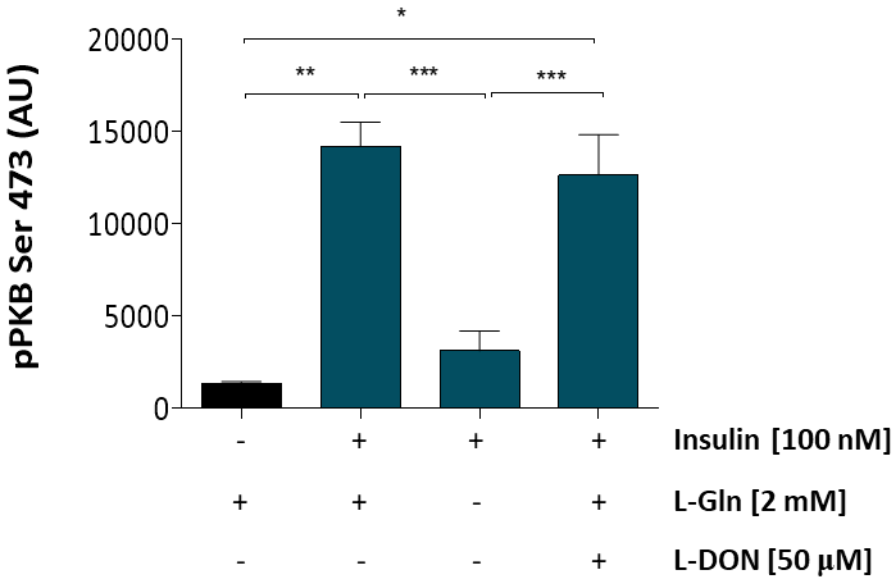
amongst its many targets, inhibits the actions of GLS enzyme (Durán *et al.*, 2012). Acivicin is another glutamine analogue, which acts to interfere with glutamate metabolism and inhibit the γ-glutamyl pathway (Hidalgo *et al.*, 1998). Finally, azaserine, another glutamine analogue, primarily acts by competitively inhibiting glutamine amidotransferase, interfering with glutamine metabolism, is the rate limiting step of the hexosamine pathway, and is an irreversible inhibitor of γ-glutamyl transferase (Rajapakse *et al.*, 2009).

I utilised these pharmacological inhibitors aiming to inhibit glutaminolysis and glutamine/glutamate metabolism, and elucidate if the inhibition of said metabolism would affect signalling to mTOR in INS1e cells, as was seen upon withdrawal of glutamine. Thus, serum-starved INS1e cells were incubated for 4 hours in either the presence or absence of 2 mM glutamine. 50 µM of the inhibitors L-DON, acivicin or azaserine were added where indicated, with insulin stimulation of mTOR signalling occurring in the final 30 minutes as described in Section 2.4. As per the phosphorylation levels of pS6K Thr389 and pPKB Ser473, the addition of the inhibitors did not affect signalling to mTORC1 and mTORC2, respectively. The addition of 50 µM of L-DON in the presence of 2 mM glutamine did not reduce phosphorylation levels of pS6K Thr389 or pPKB Ser473 (**Figure 3.10a**), an observation also seen upon addition of 50 µM of acivicin (**Figure 3.11a**) and 50 µM azaserine (**Figure 3.12a**). Withdrawal of glutamine reduces phosphorylation levels of pS6K and pPKB to those seen under basal unstimulated conditions – as expected. However, in the presence of any of the three inhibitors, signalling to mTORC1 (pS6K Thr389) and mTORC2 (pPKB Ser473) remained unaffected.

a)


- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
MWt - - - + L-DON [50 µM]
60 pPKB Ser 473
70 pS6K Thr 389
32 Total rpS6

i)


pPKB Ser 473 (AU)
20000
15000
10000
5000
0
*
**
***
***
- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
- - - + L-DON [50 µM]

ii)

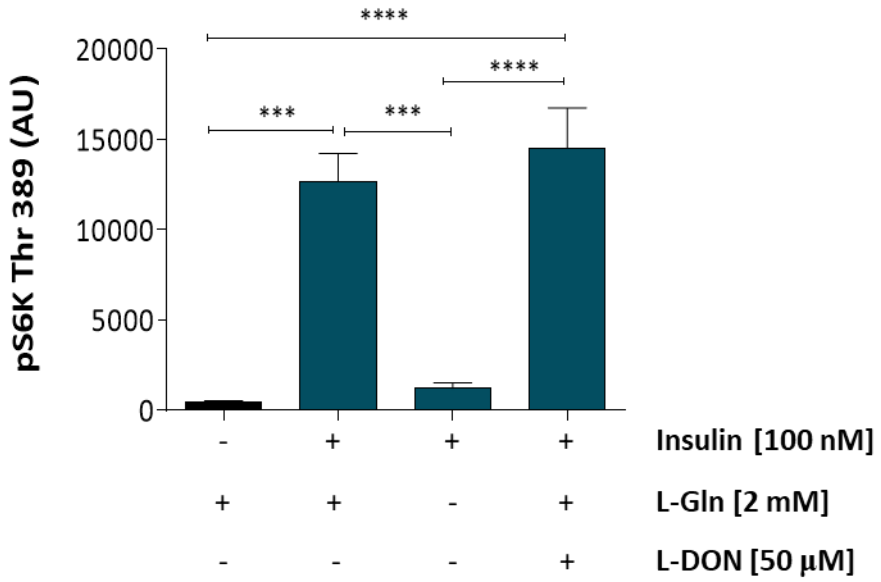

pS6K Thr 389 (AU)
20000
15000
10000
5000
0
****
****
***
***
- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
- - - + L-DON [50 µM]

Figure 3.10. Effects of inhibition of glutaminolysis with L-DON on insulin-mediated mTOR signalling.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media with 2 mM L-glutamine for 4 h as described in Chapter 2. L-DON (50 µM) was added where indicated. 100 nM insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phosphor-PKB (pPKB Ser 473) and phospho-S6K (pS6K Thr 389). Total rpS6 was used as a loading control.

a) Representative blot of changes in phospho-PKB and phospho-S6K in response to insulin.
i) Densitometric analysis of phospho-PKB responses. **ii)** Densitometric analysis of phospho-S6K responses.

The results are either representative or mean ± S.E.M. of n≥3 independent experiments. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

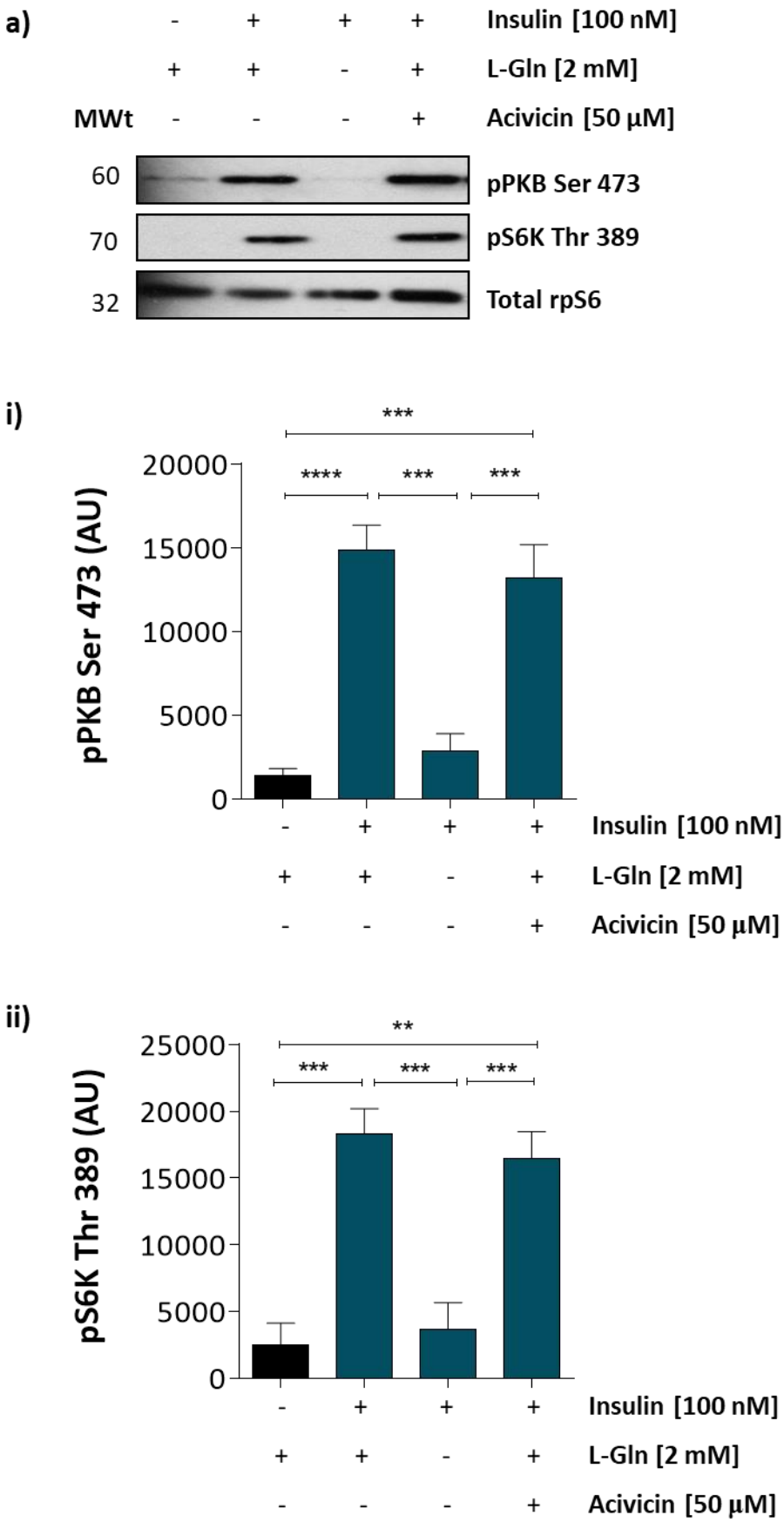

a)
- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
MWt - - - + Acivicin [50 µM]
60
70
32
pPKB Ser 473
pS6K Thr 389
Total rpS6
i)
pPKB Ser 473 (AU)
20000
15000
10000
5000
0
***
****
***
***
- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
- - - + Acivicin [50 µM]
ii)
pS6K Thr 389 (AU)
25000
20000
15000
10000
5000
0
**
***
***
***
- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
- - - + Acivicin [50 µM]

Figure 3.11. Effects of inhibition of glutaminolysis with Acivicin on insulin-mediated mTOR signalling.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media with 2 mM L-glutamine for 4 h as described in Chapter 2. Acivicin (50 µM) was added where indicated. 100 uM insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser 473) and phospho-S6K (pS6K Thr 389). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB and phospho-S6K in response to insulin.
i) Densitometric analysis of phospho-PKB responses. **ii)** Densitometric analysis of phospho-S6K responses.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

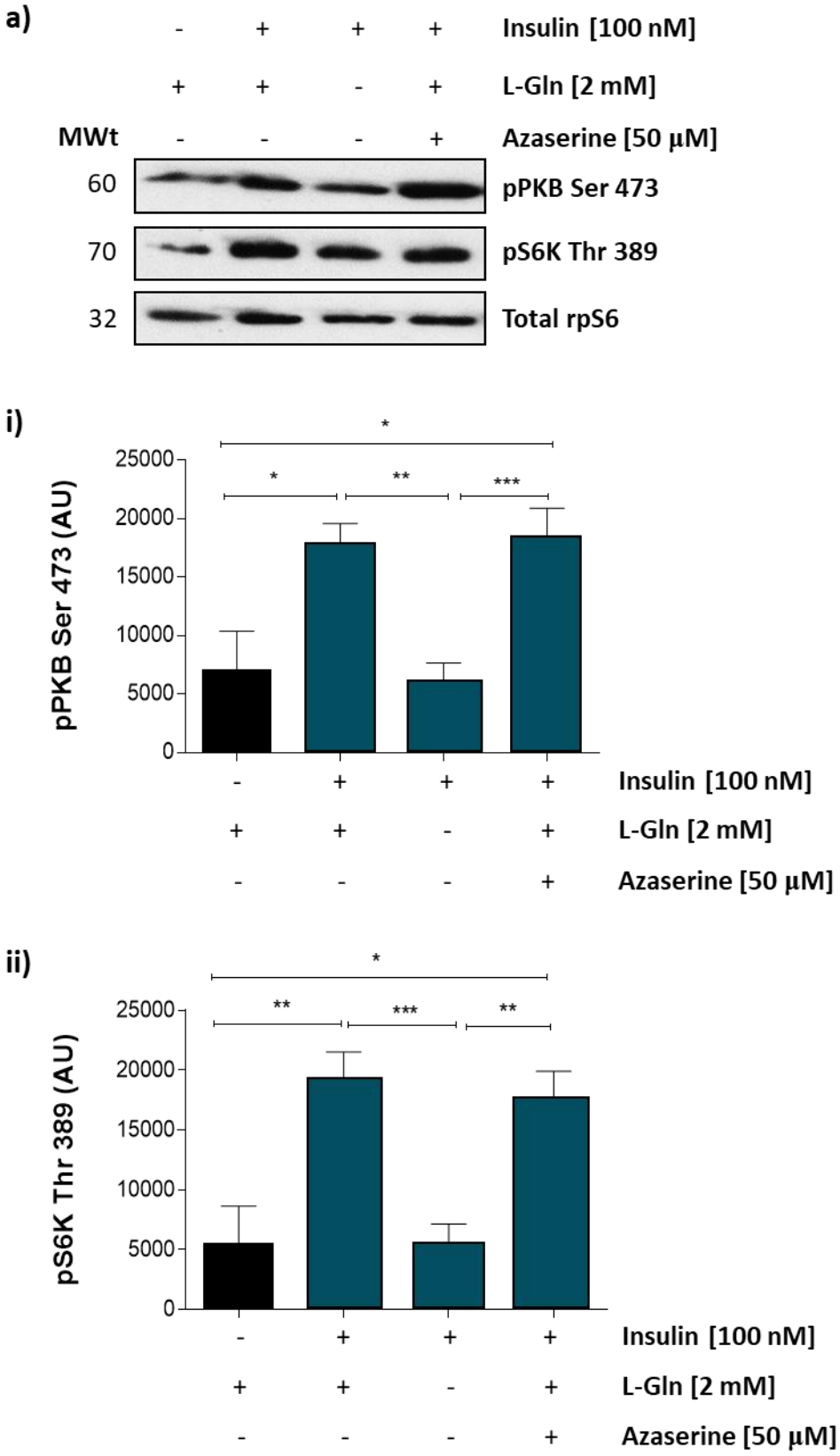


Figure 3.12. Effects of inhibition of glutaminolysis with Azaserine on insulin-mediated mTOR signalling.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media with 2 mM L-glutamine for 4 h as described in Chapter 2. Azaserine (50 µM) was added where indicated. 100 nM insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser 473) and phospho-S6K (pS6K Thr 389). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

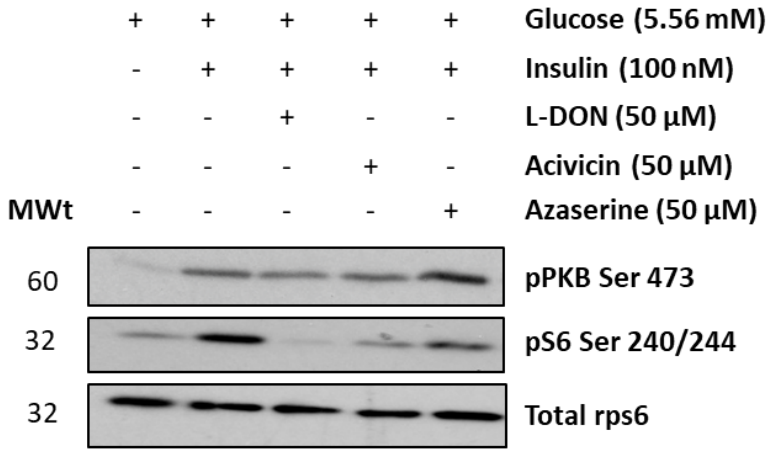
a) Representative blot of changes in phospho-PKB and phospho-S6K in response to insulin.
i) Densitometric analysis of phospho-PKB responses. ii) Densitometric analysis of phospho-S6K responses.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

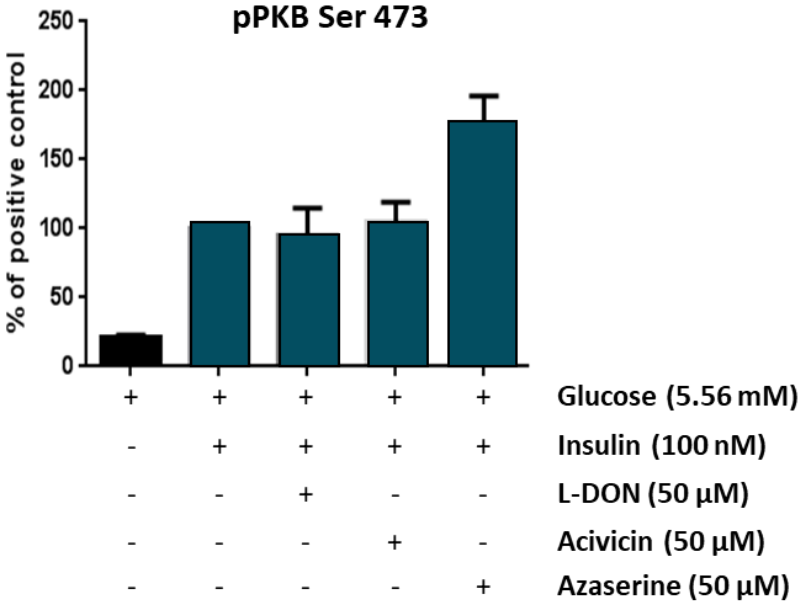
This raised concerns regarding the efficacies of these inhibitors in INS1e cells; concerns that were tackled by altering the experimental conditions. First, it was imperative to show that these inhibitors are working as intended. An experiment conducted by a previous laboratory member studying the hexosamine pathway in CHO-K1 cells (Chinese hamster ovary cells) had used the three inhibitors and shown that they can inhibit signalling to glutamine-dependent pathways. Thus, INS1e cells were serum-starved overnight in CMRL medium, then washed and incubated in DPBS test medium in the presence of 5.56 mM glucose for a period of 90 minutes, with insulin stimulation occurring in the final 30 minutes. As shown in **Figure 3.13**, the addition of L-DON inhibited signalling to mTORC1 (pS6 Ser240/244), but not mTORC2 (pPKB Ser473); acivicin and azaserine did not appear to significantly alter the phosphorylation status of either pS6 Ser240/244 or pPKB Ser473.

Having established the above, and taking into consideration the competitive nature of these inhibitors, I decided to alter the experimental conditions by first pre-incubating the inhibitors (**Figure 3.14**) to allow sufficient time for the inhibitors to bind to the glutamine-utilising enzymes, and secondly by increasing the concentrations of the inhibitors to 500 μM, whilst reducing glutamine concentration down to 1 mM (**Figure 3.15**).

a)


+ + + + + Glucose (5.56 mM)
- + + + + Insulin (100 nM)
- - + - - L-DON (50 µM)
- - - + - Acivicin (50 µM)
MWt - - - - + Azaserine (50 µM)
60 pPKB Ser 473
32 pS6 Ser 240/244
32 Total rps6

i)


pPKB Ser 473
% of positive control
250
200
150
100
50
0
+ + + + + Glucose (5.56 mM)
- + + + + Insulin (100 nM)
- - + - - L-DON (50 µM)
- - - + - Acivicin (50 µM)
- - - - + Azaserine (50 µM)

ii)

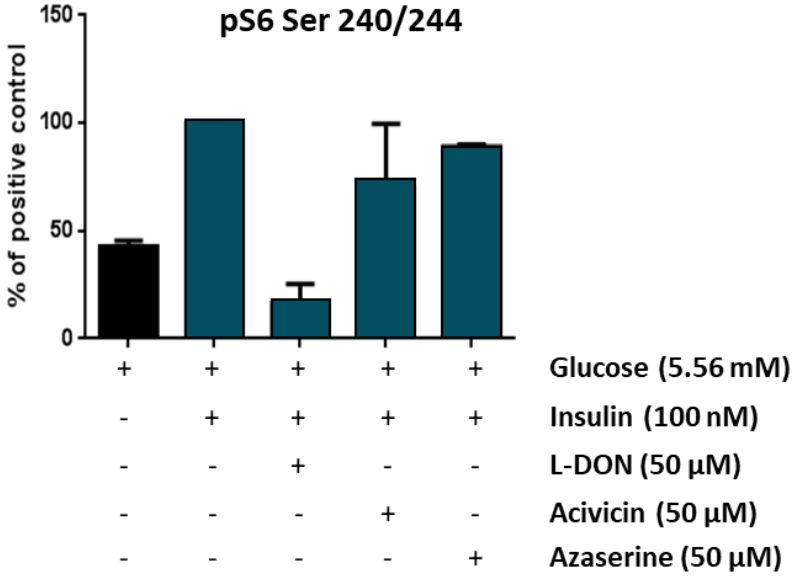

pS6 Ser 240/244
% of positive control
150
100
50
0
+ + + + + Glucose (5.56 mM)
- + + + + Insulin (100 nM)
- - + - - L-DON (50 µM)
- - - + - Acivicin (50 µM)
- - - - + Azaserine (50 µM)

Figure 3.13. Effects of inhibition of glutaminolysis with L-DON, Acivicin and Azaserine on insulin-mediated mTOR signalling in DBPS media.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in DPBS media with 5.56 mM Glucose for 90 min. L-DON, Acivicin and Azaserine (50 µM) were added where indicated. 100 nM insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phosphor-PKB (pPKB Ser 473) and phospho-S6K (pS6K Thr 389). Total rpS6 was used as a loading control.

a) Representative blot of changes in phospho-PKB and phospho-S6K in response to insulin.
i) Densitometric analysis of phospho-PKB responses. **ii)** Densitometric analysis of phospho-S6 responses.

The results are either representative or mean ± S.E.M. of n=1 experiment. AU = arbitrary units.

Figure 3.14 shows us that even when pre-incubated for 30 minutes prior to the addition of 2 mM glutamine for 4 h, the three inhibitors are still unable to significantly alter the phosphorylation status of insulin-stimulated pS6 Ser240/244 and pPKB Ser473 in INS1e cells. As expected, glutamine withdrawal for 4 h significantly inhibited insulin-dependent signalling to mTORC1 and mTORC2, relative to in the presence of 2 mM glutamine; this inhibition was not maintained when 50 µM of L-DON, acivicin and azaserine were pre-incubated for 30 minutes prior to the addition of glutamine.

Increasing the concentrations of L-DON, acivicin and azaserine to 500 µM – whilst also reducing glutamine concentration to 1 mM – did not lead to inhibition of insulin-mediated signalling to mTORC1 and mTORC2, as evident by the phosphorylation levels of pS6 Ser240/244 and pPKB Ser473, relative to that upon 4 hours of glutamine withdrawal (**Figure 3.15**).

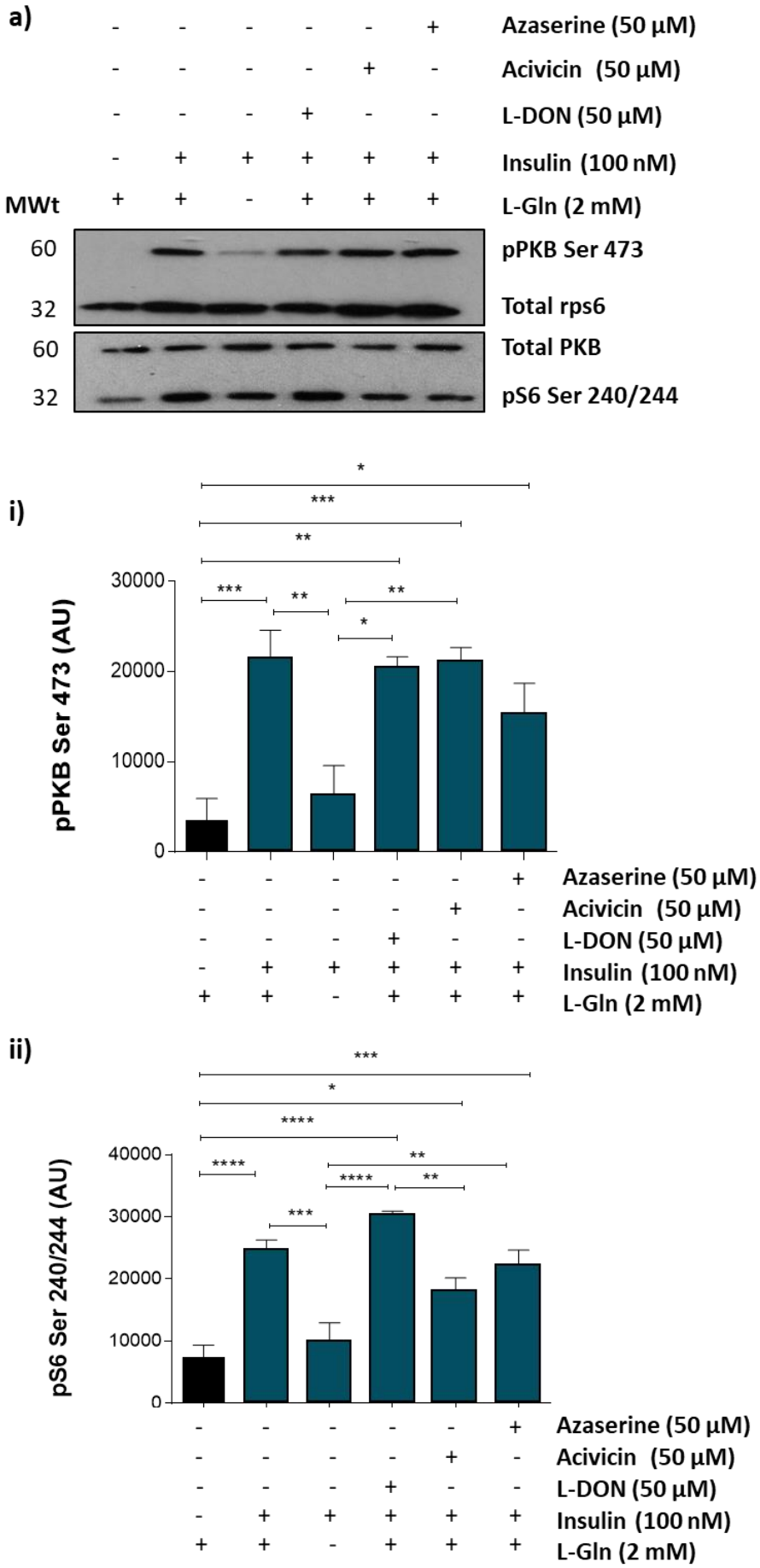

a)
- - - - - + Azaserine (50 µM)
- - - - + - Acivicin (50 µM)
- - - + - - L-DON (50 µM)
- + + + + + Insulin (100 nM)
MWt + + - + + + L-Gln (2 mM)
60
pPKB Ser 473
32
Total rps6
60
Total PKB
32
pS6 Ser 240/244
i)
pPKB Ser 473 (AU)
30000
20000
10000
0
- - - - - + Azaserine (50 µM)
- - - - + - Acivicin (50 µM)
- - - + - - L-DON (50 µM)
- + + + + + Insulin (100 nM)
+ + - + + + L-Gln (2 mM)
ii)
pS6 Ser 240/244 (AU)
40000
30000
20000
10000
0
- - - - - + Azaserine (50 µM)
- - - - + - Acivicin (50 µM)
- - - + - - L-DON (50 µM)
- + + + + + Insulin (100 nM)
+ + - + + + L-Gln (2 mM)

Figure 3.14. Effects of inhibition of glutaminolysis upon preincubation with 50 µM L-DON, Acivicin and Azaserine on insulin-mediated mTOR signalling.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media with 2mM L-glutamine for 4 h as described in Chapter 2. L-Don, Acivicin and Azaserine (50 µM) were added where indicated 30 min prior to addition of 2 mM L-Glutamine. 100 nM insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phosphor-PKB (pPKB Ser 473) and phospho-S6 (pS6 Ser 240/244). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin. **i)** Densitometric analysis of phospho-PKB response. **ii)** Densitometric analysis of phospho-S6 response.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

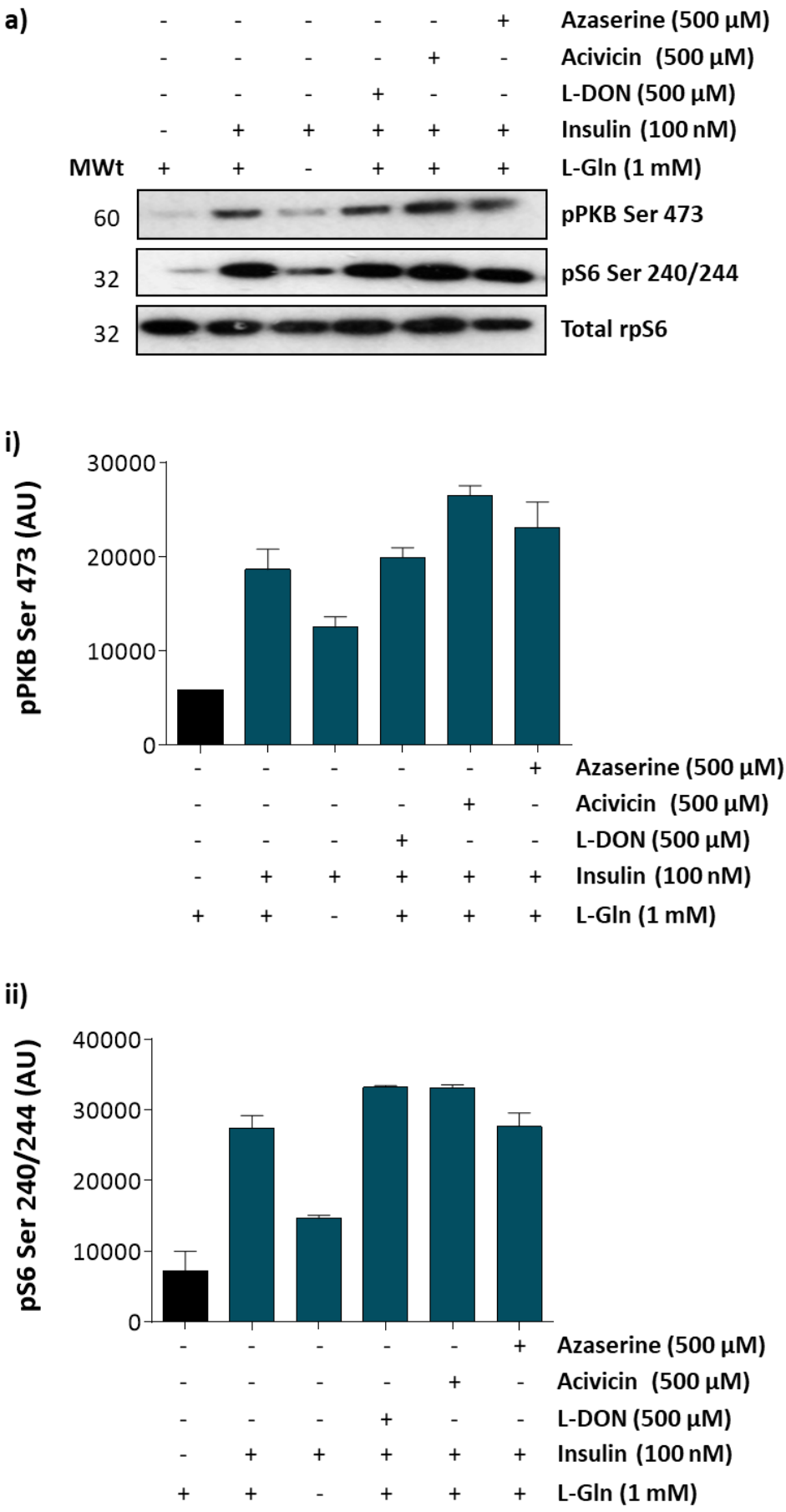

a)
- - - - - + Azaserine (500 µM)
- - - - + - Acivicin (500 µM)
- - - + - - L-DON (500 µM)
- + + + + + Insulin (100 nM)
MWt + + - + + + L-Gln (1 mM)
60
pPKB Ser 473
32
pS6 Ser 240/244
32
Total rpS6
i)
pPKB Ser 473 (AU)
30000
20000
10000
0
- - - - - + Azaserine (500 µM)
- - - - + - Acivicin (500 µM)
- - - + - - L-DON (500 µM)
- + + + + + Insulin (100 nM)
+ + - + + + L-Gln (1 mM)
ii)
pS6 Ser 240/244 (AU)
40000
30000
20000
10000
0
- - - - - + Azaserine (500 µM)
- - - - + - Acivicin (500 µM)
- - - + - - L-DON (500 µM)
- + + + + + Insulin (100 nM)
+ + - + + + L-Gln (1 mM)

Figure 3.15. Effects of glutaminolysis inhibitors L-DON, Acivicin and Azaserine on mTOR signalling.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with or without 1 mM L-glutamine for 4 h as described in Chapter 2, in the presence of 500 µM L-DON, Acivicin or Azaserine as indicated. 100nM insulin was added to all plates except the control (C) for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control.

a) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin.
i) Densitometric analysis of phospho-PKB responses. **ii)** Densitometric analysis of phospho-S6 responses.

The results are either representative or mean ± S.E.M. of n=2 independent experiments. AU= Arbitrary units.

3.3 Discussion

The major aim of this work was to investigate the role that glutamine plays in the regulation of signalling to mTOR; beginning with demonstrating that in INS1e cells, glutamine – in the presence of essential amino acids and glucose – is critical for the activation of signalling to mTORC1, as was shown previously in our laboratory in rat islets of Langerhans (Mustafa and Herbert, 2012). Withdrawal of glutamine for 4 hours surprisingly inhibited signalling to *both* mTORC1 and mTORC2 (**Figure 3.1**), with mTORC1 inhibition occurring at a much earlier time point (30 minutes) than that for mTORC2 inhibition (4 hours) (**Figure 3.1a)**. Although glutamine withdrawal impeding on signalling to mTORC1 was shown previously, the time frame at which it occurs is surprising; more surprisingly is the relatively rapid inhibition of mTORC2.

Glutamine withdrawal, in HeLa cells, had been shown to reduce the phosphorylation levels of mTORC1 markers pS6K Thr389 and pS6 Ser240/244, however this occurs at a much later time point of 24 hours (Durán *et al.*, 2012), whilst here I report that in INS1e cells, said inhibition of mTORC1 signalling occurs very rapidly. On the other hand, the observed inhibition of mTORC2 signalling upon glutamine starvation after 4 hours, was a surprising and interesting observation. Duran *et al.* (2012) did investigate the effects of glutamine and leucine deprivation on phosphorylation levels of pPKB Ser473 in U2OS cells, and reported that signalling to mTORC2 was unaffected after removal of glutamine in the presence of leucine – however, that was investigated upon withdrawal for only 60 minutes.

With intracellular concentrations of glutamine ranging from 2 mM to 20 mM, insulin-dependent activation of mTORC1 and mTORC2 signalling in INS1e cells was observed at concentrations as low as 250 µM (**Figure 3.2***).* Amino acid concentrations of selected amino acids seem to be unchanged with the varying concentrations of intracellular glutamine; that leucine and glutamate concentrations are seemingly unaffected by reduced glutamine concentrations is surprising (**Figure 3.3**). Glutamine concentration gradients are utilised by the leucine transporter LAT1 to transport leucine intracellularly and thus activate mTORC1 signalling, whilst glutamate is generated primarily from glutamine metabolism.

It is possible that minimal glutamine concentrations are sufficient to promote leucine transport. Other groups have reported that, firstly, leucine can almost fully activate mTORC1 signalling in the absence of glutamine – and that the presence of glutamine simply potentiates leucine-mediated activation of mTORC1 (Durán *et al.*, 2012) – and secondly that glutamine is the rate limiting step of mTORC1 activation, with its role in inducing said mTORC1 activation is to maintain sufficient concentrations of leucine and arginine, which together can fully activate mTORC1 signalling (Nicklin *et al.*, 2009b) (Altman, Stine and Dang, 2016). Here I show that the concentrations of leucine, which are sufficient to activate mTORC1 and mTORC2 signalling in the presence of glutamine, are the same concentrations of leucine when mTORC1 and mTORC2 signalling is inhibited (**Figure 3.2** and **Figure 3.3**). Thus, I propose that the role of glutamine in mTOR signalling in INS1e goes beyond that of mediating the transport of leucine

and potentiating leucine-dependent activation of mTORC1 and mTORC2 signalling.

Alongside glutamine's role in regulating mTOR signalling, glutamine also has an important role in regulating the MAPK signalling pathway (Ko *et al.*, 1993) (Rhoads *et al.*, 1997). I had asked how glutamine may regulate MAPK activity in INS1e cells; it had been shown that glutamine uptake and metabolism requires ERK signalling (Carr *et al.*, 2010), whilst another shows that glutamine-stimulated cell proliferation is mediated through an active ERK signalling (DeBerardinis and Cheng, 2010)(Rhoads *et al.*, 2000). On the other hand, glutamine starvation was shown to induce apoptosis in an ERK-dependent manner, and that glutamine may modulate the activity of stress-induced JNK MAPK (Paquette, Guérin and Gauthier, 2005).

In INS1e cells, I report that the glutamine-dependent inhibition of mTORC1 and mTORC2 signalling correlates with an increase in ERK1/2 phosphorylation (**Figure 3.4**), in both insulin- and PMA-stimulated INS1e cells. In fact, this induction in ERK1/2 signalling upon glutamine depletion is seen in both stimulated and unstimulated cells (**Figure 3.4b** and **Figure 3.5**, respectively). Glutamine starvation also induced stress-activated MAPKs – p38 (**Figure 3.6a)** and JNK (**Figure 3.6b**) – and increased the phosphorylation of AMPK, the cells energy sensor, a potent inhibitor of mTORC1 signalling (**Figure 3.7**).

The induction of p38 and JNK activities was shown to correlate with the promotion of cell survival under conditions of stress (Wagner and Nebreda, 2009), with one group reporting that p38 and ERK1/2 together (but not JNK) promote

proliferation in epithelial cells (Sharma, He and Bazan, 2003). Another group showed that prolonged JNK activation results in apoptosis (Ventura *et al.*, 2006).

Another laboratory reported that glutamine starvation in mouse hybridoma cells rapidly induced apoptosis, with p38 activation was detected 2 h after withdrawal of glutamine; this activation occurring after induction of apoptosis-inducing caspase proteins (Harnett *et al.*, 2013). A similar observation was reported in HL-60 cells (human leukaemia/lymphoma cell line), whereby glutamine deprivation resulted in cell shrinkage and loss of cell viability by apoptosis (Fumarola, Zerbini and Guidotti, 2001).

Does glutamine withdrawal in INS1e cells leading to the observed induction of ERK1/2, p38 and JNK signalling correlate with a compensatory mechanism whereby autophagy and cell proliferation is maintained under stressful stimuli, or does it correlate with increasing apoptosis due to mitochondrial dysfunction and oxidative stress? Is P38 and JNK activated ERK-mediated? This possibly could be answered by investigating the MAPKKK (MAP kinase kinase kinase) ASK1 (apoptosis signal-regulating kinase 1), which lies upstream of MKK3 and MKK4 – activators of p38 and JNK respectively. Another question to ask is whether there is activation of caspases (cysteine-aspartate proteases), which accelerates cell death (Shi, 2002).

Activation of AMPK signalling to TSC1/2 and therefore the inhibition of mTORC1 signalling, can arise from increased oxidative stress (Auciello *et al.*, 2014), and an activated AMPK acts to protect cells from oxidative stress-induced senescence (Han *et al.*, 2016). As glutamine is a precursor of the antioxidant

glutathione (Amores-Sánchez and Medina, 1999), it may be no surprise that glutamine deprivation in INS1e induces pAMPK Thr172 phosphorylation to protect from oxidative stress damage. It would have been interesting to investigate how the inhibition of AMPK (via its inhibitor, Compound C), in the absence of L-glutamine, could alter signalling to mTOR. Conversely, how activation of AMPK – in the presence of L-glutamine – by its activator AICAR (5-aminoimidazole-4-carboxamide ribonucleotide) could impact signalling to mTOR in INS1e cells. An induction of AMPK activation seen under glutamine withdrawal, may consequently lead to an increase in autophagy via AMPK-mTOR-Ulk1/2 (unc-5 1-like kinase 1/2) signalling, a major component of the autophagy-regulating signalling network (Alers *et al.*, 2012).

Thus, the activation of P38 and JNK – which act to control the balance between autophagy and apoptosis – may indicate a flux from autophagy to apoptosis (Qiang *et al.*, 2013) (Sui *et al.*, 2014). A time point investigation of the regulation of p38, JNK and AMPK during the 4 h of glutamine withdrawal may help in identifying if their activation arises from glutamine starvation for 4 h, or as a consequence of mTORC1 inhibition after 30 minutes of glutamine withdrawal.

The effects of amino acids on autophagy are mediated through mTORC1 (Blommaart *et al.*, 1995), and glutamine-mediated autophagy was reported by Nicklin *et al.*, where it was shown that glutamine is critical for suppressing autophagy in RT112 (human bladder) cells that exhibit glutamine-dependent activation of mTORC1 (Nicklin *et al.*, 2009b). This role that glutamine plays in regulating autophagy via mTORC1 signalling, was attributed to glutamine

metabolism through glutaminolysis (Villar *et al.*, 2015); the activation of mTORC1 by glutaminolysis, first shown by Duran *et al.* (2012), was shown to consequently inhibit autophagy. mTORC2 signalling may also regulate autophagy; modulation of glutamine metabolism by the PI3K-PKB-FOXO pathway, acts to increase cellular survival via regulating autophagy (van der Vos and Coffer, 2012). Glutaminolysis' role in regulating autophagy is further illustrated by the report that a by-product of glutaminolysis – ammonia – acts to stimulate autophagy (Eng and Abraham, 2010).

In a series of experiments, Duran *et al.* in 2012 has shown that in the presence of both leucine and glutamine, glutaminolysis is induced and consequently activates Rag-mTORC1 signalling; upregulation of glutaminolysis was shown to enhance mTORC1's activation and its localisation to the lysosome, as well as promoting GTP-loading of $Rag_{A/B}$ (Durán *et al.*, 2012). Here I report that in INS1e cells, in the absence of glutamine, the metabolic components of glutaminolysis – glutamate and αKG – were able to restore signalling to *both* mTORC1 and mTORC2 (**Figure 3.8a, i)** and **ii)**, and **Figure 3.9**), which also correlates with the reversal of AMPK activation upon glutamine withdrawal (**Figure 3.8a, iii).**

However, whilst Duran *et al.* had shown that inhibition of glutaminolysis with L-DON prevented the activation of mTORC1 in the presence of leucine and glutamine in U2OS cells, my findings regarding the effects of L-DON, Acivicin and Azaserine on glutaminolysis in INS1e cells, contradicted theirs. I report that L-DON (**Figure 3.10**), Acivicin (**Figure 3.11**) and Azaserine (**Figure 3.12**) does not inhibit signalling to mTORC1 and mTORC2. Pre-incubation of the inhibitors

(**Figure 3.14**), and altering the concentrations of the inhibitors as well as glutamine's (**Figure 3.15**), did not inhibit signalling to mTORC1. Whilst L-DON and Azaserine have the strongest effects on glutaminolysis, Acivicin is reported to have a minimal effect on glutaminolysis (Wise and Thompson, 2010).

It is a possibility that in INS1e cells, the actions of each inhibitor alone may not be sufficient to completely interrupt the generation of glutamate and αKG; concerns that may be addressed in multiple ways. Firstly, utilising combinations of inhibitors may result in an impact on glutamine-dependent signalling to mTORC1, influencing the different glutamine-metabolising pathways, such as the glutathione-producing λ-glutamyl pathway.

Secondly, usage of RNA interference (RNAi) against the glutamine-metabolising enzymes glutaminase (GLS) and glutamate dehydrogenase (GDH) may be a more direct method in implicating the process of glutaminolysis itself in modulating the activity of mTORC1 signalling in INS1e. On the other hand, enzymatic assays investigating the function of GLS and GDH in the presence of glutamine and inhibitors of its metabolism, can offer insight into how the inhibitors are influencing the activity of the glutamine-metabolising enzymes.

Finally, an α-ketoglutarate assay, in combination with HPLC analysis of intracellular concentrations of glutamate, can allow for derivation of a glutamate:αKG ratio; a high ratio of glutamate to α-KG would indicate that glutaminase activity is not impeded by the inhibitors. Similarly, a low ratio of glutamate to αKG may indicate that glutaminase is being inhibited, however αKG

is still sufficiently generated – possibly by transaminases – to mediate the activation of mTORC1 and mTORC2 pathway.

A lack of inhibition of mTORC1 signalling because of the inhibition of glutaminolysis may simply be due to the fact that in INS1e cells, the process of glutaminolysis – whilst able to activate signalling to both mTORC1 and mTORC2 in the absence of glutamine – is not critical for mTOR regulation.

3.3.1 Conclusions

- In INS1e cells, glutamine is critical for the activation of mTOR signalling.
    - Glutamine withdrawal inhibits signalling to both mTORC1 and mTORC2, and maximal activation may be achieved with glutamine concentrations as low as 250 μM.
- Glutamine withdrawal activates ERK1/2 signalling when mTORC1 and mTORC2 signalling is inhibited.
    - AMPK activation in response to glutamine starvation acts to inhibit mTORC1 and induce autophagy to maintain cell survival
    - Stress-induced, glutamine-dependent, p38 and JNK activation occurs.
- The role of glutaminolysis in mTORC1 and mTORC2 regulation implicates an important role for its metabolites glutamate and αKG in modulating signalling to mTOR
    - Inhibition of glutaminolysis in ISN1e does not impede on signalling to mTOR.

Chapter 4. Glutamine transporters

4.1 Introduction

Amino acid transporters have a vital role in mediating amino acid-dependent activation of signalling to mTORC1; their ability to regulate intracellular concentrations of amino acids, required for growth factor-induced mTORC1 activation, has produced a plethora of studies investigating the intricacies of their functions in different cell types. Two main findings of interest to this thesis involve, first, the coupling of the glutamine transporters SNAT2 (SLC38A2) or ASC2 (SLC1A5), with the leucine transporter LAT1 (SLC7A5) to mediate leucine-dependent activation of mTORC1 signalling (Nicklin *et al.*, 2009b); and secondly, the discovery that the lysosomal transporter SNAT9 (SLC38A9) mediates the lysosomal-sensing mechanism of critical amino acids that regulates Ragulator-$Rag_{A/B}$ activation of mTORC1 (Saxton and Sabatini, 2017).

As a conditionally essential amino acid, due to the many significant roles it plays, it is critical that sufficient levels of glutamine are maintained for the cell to function. Whilst the functional roles of glutamine – such as the signalling and metabolic pathways it is involved in – are an important facet in both cell proliferation and disease states, transport and sensing of glutamine constitutes another important facet. The elegant experiments conducted by Nicklin *et al.* (2009) showed that intracellular glutamine synthesis and its consequent efflux at the expense of leucine transport, at least in HeLa and MCF7 (a breast cancer cell line) cells, was the rate-limiting step in the integration of essential amino acid and

growth factor signalling to mTORC1 (Nicklin *et al.*, 2009b). Whilst these findings implicated the ASC2 (SLC1A5) transporter, other reports show such findings are also observed with transporters from the SLC38 family, specifically the System A transporter SNAT2 (SLC38A2) in L6 muscle cells (Evans *et al.*, 2007) and the System N transporters (SLC38A3 and SLC38A5) in the liver, brain and kidney (Chan *et al.*, 2016a). Most importantly, previous work in our laboratory conducted on pancreatic rat islets of Langerhans had shown that the inhibition of glutamine transport, specifically through SNAT2 (SLC38A2), antagonises signalling to mTORC1.

The glutamine-mediated modulation of both mTORC1 and mTORC2 in INS1e reported in Chapter 3 – how glutamine was shown to regulate mTOR-, MAPK- and stress-pathway signalling, as well as how the metabolites of glutamine implicated glutamine metabolism in activating mTOR signalling – raised the curiosity of how transport of glutamine may, or may not, mediate any of these effects. Amino acid signalling, a vital role by which mTOR signalling depends on, is primarily due to mechanisms of amino acid sensing in the cell. The most abundant glutamine transporter, SNAT2, is reported to have both an intrinsic transporting ability, coupled with a receptor function – whereby it can sense extracellular amino acid levels and signal accordingly.

4.1.1 Aims

To identify in INS1e cells which transporters mediate the transport of glutamine; and how regulation of glutamine transport influences mTORC1 activity.

4.2 Results

4.2.1 SNAT2 and SNAT3 may be the main transporters of glutamine in INS1e cells

The initial aim of this investigation was to elucidate the main mode of transport for glutamine in INS1e cells. To that end, three transporters belonging to the SLC38 and SLC1 families were studied, by using their preferred substrates – alongside glutamine – to compete with glutamine transport. The most abundant transporter, SNAT2 (SLC38A2), was studied by utilising its substrate and inhibitor MeAIB (α-metyhlaminoisobutyric acid). SNAT3 (SLC38A3), a member of system N transporters, transports the amino acid histidine alongside glutamine, whilst ASC2 (SLC1A5), of the system ASC transporters, transports threonine alongside glutamine. As such, by utilising the three substrates, I assayed the glutamine uptake dynamics of these transporters in INS1e cells.

^{3}H-labelled glutamine alongside unlabelled glutamine – used to provide a threshold to stimulate transport – were used in the presence of each of the three substrates MeAIB, histidine and threonine, or combinations thereof, in a transport assay. The utilisation of unlabelled glutamine was an important consideration of the experimental design, an insufficient amount prevents the activation of the transports, and therefore, inhibition of glutamine transport across the cell membrane. Thus, a starting concentration of 10 µM was used for 5 min in the initial assay. A different time-point, along with increasing concentrations of unlabelled glutamine, were used for optimisation purposes thereafter. Serum-

starved INS1e cells were incubated in EBSS test media and the transport assay was carried out as described in Chapter 2, Section 2.7.

Co-incubation of ^{3}H-glutamine and 10 μM unlabelled glutamine with saturating concentrations (10 mM) of MeAIB, histidine and/or threonine for a duration of 5 minutes, resulted in a highly significant reduction of ^{3}H-glutamine transport by SNAT2, SNAT3 and ASC2 (**Figure 4.1**), relative to control conditions. 10 mM of MeAIB (M) significantly reduced ^{3}H-glutamine transport (**Figure 4.1**, **lane 2**), a reduction which was mimicked when 10 mM MeAIB was coincubated with 10 mM threonine (T) (**Figure 4.1, lane 5**) or 10 mM histidine (H) (**Figure 4.1, lane 6**), or with both 10 mM of threonine and histidine (**Figure 4.1, lane 8**). It appears that ^{3}H-glutamine transport by ASC2 was least affected when incubated with threonine only (**Figure 4.1, lane 3**), however co-incubation with SNAT3 substrate histidine furthered the reduction of ^{3}H-glutamine transport (**Figure 4.1, lane 7**).

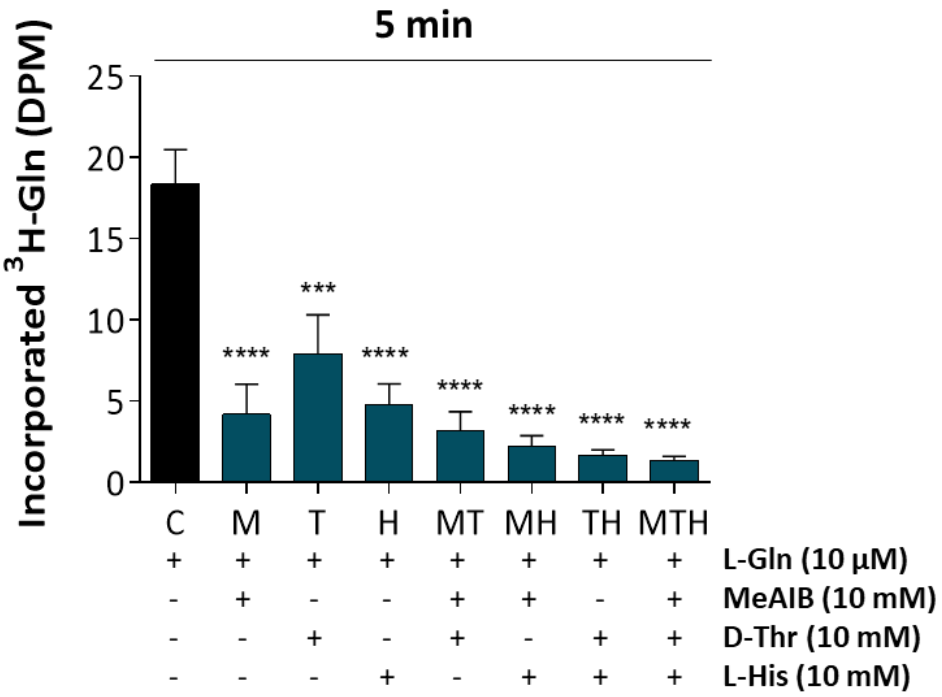


Figure 4.1. ^{3}H Glutamine transport assay upon inhibition of L-glutamine transporters by MeAIB, D-Threonine and L-Histidine, in the presence of 10 µM unlabelled L-glutamine for 5 min.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then treated in EBSS as described in Chapter 2. 10 mM of MeAIB **(M)**, D-Threonine **(T)** and/or L-Histidine **(H)** were added where indicated. Control (**C**) indicates incubation with only ^{3}H-glutamine and L-glutamine.

The results are ± S.E.M of n>3 independent experiments; * p<0.05, ** p<0.01 and *** p<0.001 calculated by Bonferroni's test following one-way ANOVA, relative to Control (C).

The apparent reduction of ^{3}H-glutamine uptake across all three transporters investigated raised a question regarding the sensitivity of the assay. Does the observed reduction in intracellular ^{3}H-glutamine correlate with an inhibition of its uptake, or is it that the time period of 5 minutes, as well as the low concentration of unlabelled glutamine (10 µM), are insufficient – or not optimal – to properly assay uptake of ^{3}H-glutamine? To explore these issues, the experiment was repeated with 250 µM and 500 µM of unlabelled glutamine, and the incubation time extended to 20 minutes.

Increasing unlabelled glutamine concentrations and incubation time led to a significant increase in the intracellular ^{3}H-Glutamine levels detected. Similarly, to what was observed in **Figure 4.1**, the presence of MeAIB or histidine, alone or in combination, led to a strong reduction of ^{3}H-glutamine uptake (**Figure 4.2** and **Figure 4.3**. **lanes 2, 4 & 6**). Interestingly, the presence of threonine alone did not result in significant reduction of ^{3}H-glutamine uptake, as opposed to what was seen previously (**Figure 4.2** and **Figure 4.3**, **lane 3 vs Figure 4.1**). The only statistically significant inhibition of ^{3}H-glutamine uptake in the presence of 250 µM unlabelled glutamine was observed in those conditions whereby both MeAIB and histidine were present (**Figure 4.2 lanes 6 & 8**)**.** With higher concentrations of unlabelled glutamine (500 µM), significant reductions of ^{3}H-glutamine uptake were seen in the presence of MeAIB (**Figure 4.3, lane 2**) or histidine (**Figure 4.3, lane 4**), or when both are present (**Figure 4.3, lanes 6 & 7**).

As mentioned above, increasing unlabelled glutamine concentrations from 10 µM to 250 µM and 500 µM, resulted in a general increase in ^{3}H-glutamine

incorporation; interestingly, by using 500 µM of unlabelled glutamine, ^{3}H-glutamine incorporation appeared to be approximately half of that seen under experimental conditions utilising 250 µM.

The ^{3}H-glutamine uptake assays shown above indicate that, in INS1e cells, glutamine transport appears to be mediated through SLC38A2 and SLC38A3 transporters (SNAT2 and SNAT3, respectively). It is possible that when the uptake of ^{3}H-glutamine was measured with a time period of 5 minutes and in the presence of 10 µM (**Figure 4.1**), the inhibition of ^{3}H-glutamine transport observed was due to the experimental conditions chosen; once extended to 20 minutes, and the concentrations of unlabelled glutamine increased 25-fold (**Figure 4.2**) and 50-fold (**Figure 4.3**), higher levels of ^{3}H-glutamine were detected. In the later experimental conditions, threonine competition with ^{3}H-glutamine was reduced, thus not significantly affecting glutamine transport; on the other hand, MeAIB and histidine competition with ^{3}H-glutamine resulted in its' reduced uptake, contributing to glutamine transport's inhibition.

These results suggest that SNAT2 and SNAT3 transporters, but not the ASC2 transporter, are the routes whereby glutamine is preferentially transported in INS1e cells. Therefore, my next aim was to investigate the functional impact the inhibition of glutamine transport and how inhibition by these transporters may affect insulin-dependent signalling to mTORC1, and mTORC2.

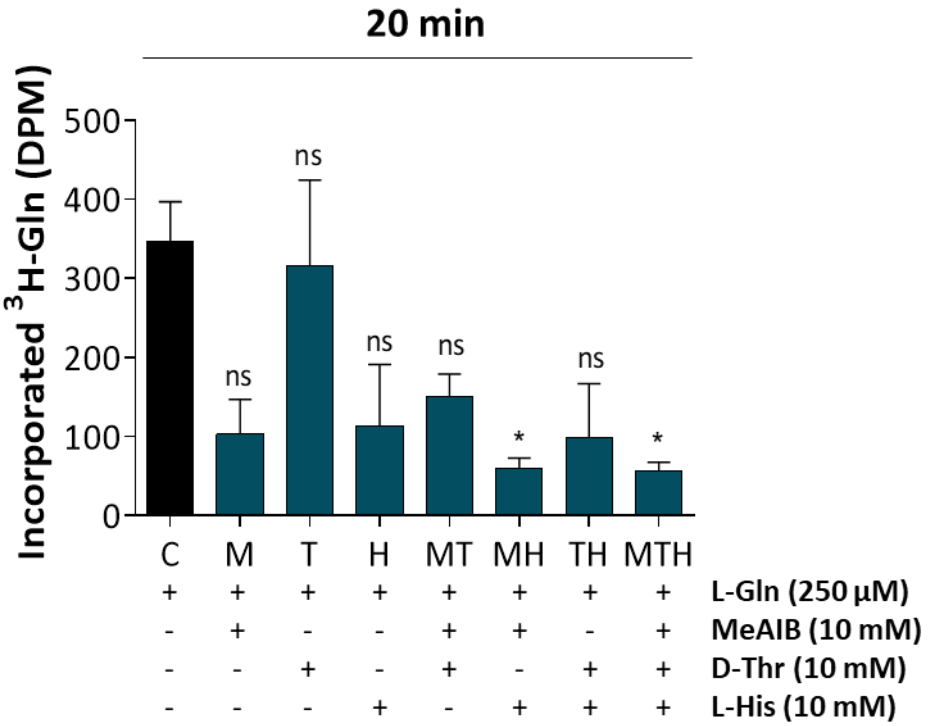


Figure 4.2. ^{3}H Glutamine transport assay upon inhibition of L-glutamine transporters by MeAIB, D-Threonine and L-Histidine, in the presence of 250 µM L-glutamine for 20 min.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then treated in EBSS as described in Chapter 2. 10 mM of MeAIB **(M)**, D-Threonine **(T)** and/or L-Histidine **(H)** were added where indicated. Control (**C**) indicates incubation with only ^{3}H-glutamine and L-glutamine.

The results are ± S.E.M of n>3 independent experiments; * $p<0.05$, ns – non-significant, calculated by Bonferroni's test following one-way ANOVA, relative to Control (**C**).

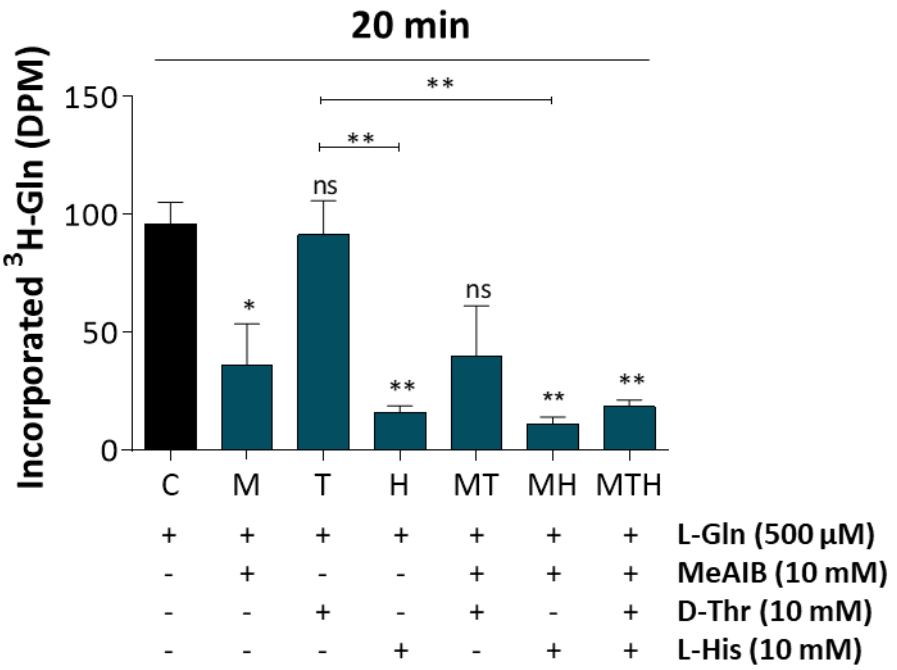


Figure 4.3. ^{3}H Glutamine transport assay upon inhibition of L-glutamine transporters by MeAIB, D-Threonine and L-Histidine, in the presence of 500 µM L-glutamine for 20 min.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then treated in EBSS as described in Chapter 2. 10 mM of MeAIB **(M)**, D-Threonine **(T)** and/or L-Histidine **(H)** were added where indicated. Control (C) indicates incubation with only ^{3}H-glutamine and L-glutamine.

The results are ± S.E.M of n>3 independent experiments; * p<0.05, ** p<0.01 and ns – non-significant, calculated by Bonferroni's test following one-way ANOVA, relative to Control (**C**).

4.2.2 Inhibition of glutamine transport does not inhibit signalling to mTOR in INS1e cells

To investigate how inhibition of glutamine transport affects mTOR signalling, serum-starved INS1e cells were incubated in EBSS test medium, in the presence or absence of 2 mM glutamine, with 10 mM of either MeAIB, histidine and threonine. Activation of mTOR pathway was instigated by the addition of insulin. Protein profiles of key components of the mTOR pathway were then investigated by western blot, in an equivalent manner to that used in the previous Chapter.

As seen in **Figure 4.4,**

Figure 4.5 and **Figure 4.6,** insulin-mediated activation of both mTORC1 and mTORC2 was maximal in the presence of 2 mM glutamine, evident by the phosphorylation levels of PKB (pPKB Ser473) and ribosomal protein S6 (pS6 Ser240/244). mTOR activation was abolished upon withdrawal of glutamine, corroborating the findings of the previous chapter. However, no significant change was observed in the phosphorylation status of either PKB or S6 upon incubation with saturating concentrations of the competitive inhibitors MeAIB (**Figure 4.4**), threonine (

Figure 4.5) and histidine (**Figure 4.6**).

When selected amino acid profiles were investigated (**Figure 4.7**), concentrations of glutamate, leucine, aspartate, lysine and alanine were

unchanged when glutamine was removed, and where glutamine was present alongside MeAIB, threonine or histidine. Concentrations of threonine and histidine were noticeably increased where they were used as competitive inhibitors of ASC2 or SNAT3 transporters, respectively.

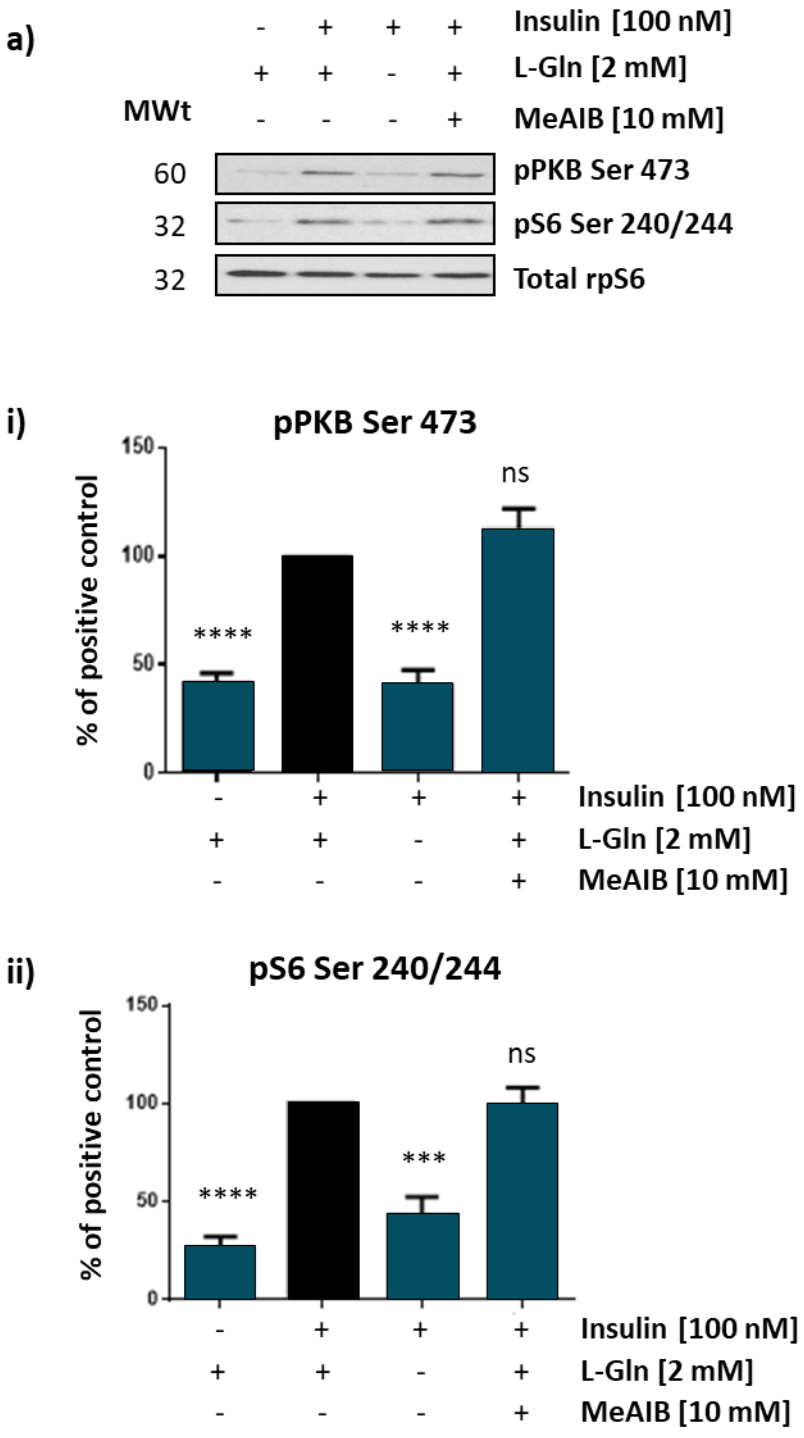


Figure 4.4. Effects of inhibition of System A transporter on mTOR signalling.

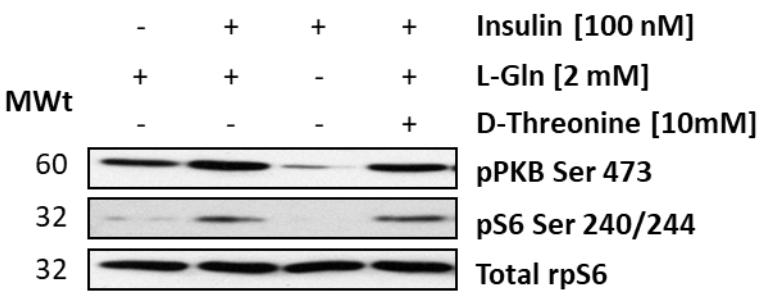
Confluent INS1e cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then incubated in EBSS media with or without 2 mM L-glutamine and 10 mM MeAIB for 4 h as described in

Chapter 2. Insulin (100 nM) was added to all plates except the control in the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

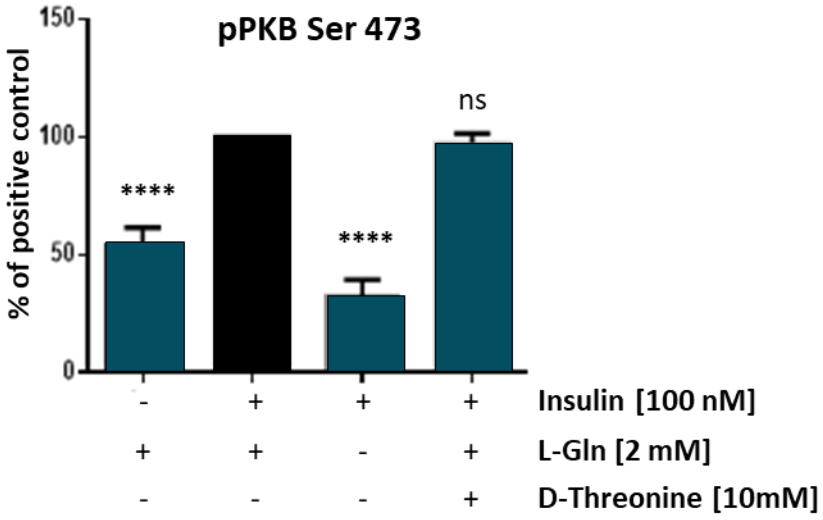
a) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin **i)** Densitometric analysis of phospho-PKB responses. **ii)** Densitometric analysis of phospho-S6 responses.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$, ns – non-significant, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

a)


MWt
60
32
32
- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
- - - + D-Threonine [10mM]
pPKB Ser 473
pS6 Ser 240/244
Total rpS6

i)


pPKB Ser 473
% of positive control
150
100
50
0
****
****
ns
- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
- - - + D-Threonine [10mM]

ii)

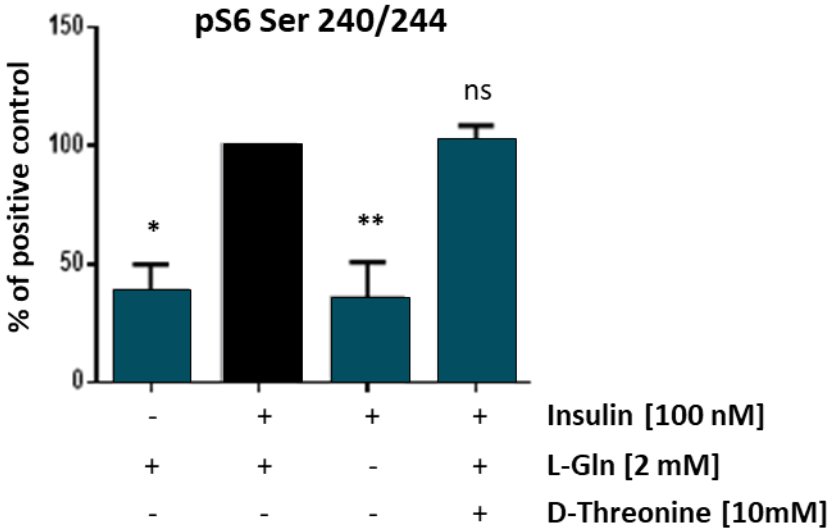

pS6 Ser 240/244
% of positive control
150
100
50
0
*
**
ns
- + + + Insulin [100 nM]
+ + - + L-Gln [2 mM]
- - - + D-Threonine [10mM]

Figure 4.5. Effects of inhibition of System ASC transporter on mTOR signalling.

Confluent INS1e cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then incubated in EBSS media with or without 2 mM L-glutamine and 10 mM D-threonine for 4 h as described in Chapter 2. Insulin (100 nM) was added to all plates except the control in the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin i) Densitometric analysis of phospho-PKB responses. ii) Densitometric analysis of phospho-S6 responses.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$, ns – non-significant, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

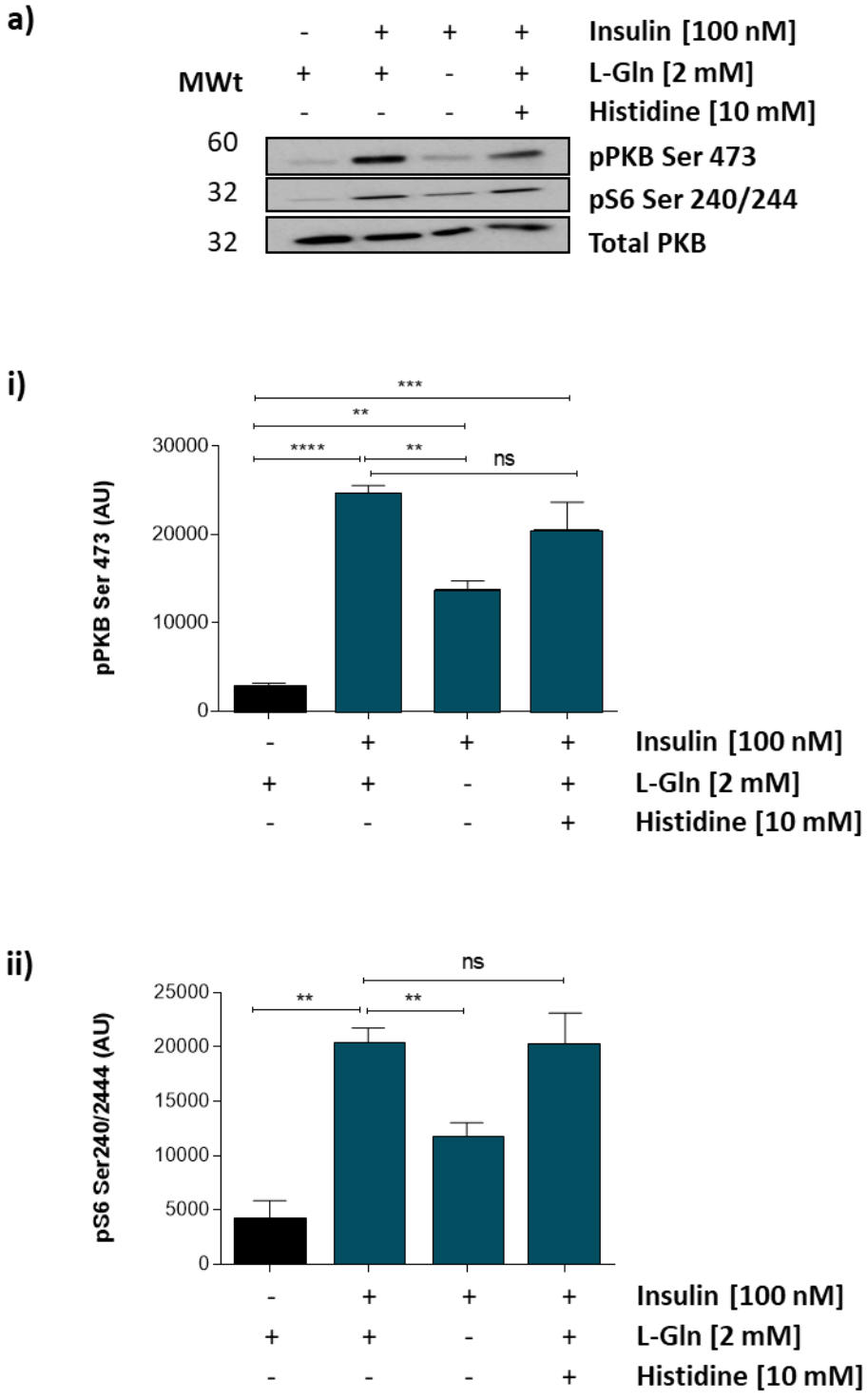


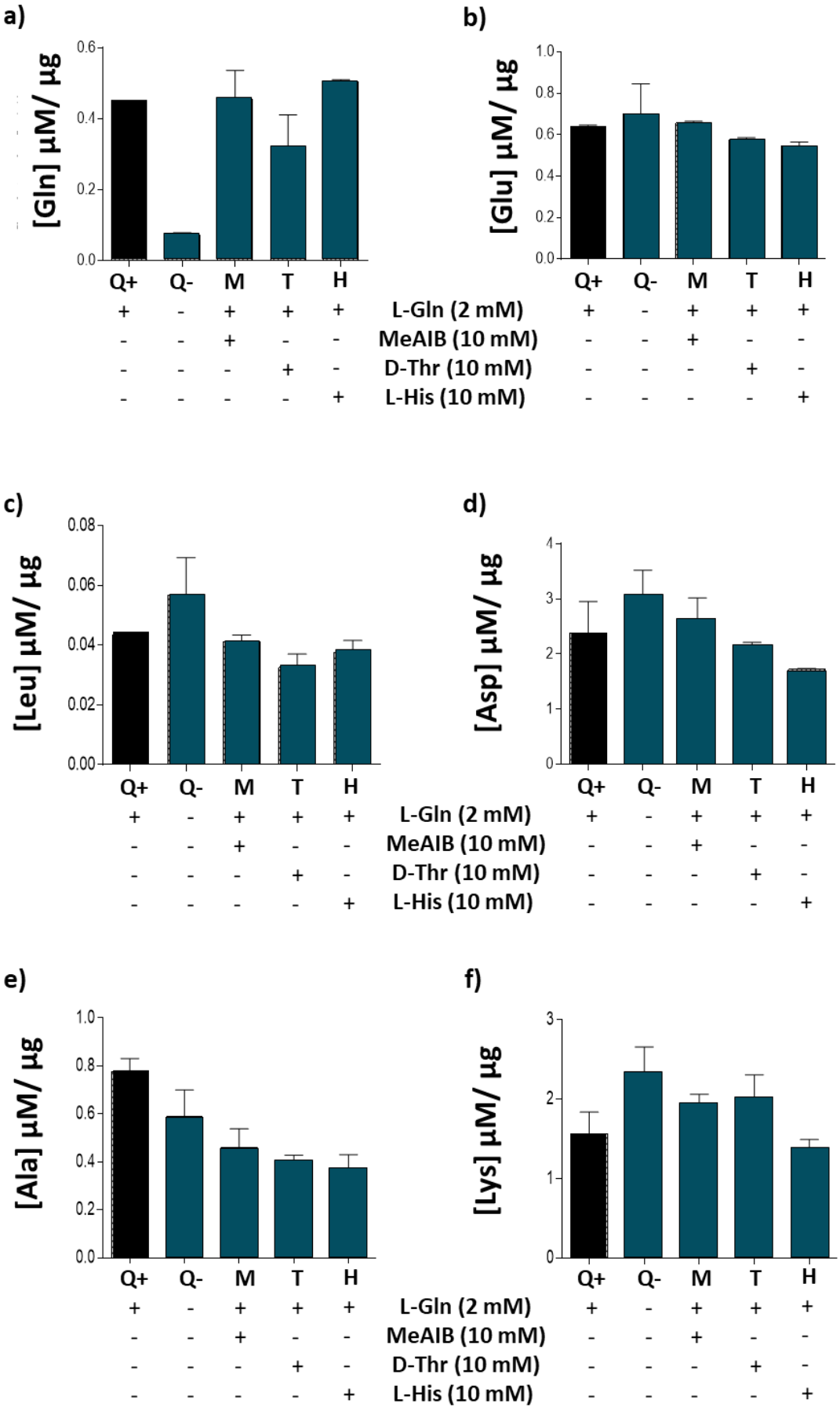
Figure 4.6. Effects of inhibition of System N transporter on mTOR signalling.

Confluent INS1e cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then incubated in EBSS media with or without 2 mM L-glutamine and 10 mM Histidine for 4 h as described in Chapter 2. Insulin (100 nM) was added to all plates except the control in the final 30 min

of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin i) Densitometric analysis of phospho-PKB responses. ii) Densitometric analysis of phospho-S6 responses.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and **** $p<0.0001$, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.


a)
[Gln] µM/ µg
0.6
0.4
0.2
0.0
b)
[Glu] µM/ µg
1.0
0.8
0.6
0.4
0.2
0.0
c)
[Leu] µM/ µg
0.08
0.06
0.04
0.02
0.00
d)
[Asp] µM/ µg
4
3
2
1
0
e)
[Ala] µM/ µg
1.0
0.8
0.6
0.4
0.2
0.0
f)
[Lys] µM/ µg
3
2
1
0
Q+ Q- M T H
L-Gln (2 mM) + - + + +
MeAIB (10 mM) - - + - -
D-Thr (10 mM) - - - + -
L-His (10 mM) - - - - +

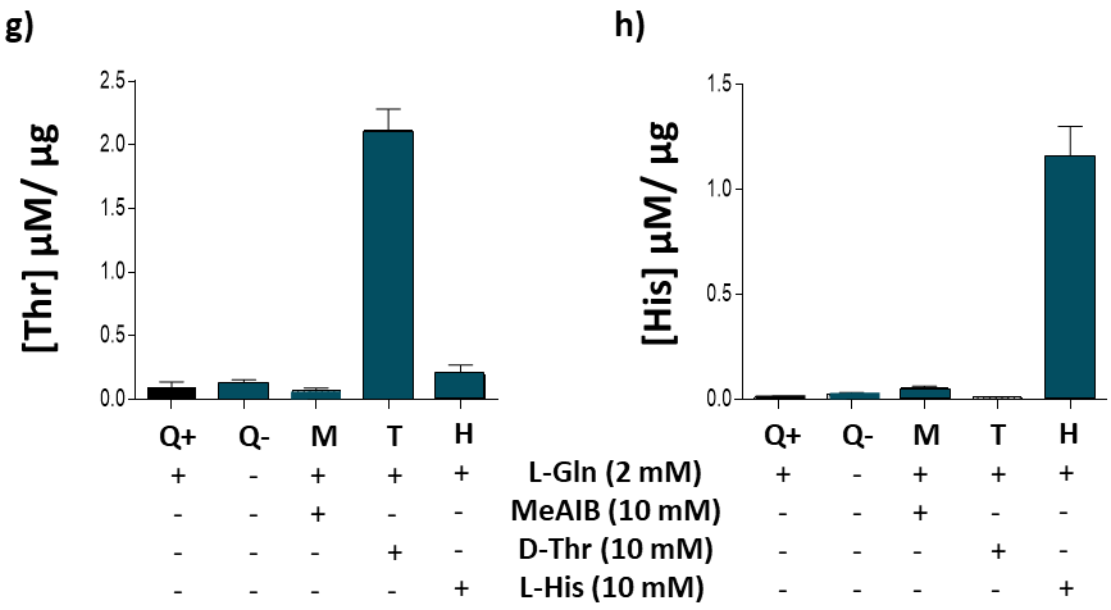


Figure 4.7. HPLC amino acid profile of INS1-E cells upon inhibition of L-glutamine transport.

Confluent INS1E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine. Cells were then washed and incubated in EBSS media with or without 2 mM glutamine, MeAIB, Threonine or Histidine. Cell monolayers were then solubilised for HPLC analysis as described in **Chapter 2**. Selected amino acid profiles were analysed: **a)** Glutamine; **b)** Glutamate; **c)** Leucine; **d)** Aspartate, **e)** Alanine, **f)** Lysine, **g)** Threonine and **h)** Histidine.

The results are mean + S.E.M. of n=2 independent experiments. Error bars represent S.E.M. AU = arbitrary units.

4.3 Discussion

As an obligate requirement for activation of mTORC1 signalling, amino acids regulate mTORC1 directly through a biochemical pathway that was only recently understood mechanistically (Saxton and Sabatini, 2017). Structural and functional studies of amino acid transporters had allowed the identification of several tissue-specific transporters that mediated glutamine- (and thus leucine-dependent) activation of mTORC1 signalling. SNAT2, the most abundantly expressed glutamine transporter belonging to the SLC38 family of transporters, is one glutamine transporter that has been heavily studied, with a plethora of functions depending on its localisation (Bröer, 2014a). As a mediator of leucine-dependent activation of mTORC1 signalling, glutamine was shown to exert such mediation through the coupling of the SNAT2 transporter with the LAT1 transporter (Nicklin *et al.*, 2009b).

One study on SNAT2 conducted in our laboratory had reported that SNAT2 inhibition in rat islet of Langerhans – achieved by either MeAIB competitive inhibition or by SNAT2 silencing with siRNA – had consequently inhibited signalling to mTORC1. Another study in L6 muscle cells, illustrated that disruption of SNAT2 function – either by competitive inhibition with MeAIB, by inducing a low pH environment or by using RNAi against SNAT2 – resulted in diminished intracellular glutamine levels, including the levels of other amino acids dependent on glutamine, which consequently lead to an abrogated mTORC1 signalling (Evans *et al.*, 2007).

System N transporters, SLC38A3 and SLC38A5 (SN1; SN2), are other SLC38 transporter members – primarily transporting glutamine and histidine – that are heavily studied. Expressed in the brain (Umapathy *et al.*, 2008) and pancreas (Gammelsaeter *et al.*, 2009) – amongst many other tissues – system N transporters function alongside SNAT2 transporters in providing sufficient intracellular glutamine levels to allow for bidirectional transport and thus glutamine-dependent activation of mTORC1 signalling. Loss of function of SNAT3 was recently reported to have a major impact on tissues expressing it; reduced levels of glutamine, glutamate and leucine were observed, that correlated with reduced signalling to mTOR (Chan *et al.*, 2016b).

ASC2 of the SLC1 family of transporters is another glutamine transporter that has been studied extensively. Verrey *et al.* (2003) first proposed a relationship between the glutamine transporter ASC2 and the leucine transporter LAT1 (Verrey, 2003); since then, many studies investigated the relationship between system L transporters and ASC2. One group reported that leucine may upregulate expression of ASC2 transporters through an mTOR- and ERK-dependent mechanism in an epithelial cell line (Zhang *et al.*, 2014), illustrating the important link between both transporters in activating mTORC1 signalling.

In this Chapter, I investigated the transport of glutamine through these three transporters. I have shown that upon competitive inhibition with MeAIB and histidine, ^{3}H-glutamine uptake in INS1e cells is significantly impeded when transported through SNAT2 and SNAT3, but not ASC2 (**Figure 4.1**, **Figure 4.2** and **Figure 4.3**). This suggests that glutamine is transported primarily through SNAT2

and SNAT3. These findings corroborate the work of Gammelsaeter *et al.,* who had reported that in β-cells, glutamine is transported through SNAT2 and SNAT3 (Gammelsaeter *et al.*, 2009). However, inhibition of those transporters has also shown that there is no functional impact on signalling to mTOR (**Figure 4.4**, ***Figure 4.5***, **Figure 4.6**)**.** This observation raised the question of compensatory transport mechanisms in INS1e cells; upon inhibition of one mode of transport, there is a possible upregulation of and increased transport through other transporters. As seen in the ^{3}H-glutamine transport assays, combinations of inhibitors elicited a stronger reduction in uptake of ^{3}H-glutamine; thus, the functional studies could have been furthered with experiments utilising combinations of inhibitors, alongside lower concentrations of glutamine.

As shown previously in Chapter 3, lower concentrations of glutamine (250 μM) can elicit an mTORC1 and mTORC2 response in INS1e cells (**Figure 3.2**). Here, the concentrations used were of 2 mM; thus, it is possible that even though the competitive inhibitors may significantly reduce glutamine transport, sufficient concentrations of glutamine (as the ones used in these assays) may be able to re-establish signalling to mTORC1 and mTORC2. In fact, this is seen in established competitive inhibitor models, in which inhibition may be surpassed by increasing substrate concentrations.

On the other hand, as system A (SNAT2) and system N (SNAT3), but not System ASC (ASC2) transporters are pH-sensitive, transport of glutamine across both transporters could be inhibited with low pH, to investigate effects on insulin-

dependent mTORC1 signalling in INS1e cells. Similarly, siRNA against the transporters could further illustrate their roles and functions in the regulation of mTOR signalling in INS1e cells.

Alternatively, glutamine transport through SNAT2 may not be a requirement to induce downstream mTOR signalling. SNAT2 possesses a "transceptor" property, whereby it may act as both a transporter and a receptor; this allows it to sense extracellular levels of amino acids, and its own occupancy, to signal downstream accordingly as to regulate mTORC1 activity. Thus, even upon inhibition of glutamine transport through SNAT2 with MeAIB – as shown in the transport assays – SNAT2 may sense the extracellular presence of glutamine, and/or MeAIB bound to it, to signal to mTORC1 and mTORC2 (**Figure 4.4**).

Interestingly, HPLC amino acid profiles in the presence or absence of glutamine and the transport inhibitors (**Figure 4.7**), indicate that whilst glutamine concentrations dropped when glutamine was withdrawn, they were unchanged in the presence of MeAIB, histidine or threonine. Glutamate and leucine levels remain unchanged upon inhibition of transport; this was observed previously, where leucine and glutamate levels remained unchanged in the presence of varying concentrations of glutamine (**Figure 3.2**). There was a reduction in alanine concentrations upon MeAIB treatment, which could be explained as alanine is a substrate for SNAT2, and may be utilised in conditions of glutamine insufficiency, as a nitrogen and carbon source.

4.3.1 Conclusions

SNAT2 and SNAT3 appear to be the preferred route for glutamine transport in INS1e cells. However, inhibition of those transporters with MeAIB and Threonine does not impede on signalling to mTORC1 or mTORC2. HPLC data shows no reduction of intracellular glutamine concentrations in the presence of MeAIB, histidine or threonine. Similarly, there were no changes in glutamate and leucine concentrations, even under conditions of glutamine withdrawal.

Chapter 5. Role of glutamine in signalling to mTORC2

5.1 Introduction

Whilst mTOR complex 1 signalling is well studied, signalling to mTOR complex 2 is poorly understood in comparison, primarily due to the lack of an inhibitor specific to mTORC2. The findings I reported in Chapter 3 include a surprising dephosphorylation of PKB on Ser473, indicative of an inhibition of mTORC2 activity towards PKB. Although inhibition of signalling to mTORC1 was previously reported to result in inhibition of mTORC2, the time frame whereby I observed this inhibition of mTORC2 in INS1e cells was surprising. Does the observed inhibition of mTORC2 arise as a consequence of a feedback loop between mTORC1 substrates and insulin signalling through the PI3K pathway? Does this inhibition, in the time frame observed, occur in other cell types alongside INS1e cells? Does the inhibition occur because of diminished kinase activity, or due to the actions of inhibitory components of mTOR complex 2?

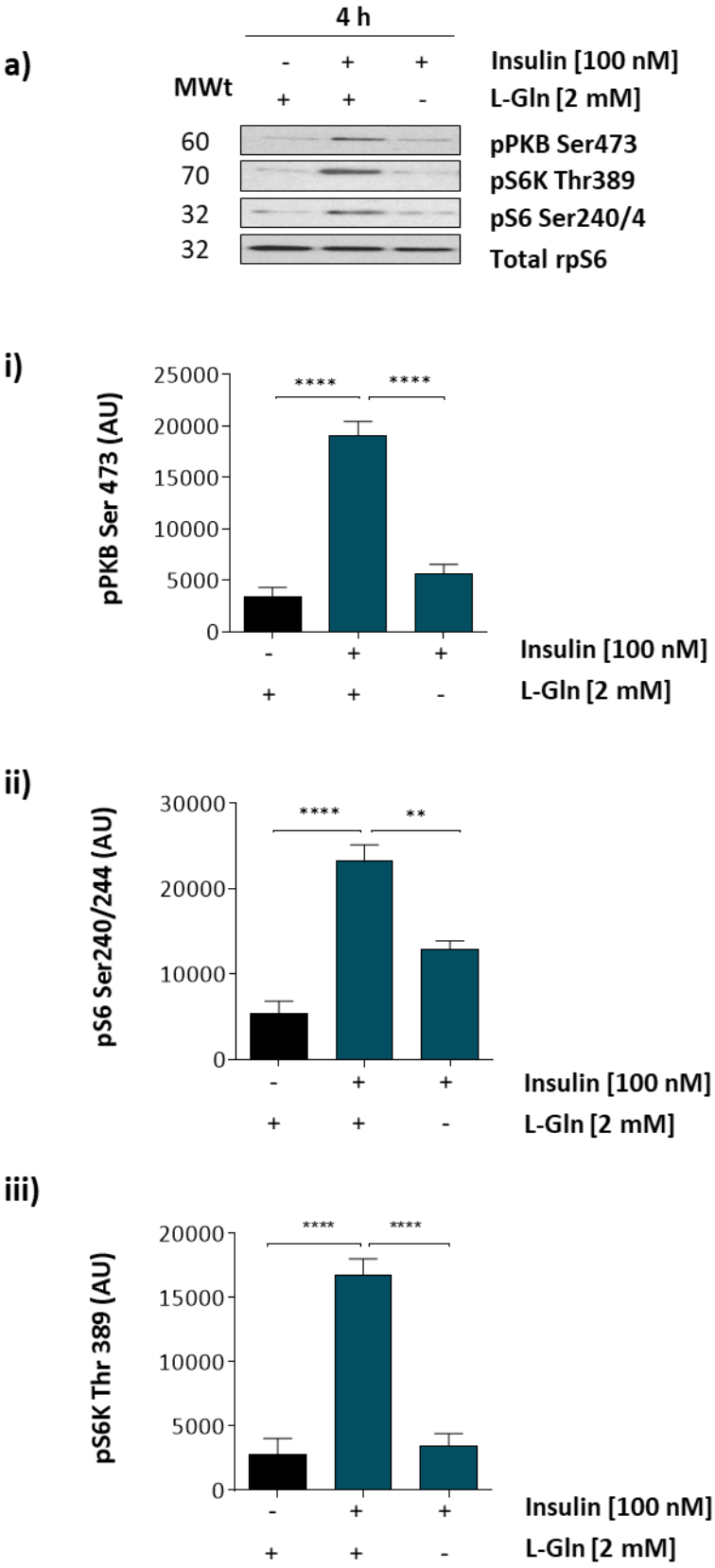
5.3 Results

5.3.1 Withdrawal of glutamine from INS1e cells inhibits mTORC2 signalling, but not PI3K signalling

Glutamine-dependent inhibition of signalling to mTORC1 and mTORC2, reported in Chapter 3, raised the question of how inhibition of mTORC2 activity was mediated by glutamine in INS1e cells. My next aim was to elucidate how such inhibition happens.

To that end, serum-starved INS1e cells were incubated in EBSS test medium in the presence or absence of 2 mM glutamine for 4 h, with insulin stimulation for 30 minutes as described in Chapter 2. The phosphorylation status of several proteins involved in mTORC2 signalling were investigated. I report that glutamine deprivation for 4 h leads to dephosphorylation of PKB on Ser473 (**Figure 5.1a)**, but not on Thr308 (**Figure 5.1b**), indicating that PI3K-mediated phosphorylation of PKB on Thr308 is unaffected by glutamine starvation in the presence of saturating concentrations of insulin. I observed a reduction in phosphorylation levels of an mTOR autophosphorylation site (pmTOR Ser2481) (**Figure 5.1c, i**); phosphorylation of Ser2481 on mTOR had been reported to be a marker of mTORC2 kinase activity (Copp, Manning and Hunter, 2009). Phosphorylation levels of mTORC1 substrate, 4E-BP1 on Ser65, shows a trend indicative of an inhibition upon glutamine starvation, like that seen in the presence of 2 mM glutamine with no insulin stimulation. Insulin stimulation in the presence of 2 mM glutamine results in

increased phosphorylation on Ser65, relative to in the absence of insulin stimulation (**Figure 5.1c, ii**).


a)
4 h
MWt
- + +
+ + -
Insulin [100 nM]
L-Gln [2 mM]
60
70
32
32
pPKB Ser473
pS6K Thr389
pS6 Ser240/4
Total rpS6
i)
pPKB Ser 473 (AU)
25000
20000
15000
10000
5000
0
****
****
Insulin [100 nM]
L-Gln [2 mM]
ii)
pS6 Ser240/244 (AU)
30000
20000
10000
0
****
**
Insulin [100 nM]
L-Gln [2 mM]
iii)
pS6K Thr 389 (AU)
20000
15000
10000
5000
0
****
****
Insulin [100 nM]
L-Gln [2 mM]

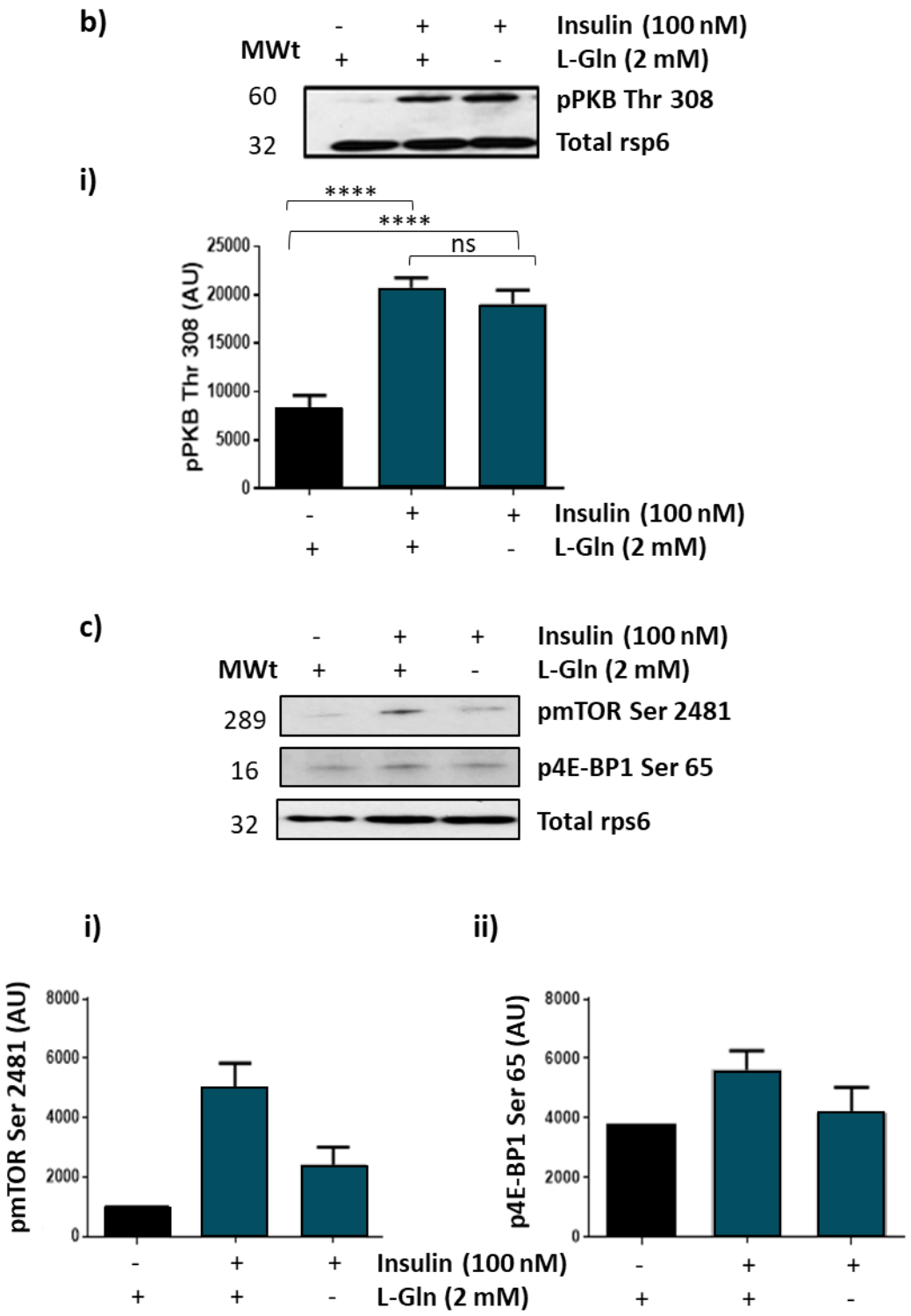


Figure 5.1. The effect of withdrawal of L-glutamine on insulin-mediated mTOR signalling for 4 hr.

Confluent INS1e cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with or without 2 mM of L-glutamine for 4 h as described in Chapter 2. Insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and lysates were immunoblotted for phospho-PKB (pPKB Ser 473 and pPKB Thr308) and phospho-S6 (pS6 Ser 240/244) and phospho-S6K (pS6k Thr 389) and phospho-mTOR (pmTOR Ser 2481) and phospho-4EBP1 (p4EBP1 Ser65). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative western blot of changes in phospho-PKB Ser473, phospho-S6 and phospho-S6K in response to insulin upon withdrawal of L-glutamine for four hours. Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB (**i**), phospho-S6 (**ii**) and phospho-S6K (**iii**).

b) Representative western blot of changes in phospho-PKB Thr308 in response to insulin upon withdrawal of L-glutamine for four hours. Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB (**i**).

c) Representative western blot of changes in phospho-mTOR and phospho-4EBP1 in response to insulin upon withdrawal of L-glutamine for four hours. Densitometric analysis was carried out to quantify relative protein levels of phospho-mTOR (**i**) and phospho-4EBP1 (**ii**). The results are n=2.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$, ns – non-significant, calculated by Bonferroni's test following one-way ANOVA. AU = arbitrary units.

5.3.2 Glutamine withdrawal inhibits mTORC2 signalling in HEK293, HepG2, but not SH-SY5Y cells

The observed requirement of the presence of glutamine for insulin-mediated signalling to mTORC2 in INS1e cells lead to questioning if this observation is INS1e-specific, or if it may be observed in other cell types. Immortalised cell lines from different tissues were investigated under the same experimental conditions as conducted in INS1e cells – serum-starved cells were treated in EBSS medium in the presence or absence of 2 mM glutamine with 30 minutes of insulin stimulation as described in **Chapter 2**.

Interestingly, U2OS, H1299 and H9C2 cells do not appear to require glutamine for insulin-mediated activation of mTORC1 and mTORC2 signalling (**Figure 5.2a**), as seen by phosphorylation levels of their respective substrates, S6 and PKB. However, signalling to mTORC1 in HepG2 and PC12 cell lines appear to be sensitive to glutamine withdrawal for 4 h, evident by dephosphorylation of S6 (**Figure 5.2b**). Interestingly, only HepG2 cells appear to require glutamine for signalling to mTORC2, evident by dephosphorylation of PKB on Ser473. Intriguingly, in CHO-K1 cell line, glutamine deprivation for 4 h appears to increase phosphorylation of PKB on Ser473 (**Figure 5.2c**).

These observations prompted further analysis of the signalling cascade in these other cell lines. HEK293, HepG2 and SH-SY5Y cell lines were chosen for this purpose. Cells were serum-starved and incubated in EBSS media in the presence or absence of 2 mM glutamine with 30 minutes insulin stimulation. Here, I report that withdrawal of glutamine for 4 hours inhibited both mTORC1 and mTORC2

signalling in HEK293 (**Figure 5.3**) and HepG2 (**Figure 5.4**) cells, but not in SH-SY5Y cells (**Figure 5.5**), and that PI3K-mediated phosphorylation of PKB on Thr308 was not affected by glutamine starvation (**Figure 5.3a, iii** and **Figure 5.4a, iii**). AMPK activity appeared to be induced in HEK293 cells upon glutamine withdrawal whilst phosphorylation of PKCα/βII was increased in either the absence of insulin or 2 mM glutamine (**Figure 5.4a**).

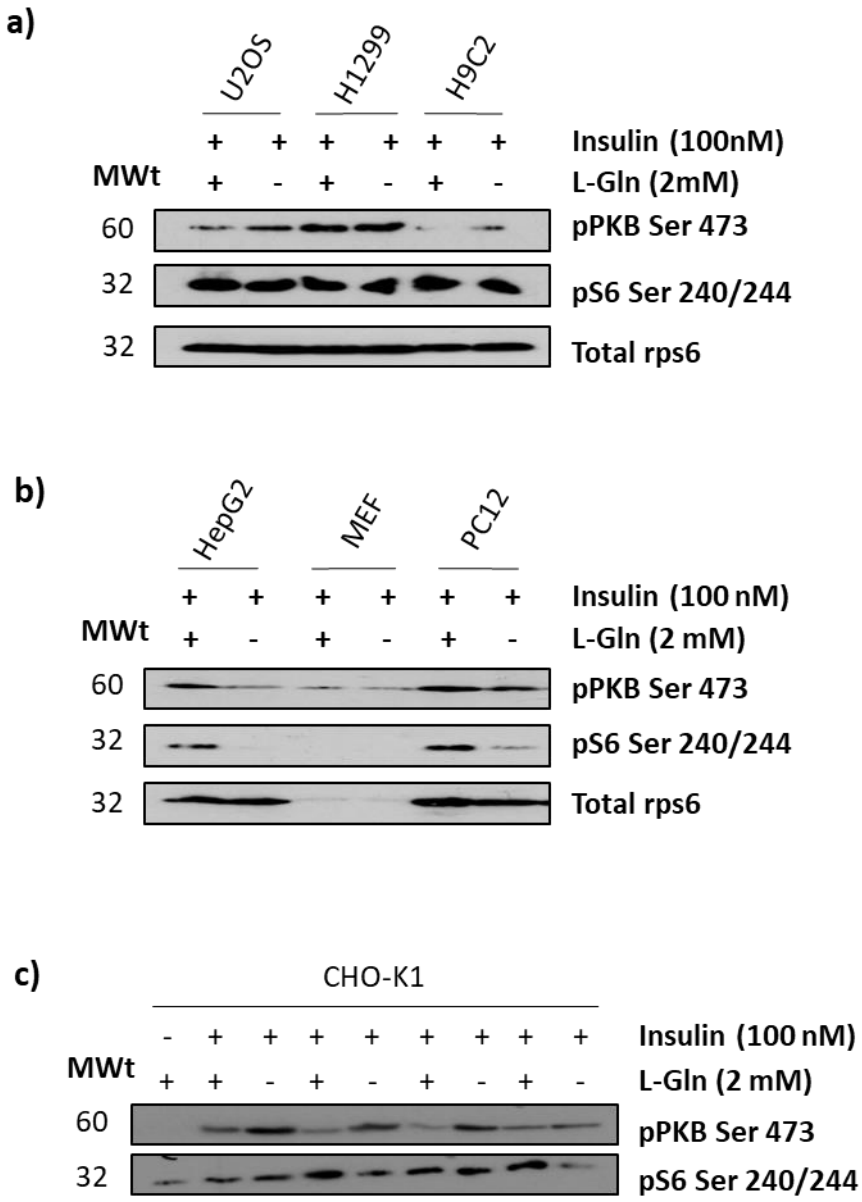


Figure 5.2. The effect of withdrawal of L-glutamine on insulin-mediated mTOR signalling for up to 4 hr in several cell lines.

Confluent U2OS, H1299, H9C2, HepG2, MEF, PC12 and CHO-K1 cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with or without 2 mM of L-glutamine for 4 h as described in Chapter 2. Insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control.

a) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin upon withdrawal of L-glutamine for 4 hours in U2OS, H1299 and H9C2 cells.

b) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin upon withdrawal of L-glutamine for 4 hours in HepG2, MEFs and PC12 cells.

c) Representative blot of changes in phospho-PKB and phospho-S6 in response to insulin upon withdrawal of L-glutamine for 4 hours in CHO-K1 cells.

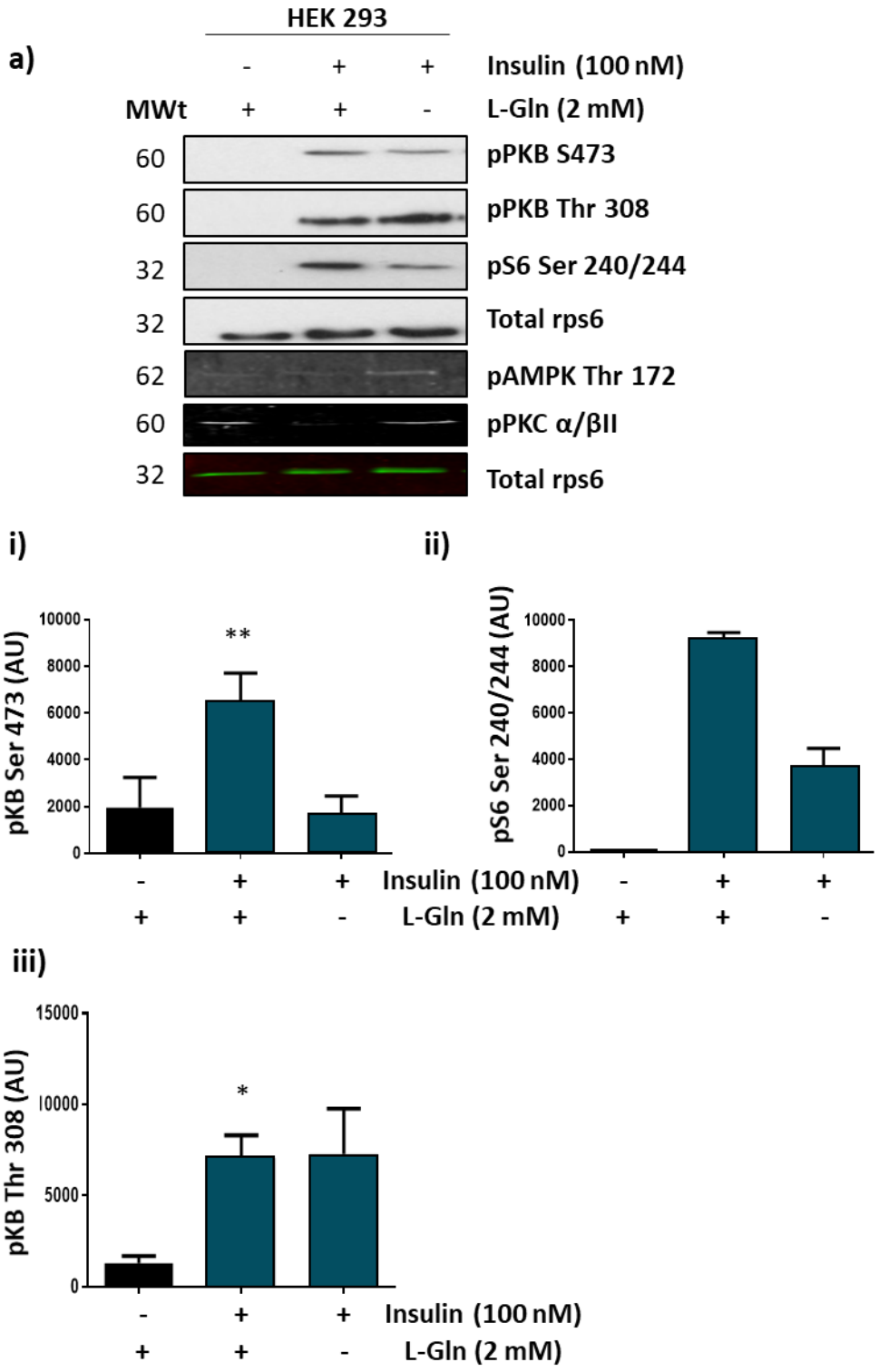


Figure 5.3. L-glutamine depletion in HEK293 cells inhibits both mTORC1 and 2 signalling, but not PI3K signalling.

Confluent HEK293 cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with or without 2 mM L-glutamine for 4 hours as described in Chapter 2. Insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473), phospho-S6 (pS6 Ser240/244), phospho-PKB (pPKB Thr 308), phospho-AMPK (pAMPK Thr172) and phospho-PKC (pPKC α/β). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB Ser473, phospho-S6 Ser240/244, phospho-PKB Thr308, phospho-AMPK and phospho-PKC in response to insulin upon withdrawal of L-glutamine for four hours.

Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB Ser 473 (**i**), phospho-S6 Ser240/244 (**ii**) and phospho-PKB Thr308 (**iii**) responses.

Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$, calculated by Bonferroni's test following one-way ANOVA relative to control (unstimulated). AU = arbitrary units.

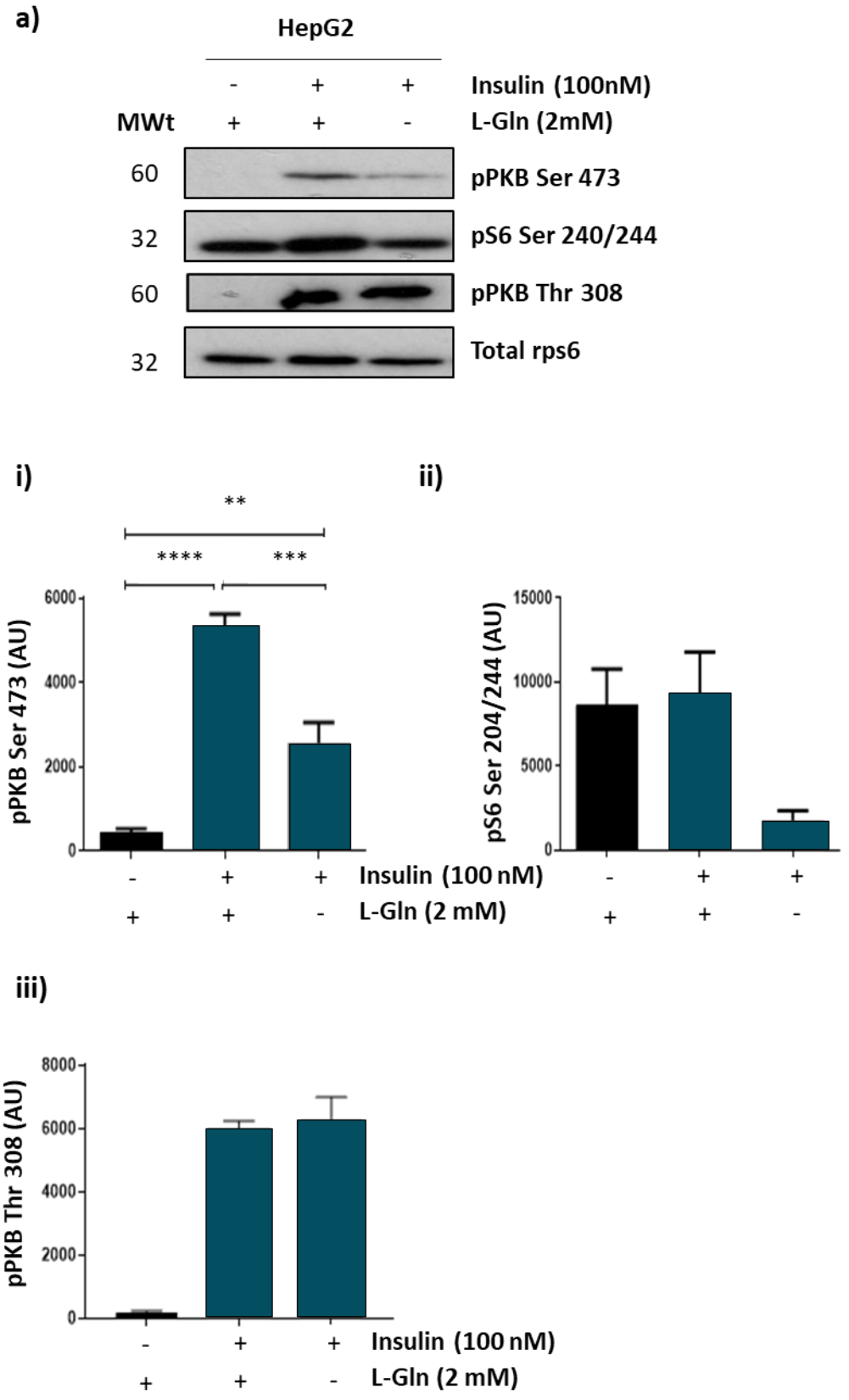


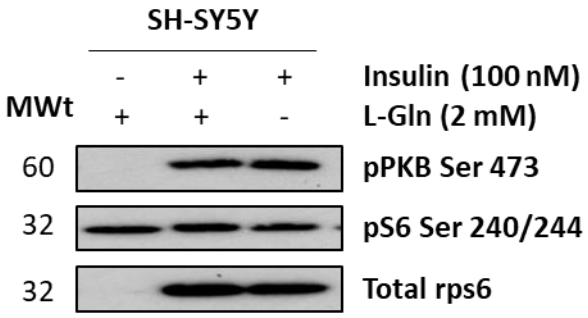
Figure 5.4. L-glutamine depletion in HepG2 cells inhibits both mTORC1 and 2 signalling, but not PI3K signalling.

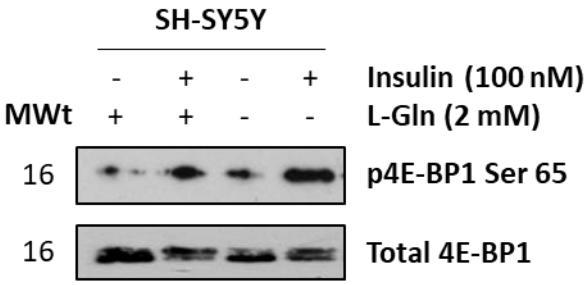
Confluent HepG2 cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with or without 2 mM L-glutamine for 4 hours as described in Chapter 2. Insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473), phospho-S6 (pS6 Ser240/244), phospho-PKB (pPKB Thr 308). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB Ser473, phospho-S6 Ser240/244, phospho-PKB Thr308 in response to insulin upon withdrawal of L-glutamine for four hours. Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB Ser 473 (**i**), phospho-S6 Ser240/244 (**ii**) and phospho-PKB Thr308 (**iii**) responses.

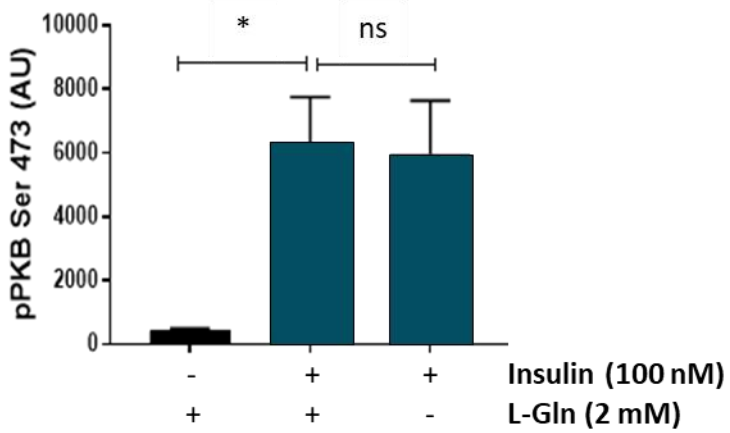
Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$, calculated by Bonferroni's test following one-way ANOVA relative to control (unstimulated). AU = arbitrary units.

a)


SH-SY5Y
- + +
MWt + + -
Insulin (100 nM)
L-Gln (2 mM)
60
pPKB Ser 473
32
pS6 Ser 240/244
32
Total rps6


SH-SY5Y
- + - +
MWt + + - -
Insulin (100 nM)
L-Gln (2 mM)
16
p4E-BP1 Ser 65
16
Total 4E-BP1

i)


pPKB Ser 473 (AU)
10000
8000
6000
4000
2000
0
*
ns
- + +
+ + -
Insulin (100 nM)
L-Gln (2 mM)

ii)

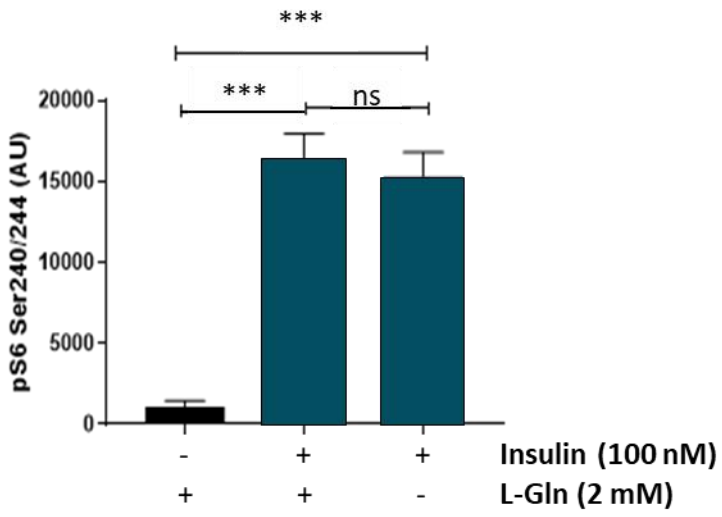

pS6 Ser240/244 (AU)
20000
15000
10000
5000
0
***
***
ns
- + +
+ + -
Insulin (100 nM)
L-Gln (2 mM)

Figure 5.5. L-glutamine depletion in SH-SY5Y cells does not inhibit mTORC1 and 2 signalling.

Confluent SH-SY5Y cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with or without 2 mM L-glutamine for 4 hours as described in Chapter 2. Insulin was added to all plates except the control for the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473), phospho-S6 (pS6 Ser240/244) and phospho-4EBP1 (p4EBP1 Ser65). Total rpS6 was used as a loading control. The results are either representative or mean ± S.E.M of n≥3 independent experiments.

a) Representative blot of changes in phospho-PKB Ser473, phospho-S6 Ser240/244 and phospho-4EBP1 (p4EBP1 Ser65) in response to insulin upon withdrawal of L-glutamine for four hours. Densitometric analysis was carried out to quantify relative protein levels of phospho-PKB Ser 473 (**i**) and phospho-S6 Ser240/244 (**ii**) responses.

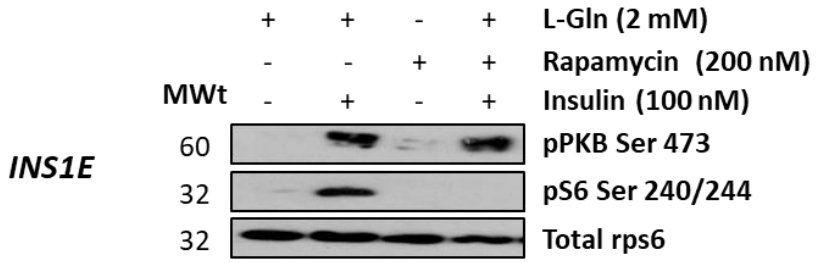
Error bars represent S.E.M. * $p<0.05$, ** $p<0.01$, *** $p<0.001$ and ****$p<0.0001$, ns – non-significant, calculated by Bonferroni's test following one-way ANOVA relative to control (unstimulated). AU = arbitrary units.

5.3.3 Inhibition of mTOR by rapamycin and torin

I next investigated the viability of mTOR and its temporal response to its inhibitors rapamycin and torin in INS1e, HepG2, SH-SY5Y, PC12 and H9C2 cells. Serum-starved INS1e, HepG2, SH-SY5Y, PC12 and H9C2 cells were treated with 200 nM rapamycin in EBSS media in the presence and absence of 2 mM glutamine for 90 minutes, and insulin-stimulated mTORC1 and mTORC2 responses were measured. As shown in **Figure 5.6**, across all five cell lines investigated, there was an inhibition of signalling to mTORC1, evident by a reduction of phosphorylation levels of its marker phospho-S6, but not to mTORC2 – with phosphorylation of PKB remaining unchanged.

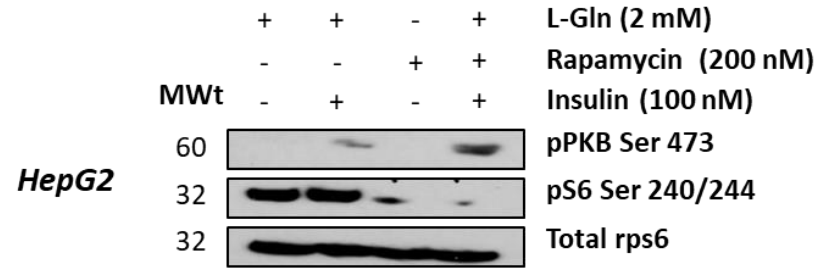
Torin treatment of INS1e cells in the presence or absence of 2 mM glutamine was also investigated, for up to 4 h (**Figure 5.7**). Insulin-stimulated signalling to both mTORC1 and mTORC2 was inhibited, evident by dephosphorylation of both pS6 Ser240/244 and pPKB Ser473, from 1 h through to 4 h. Glutamine withdrawal reduced insulin-stimulated signalling to mTORC1 after 2 and 4 h of withdrawal, whilst signalling to mTORC2 was only reduced after 4 h of glutamine withdrawal.

a)



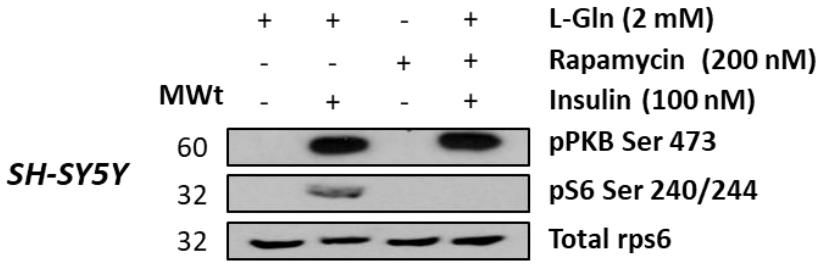
INS1E

b)



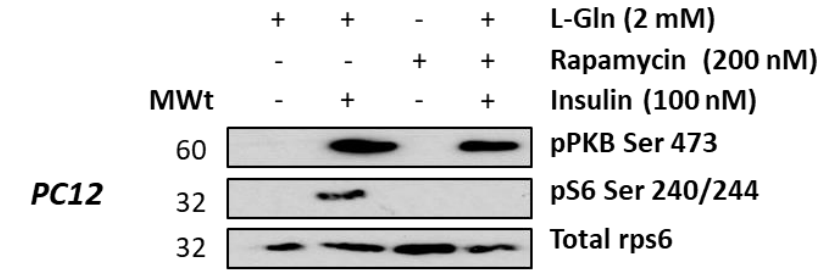
HepG2

c)



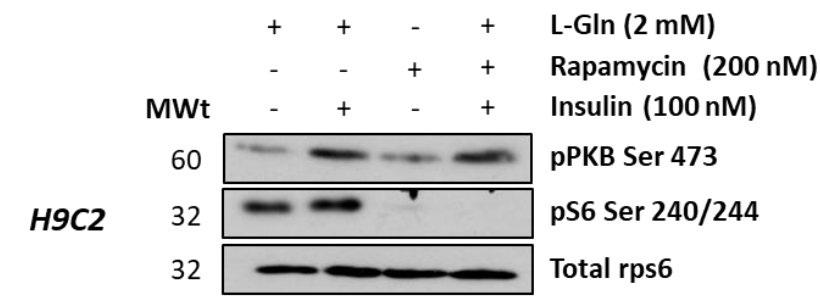
SH-SY5Y

d)



PC12

e)



H9C2

Figure 5.6. Inhibition of mTOR signalling with Rapamycin in several cell lines.

Confluent INS1-E, HepG2, SH-SY5Y, PC12 and H9C2 cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with or without 2 mM L-glutamine for 90 min as described in Chapter 2. Insulin (100 nM) and Rapamycin (200 nM) were added where indicated, with insulin stimulation occurring in the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473) and phospho-S6 (pS6 Ser240/244). Total rpS6 was used as a loading control.

Representative western blot of changes in phospho-PKB Ser473 and phospho-S6 Ser240/244 in INS1e (**a**), HepG2 (**b**), SH-SY5Y (**c**), PC12 (**d**) and H9C2 (**e**) cell lines.

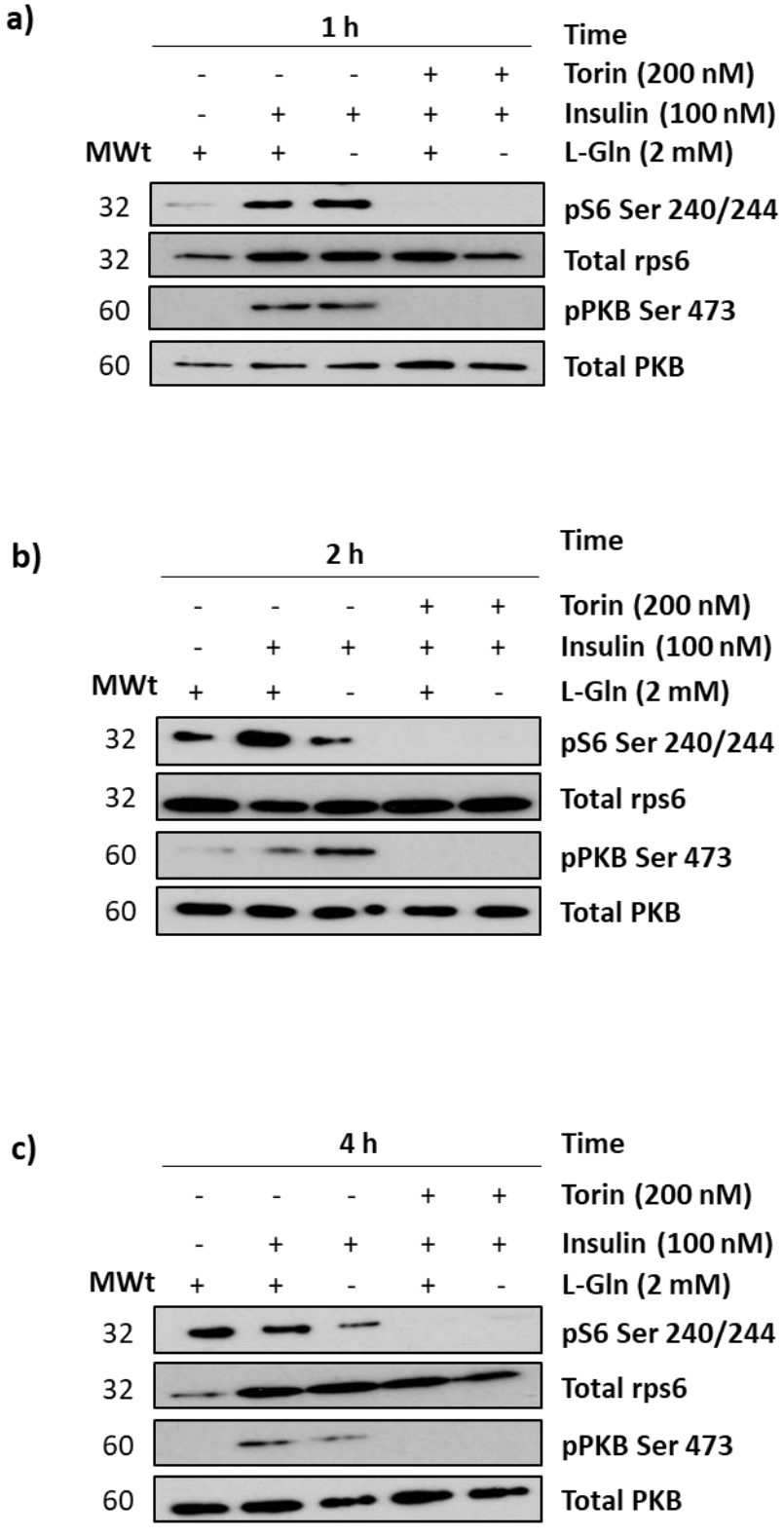

a)
1 h
Time
- - - + + Torin (200 nM)
- + + + + Insulin (100 nM)
MWt + + - + - L-Gln (2 mM)
32 pS6 Ser 240/244
32 Total rps6
60 pPKB Ser 473
60 Total PKB
b)
2 h
Time
- - - + + Torin (200 nM)
- + + + + Insulin (100 nM)
MWt + + - + - L-Gln (2 mM)
32 pS6 Ser 240/244
32 Total rps6
60 pPKB Ser 473
60 Total PKB
c)
4 h
Time
- - - + + Torin (200 nM)
- + + + + Insulin (100 nM)
MWt + + - + - L-Gln (2 mM)
32 pS6 Ser 240/244
32 Total rps6
60 pPKB Ser 473
60 Total PKB

Figure 5.7. Inhibition of mTOR signalling with Torin in INS1e cells

Confluent INS1-E cells in 40 mm plates were serum-starved overnight (16 h) in CMRL medium containing 0.25 mM L-glutamine (L-Gln). Cells were then washed and incubated in EBSS media with or without 2 mM L-glutamine for up to 4 hours as described in Chapter 2. Insulin (100 nM) and Torin (200 nM) were added where indicated, with insulin stimulation occurring in the final 30 min of the incubation. Cell monolayers were then solubilised and immunoblotted for phospho-PKB (pPKB Ser473) and phospho-S6 (pS6 Ser240/244). Total rpS6 and Total PKB were used as a loading controls.

a) Representative western blot of changes in phospho-PKB Ser473 and phospho-S6 Ser240/244 at 1 hours of Torin treatment.

b) Representative blot of changes in phospho-PKB Ser473 and phospho-S6 Ser240/244 at 2 hours of Torin treatment.

c) Representative blot of changes in phospho-PKB Ser473 and phospho-S6 Ser240/244 at 4 hours of Torin treatment.

5.4 Discussion

Whilst amino acid regulation of signalling to mTORC1 is well-established, amino acid input into mTORC2 signalling is comparatively less understood. Active mTORC2 signalling directly and fully activates PKB by phosphorylating it on Ser473 once recruited to the plasma membrane, thus coupling growth factors and environmental cues with cell proliferation and growth. This phosphorylation event requires PDK1 phosphorylation of PKB on Thr308 (Sarbassov *et al.*, 2005). Modulation of mTORC2 activity is tightly coupled to its localisation; most importantly is its plasma membrane localisation via its PH-SIN1 domain, allowing activation of PKB by mTORC2 (Liu *et al.*, 2015). However, this localisation was recently shown to be independent of growth factors and PI3K signalling (Ebner *et al.*, 2017).

Here, I report that the observed inhibition of mTORC2 activity upon glutamine starvation occurs independently of PI3K signalling. Phosphorylation of PKB on Thr308 by PDK1 is unaffected by glutamine withdrawal, whilst phosphorylation of PKB on Ser473 by mTORC2 is reduced (**Figure 5.1b**). This is corroborated by a reduction of phosphorylation on a mTOR autophosphorylation site (pmTOR Ser2481) (**Figure 5.1c**), a proposed indicator of mTORC2 kinase activity (Kroczynska *et al.*, 2017)(Copp, Manning and Hunter, 2009). This glutamine-dependent inhibition of insulin-mediated signalling to mTORC2 was also observed in HEK293 and HepG2 cells, coupled with inhibition of mTORC1 (**Figure 5.3** and **Figure 5.4**), indicative that inhibition of mTORC2 is linked – or dependent – on glutamine mediated inhibition of mTORC1.

Rapamycin treatment for 90 minutes inhibited mTORC1 in several cell types (**Figure 5.6**). Interestingly, whilst rapamycin inhibition of mTORC1 was observed in SH-SY5Y and H9C2 cells, glutamine starvation did not elicit a similar inhibition in those cells (**Figure 5.2a** and **Figure 5.5**). It would be interesting to inhibit mTORC1 with rapamycin for 4 hours, and investigate if mTORC1-dependent inhibition of mTORC2 occurs. Torin treatment for 4 h in INS1e cells, on the other hand, inhibits signalling to both mTORC1 and mTORC2, when treated for 1, 2 and 4 h (**Figure 5.7**). Unlike the inhibition of mTORC2 by torin seen from 60 minutes onwards, glutamine withdrawal only appears to inhibit mTORC2 when withdrawn for 4 h.

Collectively, the above data indicate that signalling to mTORC2 depends on glutamine, and is independent of PI3K signalling. These observations were seen in INS1e, HEK293 and HepG2 cells, but not in other cell types investigated. How this inhibition of mTORC2 activity is diminished by glutamine insufficiency is a question to be pursued. I suggest two avenues of investigation – first, if the dephosphorylation of PKB on Ser473 occurs because of increased phosphatase activity towards PKB. Protein serine/threonine phosphatase-2A (PP2A) and pleckstrin homology domain leucine-rich repeat protein phosphatase (PHLPP) are two PKB-specific phosphatases, that dephosphorylate PKB on Thr308 or Ser473, respectively (Warfel and Newton, 2012)(Xiao *et al.*, 2010). It would be of interest to investigate PHLPP specifically, as I report a reduction in phosphorylation at Ser473, but not Thr308. Second, would be investigating the equilibrium between mTORC2 kinase activity and PHLPP's phosphatase activity, which could provide

further insight into the mechanism by which these observations occur. Of course, an mTORC2-specific kinase assay could help determine whether the kinase activity of mTORC2 is diminished, or unaltered, upon glutamine insufficiency. Parallel to that, and especially if there is a diminished kinase activity, investigating the complex integrity of mTORC2 could also provide answers – co-immunoprecipitation of Rictor (mTORC2 specific component) and investigating the activities of the individual components by western blotting. DEPTOR inhibition of mTOR diminishes its kinase activity, whilst SIN1 inactivation may prevent translocation of mTORC2 to the plasma membrane where it can phosphorylate PKB on Ser473.

5.4.1 Conclusions

- Glutamine withdrawal for 4 h inhibits mTORC2 phosphorylation of PKB in INS1e, HEK293 and HepG2 cells.
    - This inhibition occurs independently of PI3K signalling – phosphorylation of PKB on Thr308 appears unaffected.
- Rapamycin inhibits mTORC1 but not mTORC2 in several cell types. Torin inhibits both mTORC1 and mTORC2.
    - Rapamycin-inhibition of mTORC1 in SH-SY5Y does not correlate with a glutamine-mediated inhibition.
    - Torin inhibition of mTORC2 occurs rapidly, whilst glutamine-mediated inhibition occurs only at 4 h of starvation.

Chapter 6. General Discussion

The importance of mTOR signalling pathway in both health and disease has generated a lot of interest and research into how mTOR signalling is regulated. By integrating several environmental and stress stimuli, growth factors and nutrients, mTOR signalling acts as a master regulator of growth and proliferation, and mTOR and its inhibitors are involved in processes such as aging and development (Cornu, Albert and Hall, 2013), as well as in disease states where aberrant growth is observed (Lien, Lyssiotis and Cantley, 2016). The multitude of benefits that can arise from manipulating and regulating the mTOR pathway can give rise to possible extensions of lifespans, reversal of neurological disorders, control of unchecked proliferation and of course, inducing proliferation during β-cell compensation (Saxton and Sabatini, 2017).

In recent years, interest in mTOR and diabetes – specifically its role in how pancreatic β-cells can adapt to increased insulin demand – has illustrated how multifaceted this regulation can be; initial activation of mTORC1 signalling seen in β-cell compensation does over time result in further peripheral insulin resistance and an eventual β-cell failure. Throughout this work, I aimed to investigate the role of glutamine in regulating mTORC1 activity in pancreatic β-cells; I had looked at how glutamine regulates general signalling pathways, and specifically the mTOR signalling pathway. My approach focused on the metabolism of glutamine, as well as the transport.

The independent regulatory arm that amino acids provide in the regulation of mTORC1 signalling is the sub-field that I attempted to further with this work. Other groups working on amino acid sensing and signalling to mTORC1 have shown that branched chain amino acids (BCAAs) – such as leucine and arginine – are the primary activators of mTORC1 signalling. BCAA transport, their regulation of the Ragulator complex through modulating the activities of Sestrin1 and CASTOR1, and lysosomal amino acid sufficiency, is widely accepted as the mechanism whereby mTORC1 is activated by amino acids. The non-essential amino acid glutamine, with its many roles, was thought to have secondary roles in amino acid activation of mTORC1; many studies have shown that glutamine mediates transport of leucine, and/or provide the nitrogen and carbon backbone for nucleotide and protein synthesis, as well as the metabolites that feed in the TCA cycle, thus assisting in energy sufficiency to promote growth.

Here, I report that glutamine activates insulin-dependent mTORC1 and mTORC2 signalling in the pancreatic β-cell line INS1e, and that its withdrawal inhibits signalling to both mTORC1 and mTORC2. In fact, glutamine appeared to elicit an "all or nothing" response, whereby chosen concentrations as low as 250 μM and as high as 2 mM fully activated signalling to both mTORC1 and mTORC2. Interestingly, HPLC data shows no change in leucine concentrations in the presence or absence of glutamine – implying the possibility that the regulation of mTOR in INS1e does not depend on leucine transport nor on leucine's activation of mTORC1, as previously described by others (Nicklin *et al.*, 2009a). Whilst inhibition of glutamine transport was reported by others to reduce leucine concentrations and therefore result in an inhibition of mTORC1 signalling (Evans

et al., 2007), my work shows that SNAT2 and SNAT3 transporters to be the two transporters utilised in INS1e cells to transport glutamine; and that inhibition of those transporters surprisingly did not cause a reduction in leucine or glutamate concentrations, nor inhibit signalling to mTORC1 or mTORC2.

Glutamine metabolism, primarily through glutaminolysis, was reported to be a potent activator of mTORC1 activation, in a leucine-dependent manner (Durán *et al.*, 2012). However, my work has shown that in pancreatic β-cells, the process of glutaminolysis itself may not be critical for mTORC1 activation. Pharmacological inhibition of glutaminolysis with L-DON did not affect mTORC1 signalling; whilst when mTORC1 and mTORC2 are inhibited in the absence of glutamine, glutamate and α-ketoglutarate restores signalling in glutamine's absence. However, HPLC data shows that intracellular glutamate concentrations do not change with intracellular glutamine concentrations, nor upon inhibition of glutamine's transport. I propose that in clonal INS1e cells, glutamate and leucine are not critical signals to activate mTORC1.

Instead, I hypothesise that glutamine-mediated inhibition of signalling to mTORC1 and mTORC2 does not arise because of diminished leucine transport or glutamine metabolism, but by inducing cellular stress and activating AMPK. The observation that glutamine metabolites restore signalling to mTORC1 and mTORC2 in the absence of glutamine, correlates with reduced phosphorylation of pAMPK Thr172. In fact, the observed inhibition of mTORC2 activity could help explain the observed AMPK inhibition; insulin-induced PKB signalling mediates

glucose uptake and its metabolism, and thus stimulating ATP production (Welsh *et al.*, 2005). This results in an indirect prevention of AMPK activation.

However, PKB can also directly phosphorylate AMPK on Ser487, a phosphorylation event that hinders activation of AMPK by LKB1 (Manning and Toker, 2017). mTORC2 inhibition and thus dephosphorylation of PKB on Ser473 (resulting in a non-fully active PKB), leads to an inability of PKB to interfere with AMPK activation by LKB1. Of interest is the localisation of AMPK and amino acid activation of mTORC1; the lysosome. The lysosomal Ragulator complex involved in mediating activation of mTORC1, is also shown to mediate AMPK activation (Zhang *et al.*, 2014). Under nutrient starvation conditions, signals initiate docking of LKB1 to Ragulator, thus allowing AMPK activation. The recent discovery of a glutamine transporter – SNAT7, previously designated an orphan transporter – in the lysosomal membrane, may implicate glutamine sensing by the Ragulator complex (Verdon *et al.*, 2017).

Collectively, I might hypothesise that the well-established Ragulator complex regulation by leucine and arginine, may also be regulated by glutamine in pancreatic β-cells. Induction of energy stress by glutamine starvation may induce cellular stress, that is sensed and potentiated by Ragulator complex – and even in the presence of sufficient leucine and glutamate levels to activate mTORC1 as previously reported – glutamine-mediated AMPK activation may occur in a Ragulator-dependent mechanism, resulting in its inability to recruit and activate mTORC1. Of course, this may occur in parallel with AMPK directly activating TSC2, thereby inhibiting mTORC1.

The evidence I have provided would need more investigation to formulate a working hypothesis. Further refinements and optimisations to the existing experimental conditions – primarily the metabolism and transporter experiments in Chapters 3 and 4 – in parallel with pursuing new avenues of investigation, are needed. Primarily, if autophagy is induced with glutamine starvation, and how stress activated AMPK may inhibit mTOR signalling; how activators of AMPK in the presence of glutamine – and conversely, inhibitors of AMPK in the absence of glutamine – might provide further insight into how glutamine regulates mTORC1 signalling. Of course, how inhibition of mTORC2 in conditions of glutamine starvation occurs is of huge interest.

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