INVESTIGATING NOVEL REGULATORS OF mTORC1 ACTIVATION IN HUMAN SKELETAL MUSCLE

By

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ABSTRACT

The mechanistic target of rapamycin (mTOR), specifically mTOR complex 1 (mTORC1) is believed to be a central regulator of muscle size, however, the upstream mechanisms which activate this kinase in response to anabolic stimuli remain to be fully elucidated. This thesis therefore aimed to investigate the role of several novel regulators of mTORC1 activity in human skeletal muscle. Chapter 3 displays that mTOR translocation to the cell periphery is mTORC1-specific and is greater following resistance exercise (RE) and protein-carbohydrate (PRO-CHO) feeding compared to feeding alone. In chapter 4 we validate and optimise a immunofluorescent staining protocol to visualise L-Type amino acid transporter 1 (LAT1) in human skeletal muscle, where it was more greatly expressed in fast twitch fibres and localised close to sarcolemma and microvasculature. Chapter 5 investigated the role of vacuolar protein sorting 34 (Vps34), a potential nutrient 'sensor', in mTORC1 activation in skeletal muscle. Here, both *in vitro* and *in vivo* experimental designs were utilised to display that changes in Vps34 cellular location, rather than kinase activity, may be important for nutrient sensing in skeletal muscle. Finally, chapter 6 reports how mTORC1 activation can occur without alterations in Vps34 protein content, kinase activity or LAT1 fibre type distribution in response to RE and/or PRO-CHO feeding. Overall, this thesis enhanced our understanding of the intricate regulation of mTORC1 activity in human skeletal muscle, identifying new potential mechanisms by which anabolic stimuli may regulate this kinase.

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LIST OF ABBREVIATIONS

10RM	10 repetition maximum
1RM	1 repetition maximum
4EBP1	Eukaryotic translation initiation factor 4E-binding protein 1
AAs	Amino acids
AKT/PKB	Protein kinase B
Ambra-1	Activating molecule in Beclin-1-regulated autophagy
AMPK	Adenosine monophosphate-activated protein kinase
ANOVA	Analysis of variance
ARL8B	ADP-ribosylation factor-like protein 8B
Atg101	Autophagy-related protein 101
Atg13	Autophagy-related protein 13
Atg14	Autophagy-related protein 14
ATP	Adenosine tri-phosphate
BCA	Bicinchoninic acid assay
BCAAs	Branched chain amino acids
Beclin-1	Coiled-coil myosin-like BCL2-interacting protein
BMI	Body mass index

C7orf59/LAMTOR4	Late endosomal/lysosomal adaptor and MAPK and MTOR activator 4
CON	Control
conA	Concanamycin A
CSA	Cross sectional area
ddH_2O	Double-distilled water
DEPDC5	DEP Domain Containing 5
DEPTOR	DEP domain-containing mTOR-interacting protein
DGKζ	Diacyglycerol kinase zeta
DMEM	Dulbecco's modified eagle media
DNA	Deoxyribonucleic acid
E2F1	E2F Transcription Factor 1
EAAs	Essential amino acids
EBSS	Earl's balanced salt solution
EDL	Extensor digitorum longus
EDTA	Ethylenediaminetetraacetic acid
eEF2	Eukaryotic elongation factor 2
eEF2K	Eukaryotic elongation factor 2 kinase

EGTA	ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid
eIF3	Eukaryotic initiation factor 3
eIF3F	Eukaryotic initiation factor 3F
eIF4A	Eukaryotic initiation factor 4A
eIF4B	Eukaryotic initiation factor 4B
eIF4E	Eukaryotic initiation factor 4E
eIF4G	Eukaryotic initiation factor 4G
eNOS	Endothelial nitric oxide synthase
ERK1/2	Extracellular regulated kinase 1/2
EXFED	Resistance exercise + protein-carbohydrate feeding
FAK	Focal adhesion kinase
FAT	FRAP, ataxia telangiectasia, transformation/translation domain
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FBS	Fetal bovine serum
FED	Protein-carbohydrate feeding alone
FIP200	Focal adhesion kinase family interacting protein of 200 kD
FNIP1/2	Folliculin-interacting protein 1/2

FoxO3	Forkhead box O3
FRB	FKBP12-rapamycin binding domain
FSR	Fractional synthesis rate
FYCO1	FYVE and coiled-coil domain containing 1
GAP	GTPase activating protein
GATOR1	GAP activity towards Rags 1
GATOR2	GAP activity towards Rags 2
GDP	Guanosine diphosphate
GEF	Guanosine exchange factor
GRp58	Glucose-regulated protein, 58-kD
GS	Grayscale
GSK3β	Glycogen synthase kinase 3 beta
GTP	Guanosine triphosphate
GTPase	Guanosine triphosphate hydrolase
GβL/mLST8	G protein beta subunit-like/ mammalian lethal with SEC13 protein 8
Hams F-10	Ham's F-10 nutrient mixture
HBXIP	Hepatitis B virus X-interacting protein

HCl	Hydrochloric acid
HEAT	Huntingtin, Elongation factor 3, Protein phosphatase 2A, TOR1 repeat
HEK293	Human embryonic kidney 293 cell line
HRP	Horseradish peroxidase
HS	Horse serum
IGF-1	Insulin-like growth factor 1
IgG	Immunoglobulin G
IRS1	Insulin receptor substrate 1
KCl	Potassium chloride
KO	Knock-out
LAMP2	Lysosome-associated membrane protein 2
LAT1	L-type amino acid transporter 1
LAT2	L-type amino acid transporter 2
LAT4	L-type amino acid transporter 4
LC3bI	Autophagy marker Light Chain 3b I
LC3bII	Autophagy marker Light Chain 3b II
LiCl	Lithium chloride

LRS	Leucyl t-RNA synthetase
LSB	Laemmli sample buffer
MAP4K3	Mitogen-activated protein kinase kinase kinase kinase 3
MEF	Mouse embryonic fibroblasts
MgC_{12}	Magnesium chloride
MHC1	Myosin heavy-chain 1
Mios	GATOR complex protein MIOS
mKO	Muscle-specific knock-out
MOPS	3-(N-morpholino)propanesulfonic acid
MP1	MEK partner 1
MPB	Muscle protein breakdown
MPS	Muscle protein synthesis
mRNA	Messenger RNA
mSIN1	Mitogen-activated protein kinase-associated protein 1
mTOR	Mechanistic target of rapamycin
mTORC1	Mechanistic target of rapamycin complex 1
mTORC2	Mechanistic target of rapamycin complex 2
Na_2VO_4	Sodium orthovanadate

$Na_4P_2O_7$-$10H_2O$	Sodium pyrophosphate decahydrate
NaCl	Sodium chloride
NaF	Sodium Flouride
NDRG1	N-Myc Downstream Regulated 1
NGS	Normal goat serum
NPB	Net protein balance
Nprl2	Nitrogen permease regulator 2-like protein
Nprl3	Nitrogen permease regulator 3-like protein
p14	ARF tumor suppressor p14
p18	INK4 protein p18
PA	Phosphatidic acid
PAT1	Proton-assisted amino acid transporter 1
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline supplemented with 0.2% Tween
PDK1	Phosphoinoisitide dependent kinase l
PI	Phosphotidylinositol
PI3K	Phosphatidylinositol-4,5-bisphosphate 3-kinase
PI3P	Phosphatidylinositol 3-phosphate

PIKFYVE	Phosphoinositide Kinase, FYVE-Type Zinc Finger Containing
PIP3	Phosphatidylinositol (3,4,5)-trisphosphate
PLD1	Phospholipase D1
PP2A	Protein phosphatase 2A
PRAS40	Proline-rich AKT1 substrate 1, 40kDa
Protor-1/2	Protein observed with Rictor-1/2
PS	Penicillin-streptomycin
Rab7	Ras-related protein Rab-7
Raptor	Regulatory-associated protein of mTOR
Rheb	Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR
RNA	Ribonucleic acid
ROIs	Regions of interest
rpS6	Ribosomal protein S6
RT	Room temperature
S6K1	Ribosomal protein S6 kinase 1
salA	Salicylihalamide A
SDS	Sodium dodecyl sulfate

SDS-PAGE	Sodium dodecyl sulfate - Polyacrylamide gel electrophoresis
Sec13	(S. cerevisiae)-13 like 1
Seh1L	SEH1 Like Nucleoporin
SGK1	Serum and glucocorticoid-regulated kinase 1
SGK3	Serum and glucocorticoid-regulated kinase 3
shRNA	Short hairpin RNA
siRNA	Small interfering RNA
SLC7A5	Solute Carrier Family 7 Member 5
SNAT2	Sodium-coupled neutral amino acid transporter 2
SR	Serum recovery
SR+SAR405	Serum recovery + SAR405
SUnSET	Surface sensing of translation
SW	Serum withdrawal
TA	Tibialis Anterior
TBST	Tris-buffered saline supplemented with 0.1% Tween
TFEB	Transcription factor EB
TLC	Thin layer chromatography
TNE	Tris-NaCl-EDT buffer

Torin-1	mTOR inhibitor 1
TSC2	Tuberous Sclerosis Complex 2
U2OS	Human Bone Osteosarcoma Epithelial Cell line U2OS
ULK1	Unc-51 like autophagy activating kinase 1
v-ATPase	Vacuolar-type H^+-ATPase
Vps15	Vacuolar protein sorting 15
Vps34	Vacuolar protein sorting 34
Vps34-IN1	Vps34 inhibitor 1
WDR24	WD Repeat Domain 24
WDR59	WD Repeat Domain 59
WGA	Wheat germ agglutinin
WT	Wild-type

Chapter 1

GENERAL INTRODUCTION

1.1 – Overview of skeletal muscle

1.1.1 Importance of skeletal muscle for optimal health

Skeletal muscle is one of the largest organs in the human body, contributing to ~40% of an individual's body mass (1). Its primary functions of posture maintenance, respiration and locomotion display this organs significance in human daily life and healthspan (1). In addition, skeletal muscle is an essential metabolic tissue implicated in glucose disposal and lipid oxidation as well as providing amino acids (AAs) to be utilised as fuel by other organs during fasting (1). The high metabolic capacity of skeletal muscle results in it being the principle contributor to basal metabolic rate in humans (2). It has long been established that increases in skeletal muscle mass and strength are associated with enhanced longevity and improved overall health (3, 4). This is in part due enhanced basal metabolic rate, which helps to stave off metabolic disease over a lifetime in addition to an enhanced capacity for glucose disposal, which by extension, reduces the risk of developing type II diabetes (5, 6). As humans age, a progressive loss of skeletal muscle mass is commonly noted (7), a phenomena termed sarcopenia, which imposes a significant financial burden on healthcare systems (8). This loss of skeletal muscle mass significantly impacts the quality of life of individuals and is therefore detrimental to overall healthspan (9). Therefore the ability to complete everyday tasks into later life is maintained if an individual has a enhanced muscle mass and strength. It is these positive effects of skeletal muscle on whole body homeostasis and health span which support the need for research examining the cellular regulation of skeletal muscle mass, to develop therapeutic strategies to prevent sarcopenia and improve health span.

1.1.2 Protein balance in skeletal muscle

Skeletal muscle is a highly plastic tissue, displaying an ability to both grow and decrease in size regularly throughout lifespan. Skeletal muscle homeostasis is maintained in adulthood through the balance of two dynamic processes, skeletal muscle protein synthesis (MPS) and breakdown (MPB), with each displaying clear day-to-day variation (10, 11). Following ingestion of protein, typically rich in AAs, increased substrate availability for MPS and the activation of signalling pathways causes MPS to rise (11-13), while the insulinogenic effects of AAs and other constituents of meals (carbohydrates) elicit a slight suppression of MPB (14). This culminates in a transient period in which MPS exceeds MPB and net protein balance (NPB) is positive. In these periods muscle proteins will be accreted. In post-absorptive states, when substrates for MPS are not readily available, MPS will lower and MPB (ubiquitin-ligase and autophagic systems) will increase in order to break down proteins to provide any needed AAs and remove damaged/dysfunctional proteins (15, 16). During these time periods, MPB will surpass MPS causing a negative NPB and muscle protein loss. In individuals who have a reasonably active lifestyle and a healthy, balanced diet, the fluctuations in MPS and MPB will be equal thereby maintaining NPB and muscle mass.

An exercise stimulus, both aerobic and resistive in nature, also stimulates MPS (17, 18) although the extent of this is much greater following resistance exercise (19). However, exercise conducted in the fasted/postabsorptive state, when no post-exercise protein/AA's ingested is unable to produce a positive NPB (18). However, exercise does serve to sensitise the muscle to nutrients, and an increase in the amount of AAs available post-exercise i.e. via a protein beverage will cause a rise in MPS that is greater than that stimulated by either exercise and nutrients alone (12, 20). Again, in this scenario, a slight

suppression of MPB is elicited via the effects of insulin (14) causing NPB to become positive and the muscle enters a state of protein accretion. If this process is repeatedly regularly such that daily NPB is frequently in a positive state then, over time, skeletal muscle hypertrophy will most likely occur (21). Although this would suggest that the time spent in a positive muscle NPB should be maximised in order to elicit the greatest hypertrophy, it is important to acknowledge that times of negative NPB are essential in order to remove damaged and dysfunctional proteins which may cause muscles to function at suboptimal standards (15, 16). These periods allow muscle remodelling permitting muscle phenotypic changes to occur in response to different stimuli (19). Overall, the control of the dynamic balance between MPS and MPB is essential in human skeletal muscle in order to permit optimal function and metabolism.

Recent research has implicated MPS as the more important contributor to NPB (22). At the molecular level, the mechanistic target of rapamycin (mTOR), in particular mTOR complex 1 (mTORC1), is noted as a significant contributor to the stimulation of MPS (23, 24). This was displayed through the administration of the mTORC1 inhibitor rapamycin to individuals prior to a bout of resistance exercise (23) or ingestion of essential AAs (EAAs) (24). On occasions where rapamycin had been ingested, MPS responses to either anabolic stimulus were unchanged from baseline values, whereas placebo conditions displayed the common significant elevations in MPS. This data displays that mTORC1 is an essential regulator of MPS in skeletal muscle, and as such the dynamic regulation of this protein complex in human skeletal muscle will be the focus of this thesis.

1.2 – mTOR Overview

1.2.1 Introduction to mTOR

The mechanistic target of rapamycin (mTOR), is an evolutionarily conserved serine/threonine kinase belonging to the PIKK family of specific serine/threonine kinases (25). mTOR contains 2549 AA residues which formulate the entire protein including its functional domains (26). At the N-Terminus resides 22 HEAT tandem repeats followed by the first of two FAT (FRAP, ataxia telangiectasia, transformation/translation) domains which are believed to be needed for the binding of regulatory components of mTOR complexes (25). The subsequent domain is the FRB (FKBP12/FK506 binding protein) domain, to which rapamycin can bind and inhibit mTOR kinase function (26). Following this is the catalytic domain of mTOR that includes an ATP binding site in addition to two sites for potential phosphorylation. Finally, the C-Terminus contains a second FAT domain (FATC) which is neighboured by two further phosphorylation sites, one of which is the site of mTOR auto-phosphorylation (27). In all, the mTOR protein has 5 sites of potential phosphorylation, each causing conformational changes in the protein structure and altering kinase activity of mTOR. The protein structure of mTOR is depicted in Figure 1.1. In most eukaryotic cells, mTOR resides as two complexes referred to as mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2), each purported to reside in different locations and exhibiting divergent functions (28).

1.2.2 mTORC1 & Components

Of the two distinct heteromeric mTOR complexes found in eukaryotic cells, mTORC1 is consistently reported as the master regulator of anabolic processes such as cellular growth and proliferation (29-31) whilst contributing to a concomitant inhibition of autophagy

(32-38). mTORC1 is comprised of several proteins (RAPTOR, PRAS40, DEPTOR, GβL/mLST8) which each individually contribute to the unique function of this complex.

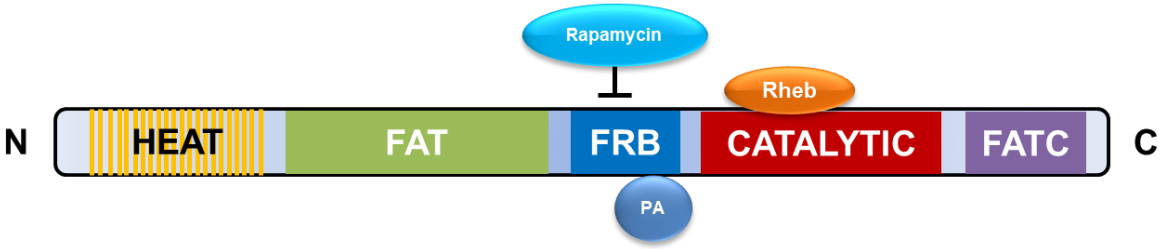

Figure 1.1. Protein structure of mTOR

At the N terminus of mTOR is 22 HEAT tandem repeats followed by the first FAT (FRAP, ataxia telangiectasia, transformation/translation) domain. The subsequent domain is the FRB (FKBP12/FK506 binding protein) domain which both rapamycin (inhibitory) and phosphatidic acid (PA, stimulatory) can bind to. The catalytic domain of this protein lies next to the FRB domain and is the main site of mTOR's catalytic activity. This domain is also the site to which Rheb binds to in order to enhance the kinase activity of mTOR. Finally, at the C terminus is the second FAT domain termed FATC.

1.2.2.1 Raptor

RAPTOR is commonly believed to be the prominent regulator of this complex, acting as a scaffold protein for mTOR and substrates such as S6K1 and 4EBP1 (39, 40) and as such the mTOR-RAPTOR interaction is critical for mTOR to complete its kinase activity (41). Several knockdown and deletion trials of the RAPTOR protein have highlighted its importance to the normal physiological function of the complex i.e. protein synthesis and cellular growth (41-43). Specifically, the utilisation of short interfering RNA strands (siRNA) which diminished RAPTOR protein expression in HEK293T cells were found to cause a reduction in leucine stimulated S6K1^{Thr389} phosphorylation, a proxy measure for mTORC1 activity. In addition, RAPTOR-deficient mice are developmentally lethal at day 7 of gestation (42), a response identical to that observed in mTOR-null animals (44). More recently, a more detailed role for RAPTOR within mTORC1 signalling has been implicated. Frey et al. (45) elegantly displayed that, in response to eccentric contractions in mouse tibialis anterior (TA) muscles, RAPTOR becomes phosphorylated at several sites (Ser696, Thr706, Ser863) and this process lies upstream of mTORC1 activation as

rapamycin treatment failed to inhibit these modifications (45). Transfection of a mutant RAPTOR protein, lacking all three phosphorylation sites, into mouse TA then revealed the crucial contribution these phosphorylation events provide to full mTORC1 activation. In muscles containing this mutant protein, a 65% reduction in the contraction-induced phosphorylation of S6K1, compared to control muscles, was reported (45). Furthermore, this decrease was accompanied by an inhibition of RAPTOR-S6K1 disassociation following contractions which may have contributed to an alteration of substrate availability to mTORC1 (45). A further postulation is that the mechanism underpinning rapamycin-induced inhibition of mTORC1 may involve RAPTOR (46). Following administration of rapamycin to HEK293T cells, co-immunoprecipitation of RAPTOR and mTOR is reduced in a dose-dependent fashion (46). Moreover, this disassociation was related to a reduction in mTOR-dependent phosphorylation of both S6K1 and 4EBP1, the two prominent mTORC1 substrates. These data combined, therefore implicate RAPTOR as an essential component of mTORC1 which can directly affect the mTORC1 physiological function through both the recruitment and proximity of mTORC1 to its substrates.

1.2.2.2 PRAS40

PRAS40 is a 14-3-3 binding protein which interacts with mTORC1 through the substrate binding site of RAPTOR (47, 48). The effects of this protein on the complex are believed to be inhibitory as the connection with RAPTOR blocks the site at which S6K1 and 4EBP1 can bind (40). Upon activation mTOR phosphorylates PRAS40 at Ser183 and causes its dissociation from the complex, relieving its inhibitory effects (49). Furthermore, PRAS40 is inhibited through phosphorylation by Akt at Thr246, which also causes the dissociation from RAPTOR due to increased binding with the cytosolic 14-3-

3 protein (50, 51). This redistribution of PRAS40 into the cytosolic fraction and away from mTORC1 is believed to be key for optimal mTORC1 function (49, 52). Consistent with its role as an mTOR inhibitor, overexpression of PRAS40 reduced insulin- and AA-stimulated phosphorylation of S6K1 and 4EBP1 in HEK293T cells (48, 53), as well as reducing overall cell size (52). Similarly, knockdown of PRAS40 increased basal mTORC1 activity in a variety of cell models (48, 54, 55). However, recent findings also suggest that mTORC1 pathway activity may be impaired due to PRAS40 silencing. Fonseca et al. (56) displayed an inhibition of insulin- and AA-mediated mTORC1 substrate phosphorylation in HEK293 cells when siRNAs targeting PRAS40 were introduced. These results have been corroborated further in both C2C12 myoblasts (57) and human primary skeletal muscle cells (58), and the extent of the inhibition of mTORC1 signalling is reported as similar to that induced by ethanol administration (51). These findings are counterintuitive due to PRAS40's believed role as an mTOR inhibitor however may be explained by the reduction in total GβL, an mTOR activator, and mTOR-GβL interaction that is noted upon PRAS40 silencing (51). Therefore, it is apparent that PRAS40 is a second component of mTORC1 that is essential in controlling this complex's action as a promoter of protein synthesis and cellular growth.

1.2.2.3 DEPTOR

Similar to PRAS40, DEPTOR acts as an mTOR inhibitor, blocking mTOR's ability to phosphorylate its substrates, S6K1 and 4EBP1 (59). During normal physiological stimulation of mTORC1, i.e. growth factors or AAs, DEPTOR becomes phosphorylated and targeted for degradation by the ubiquitin-proteasome pathway (59, 60). This breakdown releases the DEPTOR inhibition on mTORC1 and allows the initiation of protein translation and cell growth. DEPTOR knockdown, as expected from its inhibitory

function, causes hyperactivation of mTORC1, elevated phosphorylation of mTORC1 substrates (S6K1 and 4EBP1), increased protein synthesis and an enhanced proliferation rate in C2C12 myoblasts (60). Furthermore, *in vivo* knockdown of this protein in C57/BL6 mice prevented the typical muscle atrophy noted with hindlimb immobilization, an adaptation modulated by an increase rate of protein synthesis post-immobilization (60). Correspondingly, DEPTOR overexpression results in an opposite phenomena in which signalling from both mTOR complexes is inhibited (60). However, other reports suggest a catabolic consequence of DEPTOR knockdown. Zhang et al. (61) studied the effects of DEPTOR silencing in multiple myeloma cells, finding that this cell modification suppressed autophagy and induced apoptosis in this cancer cell line. Further research conducted by the same laboratory reinforced these findings showing a suppression of cell proliferation elicited by DEPTOR knockdown (62). These data suggest that DEPTOR is needed for normal mTORC1 function through the regulation of catabolic pathways and cellular replication (60-62). In addition the hyperactivity of mTORC1 induced by DEPTOR knockdown could cause unregulated cell growth, a process implicated in cancer progression (63). As such, DEPTOR also seems to be an essential component of mTORC1.

1.2.2.4 GβL/mLST8

The final component of mTORC1 complex is GβL which binds both to RAPTOR and directly to the kinase domain of mTOR (64). Several theories implicating a role of GβL in mTORC1 complex signalling have been proposed, with suggestions that it can regulate both upstream and downstream signalling of mTORC1 in addition to stabilising the RAPTOR-mTOR association (64). Despite these discrepancies, a constant finding across much of the literature regarding GβL is its positive role on mTORC1 signalling. Kim et

al. (64) utilised siRNA targeting GβL in HEK293T cells and displayed reduced mTORC1 activity (myc-S6K1Thr389 phosphorylation) in response to both serum and leucine starvation/reintroduction treatments. Furthermore, this suppression of mTORC1 signalling was similar to that noted with transfection of mTOR or RAPTOR targeting siRNA and was accompanied by a reduction in cell size, whilst overexpression elevated S6K1 and 4EBP1 phosphorylation in a dose-dependent manner (64). Several other studies have also reported this positive role as GβL-deficient mouse embryos are smaller their wild-type counterparts (42). However, this group did also report that GβL is not essential for mTORC1 signalling in response to serum deprivation/reintroduction (42). Therefore, it seems GβL is a component of mTORC1 which can positively regulate the activity of this kinase complex however; it may not be an essential factor (42, 64). Further research into this protein is needed such that its' true role in mTORC1 signalling and necessity to the complex can be elucidated.

1.2.2.5 Importance of complex formation

Each member of mTORC1 displays a unique and specific role related to the signalling of the complex such that its optimal activity cannot be achieved if any single protein is removed/inhibited. In addition to the effects of singular protein knockdown, the formation of the entire mTORC1 complex has been reported to be of importance. Ramirez-Rangel et al. (65) studied how GRp58, a protein disulphide isomerase which can regulate protein-protein binding, can affect mTORC1 complex assembly and subsequent kinase activity. Firstly, it was reported that GRp58 knockdown in HEK293T cells caused a significant reduction in the amount of RAPTOR and GβL co-immunoprecipitated alongside mTOR (65). Moreover, an opposite effect was noted when GRp58 was overexpressed as a RAPTOR-mTOR and GβL-mTOR co-immunoprecipitation was augmented (65). This

data suggested that GRp58 is implicated in the assembly of mTORC1. The authors then reported that knockdown of GRp58 elicited a reduction in the phosphorylation of mTORC1's downstream substrates S6K1 and 4EBP1, whilst overexpression caused an elevated baseline phosphorylation of these substrates but an unaltered maximal phosphorylation capacity (65). This data proposes that the assembly of the entire mTORC1 complex is vital for the full kinase activity, therefore reinforcing the notion that all components of the complex are needed for optimal physiological function of cells.

1.2.3 mTORC2 & Components

The second heteromeric mTOR complex found in many eukaryotic cells is mTORC2, implicated in glucose homeostasis and actin cytoskeleton dynamics (66). This complex is purported to be insensitive to rapamycin (67), however longer treatment has recently been shown to effectively inhibit mTORC2 (68). Several cellular locations have been observed for this kinase complex, most notably close to mitochondria (69-71), at the plasma membrane (71) and shuttling between the nucleus and cytoplasm (72). mTORC2 consists of mTOR and 5 associated proteins, RICTOR, mSIN1, Protor1/2, GβL/mLST8 and DEPTOR.

1.2.3.1 Rictor

RICTOR, similar to RAPTOR within mTORC1, is believed to be the major regulator of this complexes' kinase activity, allowing the kinase to phosphorylate a separate set of substrates to that of mTORC1. Rictor knockout mice display developmental impairments (42) and premature death 10 days into gestation. Furthermore, knocking out this protein in MEF cells reduced the phosphorylation of AKT^{Ser473}, a major mTORC2 substrate (42), in basal, serum-stimulated and insulin-stimulated states (42). Moreover, the phosphorylation of AKT substrates (Tuberous Sclerosis Complex 2 (TSC2)$^{Thr1462\ \&\ Ser939}$

and Forkhead box O3 (FoxO3)^{Thr32}) was also reduced in these cells, displaying that loss of mTORC2 kinase activity has significant downstream consequences (42). In skeletal muscle, the specific knockout of the rictor protein has a similar effect, reducing AKT^{Ser473} phosphorylation, however this effect was reversed when the mTORC1 component RAPTOR was also deleted (73). These data have been replicated in several other mouse models lacking the RICTOR protein in skeletal muscle, showing large reductions in insulin-stimulated AKT^{Ser473} phosphorylation (74), foetal growth (42) and glucose transport (74). Encapsulated, these data implicate RICTOR as essential for full mTORC2 kinase activity and suggest this component is essential for substrate phosphorylation and downstream effects of this kinase complex.

1.2.3.2 mSIN1

mSIN1 is a stress activated protein kinase which occurs in five different isoforms, three of which can occur in mTORC2 (75). Each of these isoforms generate mTORC2 complexes with varying functionalities, each responding to differing upstream signals (75). mSIN1 co-immunoprecipitates with both mTOR and RICTOR in HEK293 and HeLa cells, yet is absent from mTOR immunoprecipitates when RICTOR expression is inhibited (75), suggesting a possible association between these two proteins in the assembly of mTORC2. However, when mSIN1 expression is inhibited with shRNA, RICTOR no longer co-immunoprecipitates with mTOR proposing a possible co-dependence of the two proteins in proper mTORC2 assembly (75). Further studies have reported this ablation of RICTOR-mTOR association in mSIN1 null MEF cells (76) showing its importance to mTORC2 complex assembly. In addition, mSIN1 seems to be critical for mTORC2 kinase activity, although this may be a secondary effect following the disruption of complex assembly. When mSIN1 expression was removed through the

use of shRNA, phosphorylation of AKT^{Ser473}, a major mTORC2-regulated site, was no longer apparent (75). As previously discussed, this finding is identical to that noted when RICTOR protein expression is ablated. Interestingly, AKT phosphorylation at the second site which is implicated in its activity (Thr308) is unaltered in cells lacking mSIN1 (76) confirming the effects are due to reduced mTORC2 activity. FoxO1/3A phosphorylation was also reduced in these cells (76) suggesting AKT kinase activity, as well as phosphorylation status, is somewhat governed by mSIN1. Conversely both TSC2^{Thr1462} and GSK3β^{Ser9} phosphorylation were similar regardless of the level mSIN1 expression which suggests an intricate mechanism controlling AKT kinase activity which does not solely involve mTORC2. Furthermore, it is possible that mSIN1 acts as a scaffold for AKT allowing mTORC2 to phosphorylate this substrate as mSIN1 immunoprecipitates contain the AKT protein (76). This data provides insightful information as to how mSIN1 knockdown/inhibition reduces mTORC2 kinase activity towards AKT. Taken together these data propose that, *in vitro,* mSIN1 is critical for full mTORC2 complex assembly and kinase function. *In vivo* studies of the importance of mSIN1 to mTORC2 are rare, most likely due to the embryonic lethality caused by the knockdown of this gene (76). Nevertheless, it can be concluded that mSIN1 is vital for mTORC2 formation and activity as well as cell survival.

1.2.3.3 Protor-1 /2

Protor-1/2, also known as proline rich-protein 5 (PRR5), was first identified as a component of mTORC2 by Pearce et al. (77) in HEK293 cells through co-immunoprecipitation experiments of the major mTORC2 component RICTOR. Here, Protor-1/2 was observed alongside the other components of this mTOR complex. Further, RICTOR, mTOR and mSIN1, but not RAPTOR, were all detected in immunoprecipitates

of Protor-1 confirming its appearance only in mTORC2 (77). RICTOR was also noted to regulate the expression of Protor-1, with RICTOR-null MEF cells expressing significantly lower levels of Protor-1 compared to wild type cells. Interestingly, these novel components of mTORC2 had no apparent functional domains and as such the role of Protor-1/2 was difficult to determine. However, the use of siRNA or shRNA targeting Protor-1, and Protor-1 null MEF cells have shed some light on this matter (77-79). Reductions in Protor-1 expression had no effect on the expression of other mTORC2 components or this complex's assembly (77). In contrast, the silencing of Protor-1 with shRNA in HeLa cells did show an effect on mTORC2 kinase activity. Here, AKT^{Ser473} phosphorylation significantly decreased compared to a scrambled control (78), though this finding was not replicated in Protor-1 null MEF cells (79). This group however did observe a reduction in late-phase phosphorylation of PKCδ at its hydrophobic motif (79) suggesting some potential role of Protor-1 on mTORC2 kinase activity. More recently, Pearce et al. (80) produced Protor-1/2 single and double knockout mice in order to further elucidate the physiological role of the Protor isoforms. Again, the removal of Protor-1/2 expression had no effect on the complex assembly of mTORC2. Moreover, *in vitro* kinase assays, conducted on immunoprecipitated mTORC2 from the kidneys and liver of these mice, displayed no alterations in mTORC2 kinase activity compared to wild type controls (80). In fact, the only noticeable effect of Protor-1/2 removal was a reduction in NDRG1 and SGK1 phosphorylation in the kidneys of Protor-1 knockout and Protor-1/2 double knockout animals (80), measures which were unaffected in all other tissues analysed including skeletal muscle. This therefore suggests that, *in vivo,* only the Protor-1 isoform has an effect on mTORC2 kinase activity. Overall, it seems that Protor-1/2 may be a

dispensable component of mTORC2 in most tissues but more research is needed to fully understand the roles of these Protor isoforms on mTORC2.

1.3 – Mechanisms of mTORC1 activation

1.3.1 Direct mTOR activators

1.3.1.1 Rheb

Ras homolog enriched in brain (Rheb) is a small GTPase protein, which has been identified as a potent mTOR activator (81). Rheb's mode of action is through direct binding to mTOR's catalytic domain thereby enhancing its kinase activity (81). In a similar manner to many other small GTPases, Rheb is active when it is GTP-bound and also possesses intrinsic GTPase activity, which can drive the shift from GTP- to GDP-bound states (81). In most cell types the activation of Rheb intrinsic activity is governed by TSC2, a GTPase activating protein (GAP). When associated with Rheb, TSC2 will stimulate its GTPase activity and maintain the protein in its inactive (GDP-bound) state (82, 83). However, if TSC2 is inactive or disassociates from Rheb, intrinsic GTPase activity is no longer stimulated and Rheb is converted to the active, GTP-bound state allowing binding and activation of mTOR (81). This process has been well characterised in both *Drosophilia* and mammalian cell lines. When Rheb expression is removed in *Drosophilia* S2 cells, S6K1^{Thr389} phosphorylation is completely ablated (81). These results mirror those noted in HEK293 cells where mutations to the Rheb protein, which cause its inactivation, significantly reduce S6K1^{Thr421} phosphorylation despite adequate AAs and serum availability (81). Furthermore, Rheb overexpression can rescue S6K1^{Thr421} phosphorylation in cells which have been starved of AAs (81). Other investigations have reinforced this critical role of Rheb toward growth factor-induced mTORC1 activation confirming its importance to cell anabolism and growth (84, 85).

1.3.1.2 Phosphatidic acid

A second direct mTOR activator which has been identified is phosphatidic acid (PA), a phospholipid expressed in all mammalian cell membranes (86, 87). This compound consists of two fatty acids and a phosphate group bound to a glycerol backbone and directly activates mTOR through binding to the FRB domain (88), the same site which rapamycin exerts its inhibitory effects on mTOR. The importance of PA for mTORC1 activation has been well documented. *In vitro*, exogenous PA, combined with AAs exerted a 5-fold greater increase in S6K1 activity compared to AAs alone (88). Furthermore, the disruption of PA production through the use of 1-butanol removed any effect of serum stimulation on S6K1 activity (88). *Ex vivo* investigations have since reinforced this notion, displaying that mouse EDL muscles incubated in 1-butanol, before being subjected to eccentric contractions, exhibited no increase in $S6K1^{Thr389}$ phosphorylation (89). The production of DGKζ-/- mice provided further insight into the importance of PA to mTORC1 activation as this enzyme was identified as the major contributor to endogenous PA production in skeletal muscle (90). Here, DGKζ-/- muscle displayed a diminished anabolic response ($S6K1^{Thr389}$) to eccentric contractions compared to wild-type animals (90), emphasising how critical endogenous PA production is to mTORC1 activity.

1.3.2 mTOR and the lysosome

Lysosomes are spherical organelles that function as the waste disposal system in eukaryotic cells (91). These membrane-covered organelles contain a variety of hydrolytic enzymes which digest redundant components of the cell i.e. damaged proteins or cellular debris (91). With an internal pH maintained at ~4.5, lysosomal enzymes function in an environment similar to that of the stomach, whereby digested materials are further utilised

in the production of new cellular components (91). In addition to this role in cellular homeostasis, the lysosome has also been implicated in cellular signalling pathways (31, 92-96). Seminal work from Sancak et al. (97) using immuno-fluorescence microscopy displayed that mTORC1, when activated under conditions of AA removal and subsequent reintroduction, localised with Rab7 positive cellular structures, a protein associated with late endosomes/lysosomes (92). This work suggested that mTORC1 may need to associate with the lysosome in order to become optimally active.

Further reports have since reinforced the notion that mTORC1 interaction with the lysosome can enhance mTORC1 activity. *In vitro* work in HEK293T cells undertaken by the Sabatini laboratory (98) first displayed how the forced targeting of mTORC1 to the lysosomal surface can stimulate mTORC1 signalling and render this pathway AA insensitive. This enforced localisation of mTORC1 with the lysosome was achieved through modifying RAPTOR proteins. The final 15-17 AAs in Rheb1 or Rap1b peptide chains were fused to the RAPTOR peptide chain (coined raptor-Rheb15 and raptor-Rap1b17), whilst a control protein was produced by adding the Rheb1 peptide chain lacking the CAAX box (RAPTOR-Rheb15ΔCAAX) which should not localise to the lysosome (98). The transient expression of either raptor-Rheb15 or –Rap1b17 resulted in a constant localisation of mTORC1 to LAMP2 positive structures independent of AA availability. This continuous interaction elicited an increase in mTORC1 activity, as assessed by S6K1^{Thr389} phosphorylation, which again was insensitive to the reduction normally noted with AA withdrawal (98). A more physiological model, whereby HEK293 cells stably expressed RAPTOR-Rheb15, was also investigated by these authors (98). Identical results were found as mTORC1 remained on the lysosome irrespective of AA availability and signalling downstream of this complex was heightened. Furthermore,

cells stably expressing this mutant form of RAPTOR were larger in comparison to those expressing wild-type RAPTOR, suggesting greater growth rates in this cell line. Targeting mTORC1 to other membrane structures was insufficient to activate signalling displaying it is association with the lysosome itself which can elevate mTORC1 activation. More recent work has replicated this method *in vivo* by transfecting a plasmid encoding the RAPTOR-Rheb15 protein into mouse tibialis anterior muscles via electroporation (92). Here, the mutant RAPTOR protein stimulated a 5-fold increase in mTORC1 activity ($S6K1^{Thr389}$ phosphorylation) compared to wild-type and RAPTOR-Rheb15ΔCAAX mice phenotypes (92). Therefore, it seems that both *in vitro* and *in vivo* the association of mTORC1 to the lysosome is sufficient for mTORC1 activation and is also essential to its kinase activity.

1.3.2.1 Rheb and PA at the lysosome

In addition to residing in a location where there is an abundant supply of AAs, the presence of mTORC1 at the lysosome positions this kinase complex in close proximity to both Rheb and PA, two direct mTORC1 activators. Rheb is observed to associate with lysosomal proteins both *in vitro* (81, 93, 99) and *in vivo* in rodent skeletal muscle (90). As such, the need for mTORC1 to be associated with the lysosome for optimal kinase activity is possibly due to an increase in the association of mTOR with Rheb. This would increase the likelihood of Rheb associating with the catalytic domain of mTOR and thereby enhancing its activity. However, the close proximity of these two proteins at the lysosome is not a prerequisite for mTORC1 activation as the GAP TSC2 has also been observed at the lysosome (90). This means that in order for Rheb to be converted to the GTP-loaded state, where it can bind and activate mTOR, TSC2 must be inactivated or removed from this location.

PA is also enriched at the lysosome (100), in addition to enzymes which control its production (phospholipase D1 and DGKζ) (101, 102). Again, the close proximity of mTORC1 to its direct activator at the lysosomal membrane increases the likelihood of PA binding to the FRB domain of mTOR and enhancing its activity. Furthermore, during times of high AA availability, the activities of enzymes that produce PA are noted to increase (102, 103). This, in theory would cause an even greater abundance of PA at the lysosome and may therefore further contribute to the localisation of direct mTOR activators, at the lysosome, and contribute to maximal mTORC1 activation.

1.3.2.2 Rag GTPases

With the discovery that mTOR-lysosomal association is required for optimal mTORC1 activity, it was next imperative to understand how mTORC1 is recruited and affixed to the lysosomal membrane. This knowledge arrived through the unearthing of a family of small GTPases called the Rag proteins (97). This family of GTPases has four members (RagA/B/C/D) which bind to form stable heterodimers consisting of either RagA/B associated with RagC/D. Similarly to Rheb, as these proteins are GTPase proteins, their activity levels are governed by their guanosine loading status (97). These heterodimers are active when RagA/B are GTP-bound and RagC/D GDP-loaded, in which state they can associate with mTORC1 and recruit the kinase complex to the lysosomal surface. The specific mechanism by which this occurs is through an association of the Rag protein heterodimer with the RAPTOR component of mTORC1. This has been confirmed through several immunoprecipitation experiments *in vitro* by the Sabatini laboratory (97). Furthermore, this group displayed the importance of these proteins through the genetic variants of these GTPases, which remained permanently GTP- or GDP-loaded. Here, a mutant heterodimer containing $RagB^{GTP}$ and $RagC^{GDP}$ co-immunoprecipitated with more

RAPTOR protein than any other mutant heterodimer tested. Next, in the HEK293 cell line, this group investigated the effect of these variants on mTORC1 signalling. Interestingly, and in agreement with the increased RAPTOR association previously noted, overexpression of the heterodimer containing $RagB^{GTP}$ and $RagC^{GDP}$ not only activated mTORC1 (shown via $S6K1^{Thr389}$ phosphorylation) but caused this pathway to become insensitive to AA withdrawal (97). The knockdown of these proteins with shRNA significantly reduced mTORC1 activity in response to leucine administration, suggesting these proteins are important for mTORC1 activation by AAs. Finally, immunofluorescent staining of mTOR in HEK293 cells treated with AAs, following deprivation, resulted in mTOR-positive puncta being observed when mTOR associated with the lysosome. In contrast, when cells were treated with shRNA targeting the Rag proteins, puncta staining was no longer observed with mTOR remaining diffuse throughout the cell, a pattern normally noted in AA deprived cells (29, 97, 98, 104-108). This seminal work identified that the association of Rag protein heterodimers with RAPTOR is required for mTORC1 recruitment to the lysosome and is essential for mTORC1 kinase activity.

1.3.2.3 Ragulator

Following the identification of Rag proteins as binding partners of mTORC1 at the lysosomal surface, it was revealed that the Rag protein heterodimer's AA sequence did not contain a lipid-binding motif (98). It was therefore hypothesised that this heterodimer must have another binding partner which can bind to membrane lipid bi-layers allowing Rag protein scaffolding to the lysosome. Mass spectrometry analysis of HEK293 cells expressing active Rag mutants led to the identification of a trimeric complex associating with both the Rag proteins (98) and the lysosomal membrane (98, 104). The components of this trimeric complex were named MP1, p14 and p18. Furthermore, mTORC1 co-

immunoprecipitated with components of the ragulator when $RagB^{GTP}$ constructs were stably expressed in HEK293 cells. Genetic mutants of one component of the ragulator, p18, which targeted the protein to mitochondrial surfaces rather than the lysosome were then utilised. Here, upon AA stimulation, Rag proteins were seen to localise with the mitochondria yet mTOR did not and mTORC1 activity was low. Moreover, cells lacking either the p14 or p18 component of the Ragulator became insensitive to AAs and mTORC1-dependent phosphorylation of S6K1 remained low (98). These data display the importance of the Ragulator as a docking site for Rag proteins, and mTORC1, at the lysosome surface and also the need for this trimeric complex for full mTORC1 activation.

More recently, a further two components of the Ragulator complex have been identified (104), assembling a pentameric complex alongside MP1, p14 and p18. These two proteins, named HBXIP and C7orf59, bind to form a heterodimer which associates with p18. A second heterodimer consisting of p14 and MP1 is also associated with p18, which acts as a bridge between the two heterodimers associating them with the lysosome (104). These two novel members of the Ragulator complex are essential for mTORC1 activation in both HEK293 and *Drosophilia* cell lines, as the use of siRNA targeting HBXIP or C7orf59 greatly reduced $S6K1^{Thr389}$ in response to AAs (104). Intriguingly, this complex does not act solely as a scaffold for the Rag proteins at the lysosomal surface. Bar-Peled et al. (104) recently reported that the Ragulator complex is a guanosine exchange factor (GEF) toward RagA and RagB, catalysing the conversion of these proteins from the GDP-bound to GTP-bound active state. Furthermore, it was shown that the Ragulator had to be in its full form, the pentameric complex, for this effect to be seen (103). Therefore, the Ragulator complex's role in mTORC1 activation is two-fold, first to act as a scaffold for

the Rag proteins and second as a GEF towards RagA and RagB proteins, aiding their conversion to the active state.

1.3.2.4 V-ATPase

The vacuolar-type H^+-ATPase (v-ATPase) is a multiprotein enzyme complex primarily implicated in the transport of protons across a membrane, a process fuelled by the hydrolysis of ATP (109). This complex is comprised of two domains, the V1 domain which catalyses ATP hydrolysis, and the V0 domain which controls proton transport (109). Specifically, the process of ATP hydrolysis, by the V1 domain, generates force which rotates the V0 domain allowing for the transport of protons across the membrane (109). A potential role for the v-ATPase in mTORC1 signalling was recently proposed by Zoncu et al. (29) ^{Thr389}when they reported that deletion of several genes encoding components of the v-ATPase significantly reduced S6K1^{Thr389} phosphorylation (25). These findings were then confirmed using two polyketides, concanamycin A (conA) and salicylihalamide A (salA), which specifically inhibit the v-ATPase. Both these compounds inhibited amino-acid induced S6K1^{Thr389} phosphorylation in a concentration-dependent manner with no concomitant change in growth factor signalling. It was next displayed that the importance of this complex to mTORC1 activation was not due to the v-ATPase's role in proton-gradient assisted AA transport into the lysosome, but is downstream of intracellular AAs in this pathway. These findings then led to the hypothesis that the v-ATPase may be implicated in the recruitment of mTORC1 to the lysosomal surface. Congruent with this hypothesis, the use of conA, salA, or a shRNA targeting a v-ATPase subunit, all reduced mTORC1 translocation to the lysosome following AA stimulation (29). The forced activation of RagB (GTP-loaded) removed this effect, leading to 'normal' mTORC1 activation, suggesting that the v-ATPase lies

upstream of the Rag proteins. Subsequent co-immunoprecipitation and mass spectrometry analysis displayed a structural link between both v-ATPase domains and the Ragulator complex, and a weaker link between the V1 domain and Rag proteins (29). This implicated a role for the v-ATPase as a scaffold forming a v-ATPase-Ragulator-Rag complex allowing mTORC1 to be recruited to lysosomal membranes and therefore become activated.

1.3.2.5 Inside-Out Mechanism

In addition to the identification of v-ATPase as a component of the AA-mTORC1 pathway, Zoncu et al. (29) also displayed how mTORC1 activation is mainly driven by intralysosomal AA concentrations. This was elegantly demonstrated through the overexpression of proton-assisted transporter 1 (PAT1), an AA transporter which removes intralysosomal AAs to the cytosol, in HEK293 cells. Here, the removal of intralysosomal AAs completely abolished mTORC1 activity, however this effect was rescued via the expression of constitutively active Rag proteins (29). The inhibition of ATPase hydrolysis activity was then shown to reduce RagB-Raptor interaction and subsequent mTORC1 activation, suggesting the v-ATPase may signal intralysosomal AAs levels. This led to the authors proposing an inside-out mechanism of mTORC1 activation at the lysosome. They suggest that, upon AA exposure, intralysosomal AAs are elevated. This activates the v-ATPase which signals the alteration in AA levels to the Ragulator complex, activating this complex's GEF activity toward Rag A/B. Subsequently Rag proteins are activated (through GTP loading of RagA/B) and recruit mTORC1 to the lysosomal surface where it can become active (97). Overall the data supporting this mechanism implicate the v-ATPase as an integral component in the AA induced activation of mTORC1 and cell growth.

1.3.3 Other regulators of mTORC1

1.3.3.1 *Folliculin*

Folliculin is a tumor suppressor protein that interacts with folliculin interacting proteins 1 and 2 (FNIP1/2), however the specific function of these proteins is poorly defined. This protein complex's role in mTORC1 activation was identified upon the discovery that the activation of the Rag protein heterodimer, involved in mTORC1 recruitment to lysosomes, is dependent on the GDP-loading status of RagC (105). The forced di-phosphate loading of RagC co-immunoprecipitated a greater amount of RAPTOR than any other Rag heterodimer mutant, and caused mTORC1 activity to be resistant to AA starvation. Therefore, it was important to identify potential GEF or GAP proteins for RagC which could be regulators of mTORC1 activation. Mass spectrometry screens of RagC-associated proteins displayed a regular association with Folliculin and FNIP1/2 (105). Co-immunoprecipitation analysis then confirmed this association, mTORC1 association only occurred when folliculin and FNIP2 were stably co-expressed. The use of shRNA targeting folliculin in HEK293 cells inhibited mTORC1 activation ($S6K1^{Thr389}$) in response to AAs, and mTORC1 did not associate with lysosomes in these cells (105). In contrast, the knockdown of folliculin expression had no effect on the localisation of Rag proteins to the lysosome, thus only their ability to recruit mTORC1 was affected.

In non-transfected cells, folliculin was observed to localise to Rag proteins and the lysosomes during AA starvation. Upon AA exposure, folliculin dispersed into the cytosol in a mTORC1-independent fashion as Torin-1 had no effect on these measures (105). Following this, the function of the folliculin-FNIP2 complex was discovered. This complex was seen to have GAP activity toward RagC/D, hydrolysing GTP into GDP on

these proteins and allowing the Rag heterodimer to convert to the active state (RagA/B^{GTP}-RagC/D^{GDP}) (105). This was confirmed through the use of various Rag protein mutants, displaying how folliculin-FNIP2 can contribute to the activation of the Rag heterodimer allowing mTORC1 to be recruited to the lysosomal membrane. It is purported that, as Rag proteins leave the lysosome to recruit mTORC1, folliculin may be removed from the heterodimer and this results in the diffuse cytoplasmic staining pattern of folliculin under AA rich conditions (105). Such, data has since been replicated elsewhere (110), indicating that folliculin recruitment to the lysosome during AA deprivation is integral to mTORC1 activation upon subsequent AA reintroduction. Therefore, it seems, the folliculin-FNIP2 complex is a further integral component in the AA-mTORC1 pathway regulating protein synthesis and cell growth.

1.3.3.2 *GATOR Complexes*

The Sabatini laboratory next undertook several investigations into possible negative regulators of the Rag protein heterodimer. This led to the identification of 8 proteins, divided into 2 sub-complexes and coined the GATOR complexes (106). GATOR1, comprised of DEPDC5, Nprl2 and Nprl3 associates strongly with Rag proteins compared to GATOR2, consisting of Mios,Seh1L, WDR24, WDR59 and Sec13, which only appears weakly in Rag protein immunoprecipitates (106). The reduction in expression of each complex surprisingly had varying results. When those proteins included in GATOR1 were targeted with shRNA, mTORC1 became constitutively active and became resistant to AA withdrawal. In contrast, when the expression of GATOR2 components was reduced, mTORC1 activation in response to AAs was attenuated (106). These data therefore suggested opposing roles of the GATOR complex in mTORC1 activation. Inhibiting GATOR2, alongside concomitant reductions in GATOR1 expression, had no

effect on mTORC1 activity displaying a possible role for GATOR2 as an inhibitor of GATOR1 and as such explains GATOR2's positive regulation of mTORC1 activity.

The same group then displayed how GATOR1 functions as a GAP for RagA/B proteins, increasing GTP hydrolysis at its targets and causing the conversion to the GDP-bound (inactive) state (106). This displays how GATOR1 performs its inhibitory role toward the AA activation of mTORC1. Interestingly, mutations to this part of the mTORC1 are apparent in several cancers, where mutations to GATOR1 causes its inhibition and dysregulation of mTORC1 resulting in tumorogenesis (106). It therefore seems that GATOR1 acts as a negative regulator of mTORC1, primarily during nutrient starvation. Furthermore, a second GATOR complex acts as an inhibitor to GATOR1 during times of AA sufficiency. This would remove the inhibitory effects of GATOR1 on RagA/B, allowing mTORC1 to be recruited to the lysosome and become active.

1.3.3.3 *Sestrins*

In an attempt to further elucidate potential regulators of mTORC1, mass spectrometry analysis has been conducted in order to identify GATOR2-interacting proteins. This led to the identification of Sestrin2, which consistently appeared to be associated with GATOR2, along with Sestrin1 and 3, although these appeared at lower concentrations (107). Again, co-immunoprecipitation experiments were conducted in order to confirm these findings, which demonstrated that Sestrin2 immunoprecipitated with components of GATOR2 and vice versa. Sestrin2-GATOR2 association seemed to occur in an AA dependent manner, with a stronger association observed under conditions of AA deprivation (107). Overexpression of Sestrin2 caused a significant blunting of S6K1^{Thr389} phosphorylation and cell size congruent with its purported role as an mTORC1 inhibitor. Furthermore, the concomitant reduction in expression of Sestrin1 and Sestrin2 resulted in

elevated mTORC1 activity during AA starvation although the response to AA sufficiency was largely unchanged (107).

Such data have been replicated in other investigations, where Sestrin2 was also identified as a GATOR2-interacting protein (111). Here, increased levels of Sestrin2 were shown to reduce the association between GATOR1 and GATOR2 complexes, leading to the increased GAP activity of GATOR1 to RagA/B, even whilst AAs levels are elevated. Moreover, Sestrin2 was not present on lysosomal membranes yet could control mTOR cellular localisation, through a mechanism which was GATOR1-dependent. Interestingly, another investigation found that Sestrin2 did not affect GATOR1-GATOR2 association however still inhibited mTORC1 activation in a GATOR-dependent manner (112). This suggests a need for further investigation regarding the effect of Sestrin proteins on the formation of the GATOR1/2 supercomplex.

Recently, a leucine sensing role for Sestrin2 has been identified (108). The AA leucine is known to be the most potent activator of mTORC1, rapidly and transient elevating mTORC1 activity in many cell types (108, 113, 114). Wolfson et al. (108) elegantly displayed that the removal of leucine alone from HEK293 cells was sufficient to increase the association of Sestrin2 and GATOR2, thereby causing the activation of GATOR1. Furthermore, the re-addition of leucine rapidly reversed this effect, reducing Sestrin2's binding to GATOR2 (108). These findings were also apparent for Sestrin1 but not Sestrin3 revealing a possible diverse regulation of this homologous set of proteins. Interestingly, it was then shown that leucine could bind directly to Sestrin1 and Sestrin2 and that this binding was needed in order for mTORC1 to be activated in response to leucine exposure (108). Therefore, taken together, these data identify the Sestrin family of proteins, in particular Sestrin2, as a negative regulator of mTORC1. These proteins act

as inhibitors of GATOR2, removing its inhibitory effect on GATOR1 (possibly via decreased association) and increasing the GDP-loaded state of RagA/B. As the Rag heterodimer would then be inactivated, mTORC1 recruitment to the lysosome will no longer occur. Moreover, it seems the Sestrins can act as leucine sensors, binding to this AA directly and removing its inhibition on GATOR2. GATOR2 is then free to inhibit GATOR1, allowing RagA/B to be GTP-loaded and recruit mTORC1 to the lysosomal surface.

1.3.3.4 *Leucyl-tRNA Synthetase*

Leucyl-tRNA synthetase (LRS) is an enzyme which governs the charging of leucine to its corresponding tRNA ready for translation into a peptide chain at active ribosomes (115). In this canonical role, LRS is one of 9 total tRNA synthetases which form the multi-tRNA synthetase complex allowing efficient translation of new proteins in addition to controlling rRNA biogenesis and anti-apoptotic signalling (116). More recently, a potential role in AA sensing of LRS has been described. Firstly, through both immunofluorescent imaging and immunoprecipitation techniques, LRS was observed to associate with mTOR and RAPTOR at the lysosome, though only in the presence of leucine sufficiency (116). Subsequent reductions in LRS expression through the use of siRNA inhibited mTOR activity ($S6K1^{Thr389}$), lysosomal recruitment of mTOR and overall cell size (116) suggesting LRS is implicated in AA induced mTOR signalling. Further to this it was revealed that LRS possesses GAP activity towards RagD, enabling the conversion to its GDP-loaded, active form (116). A second group have also recently displayed how the leucine-dependent increase in LRS-lysosome localisation serves to negatively regulate autophagy (117). Therefore it seems LRS positively regulates

mTORC1 activity through its GAP activity toward RagD, and this effect may negatively affect autophagy possibly through mTOR-dependent means.

Similar investigations in yeast have replicated these findings, reporting LRS to associate with Gtr1, a GTPase yeast analogue, in a leucine dependent manner (118). Further, this association seems essential for full mTORC1 activation in response to leucine administration. Interestingly, Gtr1 is the analogue of RagA/B in human cells and as such this mechanism varies from that previously described (116, 118), where LRS affects the other component of the Rag heterodimer (RagD). This suggests that the LRS-Gtr1(RagA/B) axis may not be conserved from yeast to eukaryotic cells, yet may have adapted to have an effect on a different component of the mTORC1 activation pathway. Adding further confusion to the potential role of LRS in mTORC1 activation is work from the Sabatini lab. This group have consistently found LRS to possess no GAP activity toward the Rag proteins (105, 106), and as such may not be implicated in leucine-induced mTORC1 activation. Most recently, it has been proposed that LRS may mediate it's action on mTORC1 through the vesicle sorting protein Vps34, a potential AA sensor (119). This potential mode of action will be discussed in subsequent sections of this thesis.

1.3.4 Growth Factor Activation of mTORC1

It is well known, particularly *in vitro*, that growth factors activate mTORC1 and subsequently regulate cell size/growth (120). The intricate mechanism by which this activation occurs has been well documented, beginning at a cell's plasma membrane and culminating at the lysosome where mTORC1 associates with its direct activator Rheb. Specifically, growth factors i.e. insulin/IGF-1 bind to their corresponding tyrosine kinase receptors on the cell membrane (121, 122). This causes the activation of the Class I phosphatidylinositol-3-kinase (PI3K) signalling pathway involving several kinase

enzymes, catalysing the conversion of phosphatidylinositol to PI 3,4,5-triphosphate (PIP_3) (122, 123). This compound then binds to AKT, allowing a conformational change in this protein's structure revealing potential phosphorylation sites which govern AKT's kinase activity (124). AKT can then be phosphorylated at two sites, AKT^{Thr308} by phosphoinositide-dependent kinase 1 (PDK1) and AKT^{Ser473} by PDK2 and integrin-linked kinase (ILK) (124). Activated AKT is then able to phosphorylate downstream targets and initiate a two-fold effect on mTORC1 activity. Firstly, AKT can phosphorylate TSC2 at Thr1462 and Ser939, a process which was initially thought to inhibit the GAP activity of TSC2 toward Rheb (125). However more recent work suggests that the GAP activity is not affected by this, yet the cellular location of TSC2 is altered in response to this post-translational modification (92, 126). This results in the removal of TSC2's association with Rheb, removing its GAP activity toward this protein and allows Rheb to become GTP-loaded. This allows Rheb to be active and bind to mTORs catalytic domain to enhance its kinase activity (81).

The second effect of AKT kinase activity on mTORC1 is through a component of mTORC1, PRAS40. As previously discussed, PRAS40 inhibits the kinase activity of mTOR by blocking the substrate binding site of RAPTOR. AKT catalyses the phosphorylation of $PRAS40^{Thr246}$, which has been shown to causes the dissociation of PRAS40 from mTORC1 (40, 48, 52). This will then clear RAPTOR's substrate binding site, allowing S6K1 and 4EBP1 to associate with the complex and be phosphorylated by mTOR (40, 56). As such, AKT plays a dual role in mTORC1 activation in response to growth factors both through the increased association of mTOR with its direct activator

(Rheb) and the removal of the mTORC1 inhibitor PRAS40 from this complex. This complex signalling pathway is depicted in Figure 1.2.

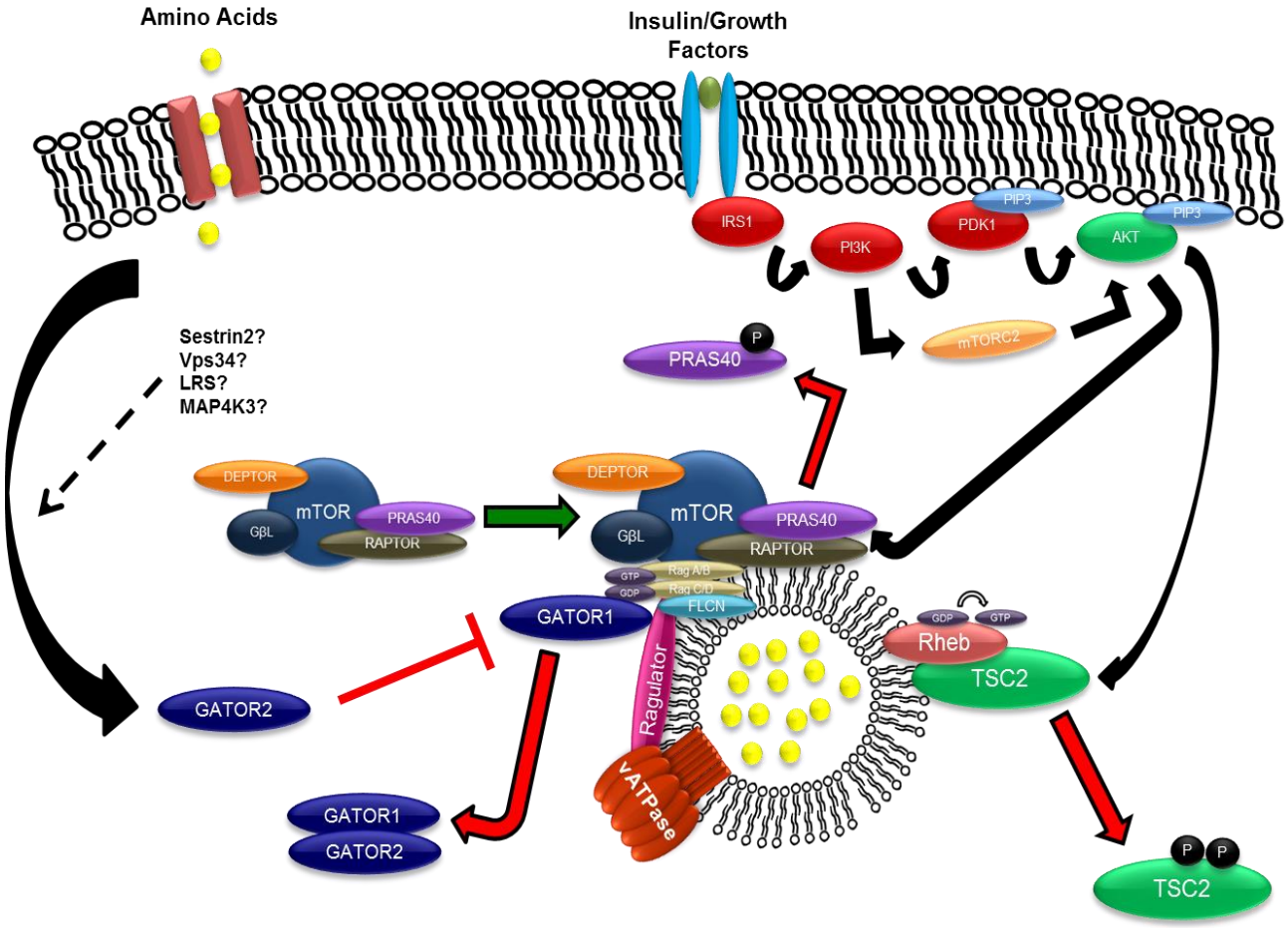

Figure 1.2. Regulation of mTORC1 by growth factors/amino acids.

Insulin/growth factors bind to their corresponding receptor on the cell membrane, initiating a signalling cascade which, via PI3K and mTORC2, results in the phosphorylation and activation of AKT at Thr308 and Ser473. AKT is then able to phosphorylate TSC2 at Ser939 and Thr1462, causing its translocation away from Rheb, lifting its GAP activity toward Rheb and allowing Rheb to become active in the GTP-loaded state. This will then allow Rheb to bind to mTOR's catalytic domain and activate mTORC1. Simultaneously, AKT phosphorylates PRAS40 at Thr246, removing it from its association with Raptor and allowing Raptor to bind mTORC1 substrates. A rise in intracellular amino acids (AAs) relieve the inhibition on GATOR2, allowing it to bind and inhibit GATOR1. This removes GATOR1's GAP activity toward RagA/B and allow it to become GTP-loaded. Simultaneously folliculin is activated, exhibiting its GAP activity toward RagC/D and converting it to its active GDP-loaded state. At this time, the elevated intralysosomal AA concentrations activate ATPase which activates Ragulator to act as a scaffold for the active Rag heterodimer and express GEF activity toward RagA/B, aiding their conversion to the GTP-loaded state. The Rag heterodimer then recruits mTORC1 to the lysosomal surface through binding to Raptor. This then places mTORC1 close to its direct activators, Rheb and phosphatidic acid, and an ample supply of AAs.

1.3.5 AA activation of mTORC1

Another widely described pathway of mTORC1 activation in many eukaryotic cells is that of AA induced mTORC1 activation. This pathway centres on mTORC1 recruitment to the lysosomal membrane and includes many of the proteins/complexes described in previous sections of this chapter. Intriguingly, the processes by which AAs are initially sensed in cells are yet to be fully elucidated. As previously discussed, potential AA sensors including the Sestrins and LRS have been purported yet direct evidence for this role remains inconclusive. Several other enzymes are also alleged to be AA sensors i.e. Vps34 and MAP4K3 and these will be discussed in detail in later chapters of this thesis. Nevertheless, it is now well established that when there is an increase in intracellular AAs, several differing pathways converge to recruit mTORC1 to the lysosomal membrane where it is in close proximity to Rheb and PA as well as an abundant supply of free AAs. The GATOR complexes, GATOR1 and GATOR2, function in opposing roles to negatively and positively regulate AA activation of mTORC1 respectively. During times of low AA availability, GATOR2 is not associated with GATOR1, which is active and has GAP activity toward RagA/B (106). This stimulates the Rags intrinsic GTPase activity and keeps RagA/B in the inactive, GDP-loaded state (97, 106). Upon AA stimulation, GATOR2 is able to complex with GATOR1 and inhibit this complex, possibly through the removal of Sestrins, from GATOR2, by an association with leucine (106, 107, 111, 112). The inhibition of GATOR1 removes its GAP activity from RagA/B, reducing its GTPase activity and allowing a conversion back to the active GTP-loaded form. Simultaneously to this, and also affecting RagA/B, the v-ATPase-Ragulator-Rag protein axis is activated through and inside-out mechanism (29, 97, 98). Here, the v-ATPase recognises an increase in intralysosomal AAs and causes the activation of the Ragulator to act as a scaffold for the Rag proteins. These proteins can then recruit

mTORC1 to the lysosome through binding with RAPTOR (97). Further, the Ragulator complex has a secondary role in which it possesses GEF activity toward RagA/B aiding in the conversion of this protein from the GDP- to the GTP-loaded state when GATOR1 has been inhibited. The second half of the Rag heterodimer, comprised of RagC/D is also affected by AAs. The folliculin-FNIP2 complex becomes activated by AAs and possesses GAP activity towards RagC/D (105, 110). This means that under AA stimulation, folliculin activates the conversion of RagC/D from the GTP- to GDP-loaded state and combined with the effects of other GAPs and GEFs allows the Rag heterodimer to be fully active (RagA/B^{GTP}-RagC/D^{GDP}). mTORC1 is then recruited to the lysosomal surface where both Rheb and PA are able to bind with the catalytic and FRB domains of the mTOR protein respectively (83, 89, 103, 127, 128) and activate its kinase activity. Interestingly, enzymes which produced PA such as PLD1 are noted to elevate in activity when levels of AAs are high, therefore producing more of this direct mTOR activator (89, 102, 103). This pathway of AA-induced mTORC1 activation is also depicted in Figure 1.2.

1.4 – mTORC1 activation in skeletal muscle

1.4.1 Growth Factors/Hormones

Both growth factors and hormones are noted to induce hypertrophy of muscle cells both *in vitro* and *in vivo* (120, 129, 130). Seminal work in C2C12 myotubes displayed that incubation with IGF-1 (10ng/ml) for 15 minutes elicited a substantial increase in S6K1^{Thr389} phosphorylation, an alteration which was subsequently blocked when rapamycin was co-incubated (120). This work suggested that IGF-1 stimulated the phosphorylation of S6K1 in an mTORC1 dependent matter. Furthermore, a second downstream target of mTORC1, 4EBP1, exhibited an upward gel shift suggesting an

increased phosphorylation status (120). The authors then demonstrated that expression of a dominant-negative form of the phosphatase SH2 domain containing inositol 5-phosphatase 2 (SHIP2), which dephosphorylates AKT elicited a greater hyperphosphorylation of S6K1, in response to IGF-1, when compared to a scrambled vector (120). Therefore, it was apparent that in muscle cells, IGF-1 stimulates mTORC1 through the canonical pathway. In subsequent studies, this group demonstrated that 48h of IGF-1 incubation elicited hyperphosphorylation of S6K1, an effect again blocked by rapamycin (121). Moreover, this effect inversely regulated atrophy-related genes (121), contributing to a shift toward cellular anabolism. Other reports also concur these findings, displaying that 2h IGF-1 exposure significantly elevated mTORC1 activity in C2C12 myotubes, possibly via a mechanism dependent on Focal Adhesion Kinase (FAK) (131). Additionally, myotubes exposed to a stretching stimulus were noted to release IGF1, which in turn phosphorylated S6K1 (132). Overall, it is well established that growth factors such as IGF-1 can activate mTORC1 through the PI3K/AKT pathway, resulting in the phosphorylation, and subsequent removal of TSC2 from Rheb.

Intriguingly, however, it has now become well established that growth factor mediated stimulation of mTORC1 is not a prerequisite for load-induced mTORC1 activation *in vivo*. Original research purported a paradigm by which mechanical strain induced an increase in IGF-1 production, which then activated the PI3K/AKT/mTOR axis, stimulating MPS and allowing hypertrophy to occur (133). Several models have since rejected this hypothesis, finding that the inhibition or genetic removal of IGF-1 signalling pathways did not diminish the activation of mTORC1 in response to mechanical stimuli (134-136). Furthermore, an elegant *in vivo* model in human skeletal muscle has echoed these findings. West et al. (137) utilised a unique study design where by participants

completed a single arm, unilateral resistance exercise bout either alone or following a heavy bilateral lower body resistance exercise bout. This produced two distinct systemic growth factor milieu, where significant elevations in systemic IGF-1 levels were only noted when the lower body exercise bout was completed (137). Interestingly, mTORC1 activation and MPS were similar in response to each exercise bout suggesting systemic levels of growth factors did not govern the anabolic response of skeletal muscle. Therefore, it is apparent that although growth factors can activate mTORC1 through the PI3K/AKT/mTOR axis, they most likely do not play a significant role *in vivo* in human skeletal muscle.

In addition to growth factors such as IGF-1, hormones are also known to activate mTORC1 (130, 138). Testosterone is considered the main 'anabolic' hormone and has been shown to initiate increases in mTORC1 signalling *in vitro* in as little as 5 minutes (138). Testosterone also elicits dose- (139) and time-dependent (130, 138) increases in mTORC1 activity and causes hypertrophy *in vitro* in L6 myoblasts (130) and isolated primary rat myotubes (138). Several studies have also investigated the mechanisms by which this stimulation of mTORC1/hypertrophy occurs. In rodent primary myotubes, testosterone administration for 60 minutes elicited 3-fold elevations in $S6K1^{Thr389}$ phosphorylation (138). Furthermore, incubation with testosterone for 12h caused significant hypertrophy, however both these effects did not occur in the presence of PI3K, AKT or mTOR inhibitors (138). These findings suggested that the effects of testosterone on muscle anabolism occurred through the PI3K/AKT/mTORC1 pathway, similar to many other growth factors. Other studies however, contradict these findings displaying that varying lengths of testosterone incubation stimulate rises in $S6K1^{Thr389}$ phosphorylation without concomitant elevations in AKT phosphorylation (130). These

authors suggested that testosterone may signal through extracellular signal-regulated kinase 1/2 (ERK1/2) as elevations in ERK1/2$^{Thr202/Tyr204}$ phosphorylation occurred alongside changes in S6K1^{Thr389} phosphorylation upon testosterone administration. Furthermore, when testosterone was removed, the phosphorylation of both of these proteins returned rapidly to baseline. In agreement with these results, White et al. (139) noted increases in AKT^{Ser473} phosphorylation only when testosterone concentrations of 500nM were administered to C2C12 myoblasts, whereas lower concentrations (5nM & 50nM) elicited significant elevations in ribosomal protein S6 (rpS6)$^{Ser235/236}$ phosphorylation, a common readout of mTORC1 activity. Here, however, the phosphorylation of downstream substrates of AKT (GSK3β^{Ser9} & FoxO3a^{Ser253}) were observed to rise with lower testosterone concentrations suggesting earlier AKT activity may have been missed (139). Nevertheless, further research is needed in order to elucidate the full mechanism which underpins testosterone mediated mTORC1 activation, a process probably occurring through both AKT and ERK1/2 pathways.

In vivo experiments have also examined the effects of testosterone on mTORC1 activity in skeletal muscle. An elegantly designed study in rodents utilising 42 days of castration significantly reduced AKT^{Ser473}, S6K1^{Thr389} and 4EBP1$^{Thr37/46}$, occurring alongside reductions in both fibre cross-sectional area (CSA) and MPS (139). This implicated a role for systemic testosterone in the maintenance of muscle anabolism and mass and again suggested testosterone may act through the canonical growth factor signalling pathway. In humans however, systemic levels of testosterone do not seem to be important for load-induced skeletal muscle anabolism. The work of West et al. (137), as described earlier, created an exercise protocol which elicited two varying systemic 'anabolic' milieu whereby unilateral bicep exercise, conducted after lower-body resistance exercise,

occurred in the presence of elevated levels of growth factors and testosterone. In contrast, bicep exercise conducted alone occurred with no elevated levels of these factors yet displayed no differences in skeletal muscle anabolism (mTORC1 signalling and MPS) when compared to the bicep and lower body exercise trial (137). Moreover, when this resistance exercise paradigm was extended to a long-term training study, no differences in skeletal muscle hypertrophy, between the conditions, was observed (140). This therefore suggested that physiological rises in systemic hormone levels do not contribute to skeletal muscle anabolism in humans. It is apparent however, that alterations in hormone levels outside of the physiological range do affect skeletal muscle anabolism as previously purported (129, 141-143).

1.4.2 AAs

As previously discussed, AAs possess an ability to both transiently and potently activate mTORC1 to stimulate protein translation and cell growth in various eukaryotic cell lines (29, 97, 98, 107, 122). Much research has since focussed on the translation of this research into skeletal muscle both *in vitro* and *in vivo* in order to elucidate the signalling pathways by which AAs stimulate skeletal muscle hypertrophy. Correspondingly, AA solutions, in particular leucine, have been consistently observed to activate mTORC1-dependent signalling pathways (113, 144-148), often in a dose-dependent manner (144, 145) in C2C12, L6 and human myotubes. Interestingly, in several investigations, leucine is reported to be the only AA able to initiate a greater than 2-fold increase in S6K1^{Thr389} phosphorylation *in vitro* (113, 144, 145). Furthermore, AAs do not elevate AKT^{Ser473} phosphorylation (113) suggesting AAs activate mTORC1 via a pathway other than that of growth factors, an observation concurrent with that noted in other cell lines (29, 30, 97, 98, 122). Currently, however, no further investigations into the precise mechanisms

by which mTORC1 is activated by AAs in skeletal muscle cell lines has been conducted. Although it is probable that these mechanisms will be similar to those discovered by the elegant work from the Sabatini laboratory (29, 30, 42, 97, 98, 104-108), research confirming this is required.

The anabolic potential of AAs toward mTORC1 and its associated pathways have also been reported *in vivo* in both rodent and human skeletal muscle. In rodents, the infusion of an AA mixture (149), or the ingestion of protein (150), elicit skeletal muscle mTORC1 activation as measured by 4EBP1$^{Thr37/46}$ and S6K1^{Thr389} phosphorylation. Following this, several other laboratories repeated these results showing that various methods of elevating skeletal muscle AA levels caused an increase in mTORC1 activity (151-153). These effects were again observed to be primarily driven by leucine (153-155) and occur in a dose-dependent manner (153) as reported *in vitro*. Rapamycin administration attenuated the increase in mTOR-target phosphorylation, confirming AAs exert their anabolic effects on these targets via mTORC1 (151). Therefore, these data confirm that AAs can potently activate mTORC1 in rodent skeletal muscle.

In humans, the positive and dose-dependent effects of AAs are regularly reported (24, 114, 156-162), however in some instances dose-dependent relationships are not apparent (163). Seminal work from the laboratory of Eugene Barrett utilised AA infusions in human participants to understand their effects in skeletal muscle (164, 165). Here, infusions of AAs (165) or branched chain AAs (BCAAs) alone (164) elicited significant upward gel shifts in both 4EBP1 and S6K1 proteins, indicative of greater phosphorylation status. Interestingly, these elevations occurred with no change in AKT^{Ser473} phosphorylation (165), resonating the notion that AAs activate mTORC1 in a growth factor-independent manner (30). Further work by several prominent laboratory groups

exhibited how the simple ingestion of protein or AAs has a very similar effect of mTORC1 activity to that of infusions (157, 160, 166-168). In human skeletal muscle, leucine is again observed to be the most potent activator of mTORC1, with the ingestion of a leucine-enriched essential AA supplement prompting rapid, transient and significant increases in the phosphorylation of downstream mTORC1 targets (166). Furthermore, the addition of leucine to lower amounts of 'whole protein' sources can rescue phosphorylation of some mTORC1 targets to comparable levels to those noted with higher 'whole protein' doses (114). Through the unique use of rapamycin administration in humans, Dickinson et al. (24) displayed a complete attenuation of mTORC1-dependent signalling following essential AA ingestion, reiterating the concept that these proteins are phosphorylated in a mTORC1-dependent manner. In summary, it is well reported and accepted that AAs, and in particular leucine, can activate mTORC1-dependent signalling pathways *in vivo* in human skeletal muscle.

1.4.3 Mechanical Loading

One particular anabolic stimuli known to activate mTORC1 in skeletal muscle is that of mechanical loading. This paradigm is comparatively understudied *in vitro* as it does not reflect a valid physiological stimulus in the organs from which many cell lines are derived. Nonetheless, in skeletal muscle, the primary organ controlling locomotion, this stimulus occurs regularly in varying intensities and frequencies. Resistance exercise has garnered much attention in relation to mTORC1 as periodic repetitions of this exercise format often result in skeletal muscle hypertrophy (140, 169-179). In accordance with this, acute bouts of mechanical stretch, electrical stimulation and resistance exercise consistently elevates mTORC1-related signalling in rodent and human skeletal muscle (180). As previously discussed, the initial hypothesis of how mTORC1 was activated in

response to mechanical loading centred on an increase in circulating growth factors (132, 133). However, through the elegant use of the PI3K inhibitor wortmannin (134), genetic models (135) and unique resistance exercise models (137, 140), mTORC1 activation following a load stimulus is now believed to occur independently of growth factors and AKT signalling. These observations led to further investigations into the precise mechanisms by which mechanical loading activates mTORC1.

Pivotal work conducted in the Hornberger laboratory utilised immunofluorescent microscopy to study mTORC1 activation (90). Surprisingly, an event that occurs in response to growth factors, the removal of TSC2 from Rheb, also occurred following eccentric contractions in mouse skeletal muscle (90). This therefore suggested that proteins other than AKT could also elicit TSC2 removal and subsequent elevations in GTP-bound Rheb. Moreover, eccentric contractions were observed to increase the colocalisation of mTOR and a marker of the lysosome (LAMP2) (90), an event commonly reported to occur due to an elevation in AA availability (29-31, 97, 98, 104, 122). This notion may in fact be true in this context as resistance exercise is known to increase intracellular AA levels either through increased autophagy/breakdown or AA influx (181). Nevertheless, these results suggested a new mechanism whereby a currently unknown kinase phosphorylated TSC2 within its RxRxxS*/T* consensus motif (90), removing it from its association with Rheb and allowing Rheb to become GTP-loaded and active. Simultaneously mTORC1 may be recruited to the lysosomal membrane, where it can associate with Rheb, either due to an increase in intracellular AAs or by a hitherto undefined mechanism specific to skeletal muscle. Further work by this laboratory utilising inducible skeletal muscle-specific knockout models for both TSC2 and Rheb proteins identified 6 phosphorylation sites on TSC2 that were regulated by eccentric

contractions, the majority of which had not been reported previously (182). These phosphorylation sites were also distinct from those which had been identified in the canonical growth factor pathways adding credence to the notion that mechanical activation of mTORC1 occurs independently of IGF1/PI3K/AKT. All 6 of these phosphorylation sites were then shown to be required for mTORC1 activation following eccentric contractions, an effect that was no longer noted when TSC2 was mutated to become partially phospho-defective (182). This data would therefore suggest that it is the combination of TSC2 phosphorylation at these sites which stimulates its removal from Rheb following contraction. Intriguingly, the effects of eccentric contractions on mTORC1 activity were only reduced by ~50% in either genetic model (182) suggesting pathways other than the TSC2-Rheb axis may contribute to mTORC1 activation in this scenario.

A possible candidate for this contribution is the second mTOR direct activator, PA. PA concentrations in rodent skeletal muscle are observed to rise following eccentric contractions (89, 102, 103), and this occurs upstream of mTORC1 itself (89). PA is believed to accumulate at the lysosome where it can be in close proximity to mTORC1, allowing efficient activation through binding of the FRB domain (103). *In vitro*, PA exhibits a dose-dependent relationship with S6K1^{Thr389} phosphorylation in C2C12 myoblasts and exerts its effect in an insulin/growth factor independent manner, suggesting PA alone activates mTORC1 (89). The inhibition, or genetic deletion, of the two main enzymes which produce PA, PLD1 and DGKζ, significantly reduces S6K1^{Thr389} phosphorylation in response to eccentric contraction or stretch (89, 102). Again, in agreement with previous data, this inhibition/deletion did not completely abolish the effects of mechanical load on mTORC1 activation, confirming the belief that multiple

pathways regulate mTORC1 following contraction (102, 182). Therefore it seems that in response to mechanical load/muscle contraction TSC2 becomes phosphorylated and is removed from Rheb, Rheb's intrinsic GTPase activity is no longer activated and then becomes GTP-loaded and active. Simultaneously, PA production is stimulated, now believed to be mainly catalysed by DGKζ (102) , and accumulates at the lysosome. mTORC1 is then recruited to the lysosome, most likely as a result of an elevation in intracellular/intralysosomal AAs, where it is in close proximity to both of its direct activators. Even though this mechanism is now well characterised in rodent skeletal muscle, research regarding this topic in humans is scarce and as such requires further investigation.

1.5 – Downstream effects of mTORC1 activation

Once activated, the mTORC1 kinase complex is able to phosphorylate a myriad of downstream targets, many of which are encompassed in the areas of protein translation and autophagy. The next section of this thesis will describe how mTORC1 affect both of these cellular mechanisms, focussing on how these apply to skeletal muscle plasticity.

1.5.1 Stimulation of protein translation

1.5.1.1 S6K1

The most characterised consequence of mTORC1 activation is the stimulation of protein translation. This effect is mediated through the phosphorylation of mTORC1's most well-known substrates, S6K1 and 4EBP1, which as previously discussed, are often used as readouts of mTORC1 activity (25). When phosphorylated, specifically at Threonine residue 389, S6K1 kinase's activity is elevated. S6K1 has several downstream targets which all contribute toward efficient protein translation. Firstly, S6K1 will phosphorylate

rpS6 at several different serine residues (including Ser235/236 and Ser240/244) (25, 183). The specific function of this phosphorylation was initially believed to allow enhanced recruitment of 5'TOP mRNAs to ribosomes (183). This was concluded as S6 phosphorylation correlated highly with the translation of these mRNAs (183). More recently, however, this mechanism has been questioned as mice expressing a phospho-deficient form of rpS6 are still able to alter the translation of 5'TOP mRNAs (184). Interestingly, a physiological role for rpS6 phosphorylation remains elusive, with some reports even suggesting it negatively regulates protein synthesis (184). Nevertheless it still serves as a valid and accurate readout of S6K1 activity (25).

A second target of S6K1's kinase activity is eIF4B, a translation pre-initiation factor. This protein is phosphorylated on Serine residue 422 (185), an event which seems to positively regulate translation. When phosphorylated, eIF4B is able to associate with eIF4G/eIF4A/eIF3, a complex which recruits the 40S ribosomal subunit to mRNA strands (186). Here eIF4B activate the helicase activity of eIF4A, which acts to unwind the mRNA's secondary structure permitting more efficient binding of the ribosome to mRNA strands (187). Ribosomal footprinting assays have been utilised to confirm that eIF4B is needed for the translation of mRNAs containing a secondary structure (188), whilst RNA interference targeting eIF4B inhibits the translation of these mRNAs (189). Furthermore, recombinant, unphosphroylated forms of eIF4B are unable to enhance translation, suggesting eIF4Bs activity is dependent on its phosphorylation status (188). Therefore, it can be postulated that this is a mechanism by which mTORC1 elicits S6K1-dependent effects on protein translation.

The translation elongation factor eEF2 is also regulated in an mTORC1 dependent fashion. When mTORC1 is activated, the phosphorylation of eEF2 at Thr56, an inhibitory

site, is reduced (190). This reduced inhibition allows eEF2 to bind with ribosomes and accelerate translation elongation (190-192). The phosphorylation of eEF2 at this site is governed by eEF2 kinase, which in turn can be phosphorylated, and inhibited, at 3 different sites in times of high mTORC1 activity (193-195). Of note, one particular phosphorylation site on eEF2 kinase, Ser366, is believed to be phosphorylated by S6K1 (193). Following this phosphorylation eEF2 kinase is inactivated and eEF2 is free to bind to ribosome and enhance protein translation (190). Therefore, characterisation of the unknown kinases controlling the phosphorylation of the further phosphorylation sites on eEF2 kinase will provide extra insight into the regulatory role mTORC1 demonstrates towards protein translation.

1.5.1.2 4EBP1

The second direct target of mTORC1 implicated in protein translation is 4EBP1, however, unlike S6K1, mTORC1-dependent phosphorylation of this target exhibits inhibitory effects (196). 4EBP1 is a binding protein of eIF4E, a translation initiation factor whose role involves the recruitment of the 40s ribosomal subunit to the 5' end of nuclear transcribed mRNA strands (197). The binding of 4EBP1 to eIF4E is inhibitory as it competes for the binding site at which eIF4G associates with eIF4E allowing the recruitment of the 40S ribosomal subunit to mRNA strands (198). mTORC1-dependent phosphorylation of 4EBP1 occurs at 4 different serine residues, which are phosphorylated in a hierarchical manner and are all required for the removal of 4EBP1 from eIF4E (196, 199). Initially, serine residues 37 and 46 are phosphorylated (196), acting to prime 4EBP1 for subsequent phosphorylation of Ser65 and Ser70 and removal of 4EBP1 from eIF4E (196, 198). These phosphorylation sites are noted to be in close proximity to the site at which 4EBP1 binds to eIF4E, and as such may create electrostatic repulsion of eIF4E due

to the additional phosphate molecules (25, 200). Hereafter the binding site for eIF4G is unblocked and the 40S subunit can be recruited to the mRNA strand where translation can be initiated (199).

1.5.1.3 Other Targets

Several other proteins display phosphorylation events which seem to be mTORC1-dependent. The two proteins which comprise the eIF4G scaffolding complex, eIF4GI and eIF4GII, become phosphorylated on 3 different serine residues (1148, 1188 & 1232) when mTORC1 activity is high (201). The precise mechanism and role of this phosphorylation is unclear, however it most likely causes a conformational change in these proteins which allows more efficient construction of the ribosomal pre-initiation complex (201). Therefore, eIF4G is the third component of the pre-initiation machinery which is regulated by mTORC1 activity. The most intriguing of potential mTORC1 targets is PP2A, a phosphatase which catalyses the dephosphorylation of mTORC1 substrates (202). mTORC1 is observed to phosphorylate this phosphatase *in vitro*, seemingly inhibiting its activity and preventing the dephosphorylation of S6K1 and 4EBP1 (200). Thus mTORC1 may exert positive effects on substrate phosphorylation via a two-fold mechanism whereby substrates are phosphorylation occurs directly by mTORC1 itself and dephosphorylation is reduced indirectly via inhibition of PP2A. The positive effect of mTORC1 on protein translation seems to predominantly occur through enhancing the recruitment of the 40S subunit to mRNA, via the efficient construction of the pre-initiation complex, whilst also enhancing translation elongation by inhibiting eEF2 kinase. This intricate mechanism of the regulation of protein translation by mTORC1 is depicted in Figure 1.3.

1.5.1.4 mTORC1-dependent protein translation in skeletal muscle

As previously discussed, mTORC1 is a popular topic of investigation in skeletal muscle due to its role in augmenting cellular growth potentially contributing to hypertrophy. Therefore, the downstream effects of mTORC1 are regularly measured in response to anabolic stimuli in this tissue. Mechanical loading and AA ingestion are known to elevate the phosphorylation of downstream mTORC1 substrates in both rodent and human skeletal muscle (156, 160, 162, 166, 169, 176, 178, 203-207). For example, the phosphorylation of both S6K1 (23, 24, 162, 166) and 4EBP1 (24, 166) is elevated transiently following contraction and/or feeding, although the extent of this phosphorylation is not always congruent between the two substrates (23, 24, 162). In addition, the phosphorylation levels of differing 4EBP1 residues often do not align (208), possibly due to the hierarchical fashion in which this protein is phosphorylated and the inability to measure rapid perturbations in phosphorylation in skeletal muscle. Downstream of S6K1, rpS6$^{Ser235/236}$ & rpS6$^{Ser240/244}$ phosphorylation is a measure often utilised by muscle physiologists as a readout of S6K1 activity (205, 209), and is noted to significantly increase in response to various muscle loading and feeding protocols (167, 205, 209-212). Translation elongation, measured indirectly through the phosphorylation of eEF2, has also been shown to be altered in response to muscular contraction and/or protein feeding (23, 162, 166, 208, 213). Therefore, it is apparent that the downstream effects of mTORC1, on proteins implicated in protein translation, extend to skeletal muscle and allow valid measurements of mTORC1 activity in this tissue.

The culmination of these signalling pathways, protein translation, is also regularly measured in skeletal muscle via the use of stable isotope methodology (22). Here, the infusion of an AA with a higher mass, due to increased neutrons within its nucleus, than

its regular form produces a scenario whereby the incorporation of this AA into newly synthesised proteins can be measured using mass spectrometry (22, 214). Comparing the incorporation of this AA between time points can then be used to infer the amount of protein translation within a given tissue in this timeframe, a paradigm referred to as fractional synthesis rate (FSR) (215). Early studies displayed that mixed muscle FSR was enhanced by an acute bout of mechanical loading (18, 216-218), and this effect remained for 36-48h (18, 218), albeit in a small sample size. Since reporting these seminal findings, research conducted in larger subject populations has replicated these findings in specific functional fractions of human skeletal muscle (137, 162, 206, 213, 219, 220). Specifically, acute bouts of resistance exercise elicit large elevations in MPS/FSR in the initial recovery period (up to 6h) (137, 219, 221-223), decreasing progressively and returning to baseline values by 24-48h post-exercise, dependent on training status (18, 218, 224). Oral ingestion of the mTORC1 inhibitor, rapamycin, prior to a resistance exercise bout completely abolished the post-exercise augmentation of MPS/FSR in human skeletal muscle (23), providing direct evidence that mTORC1-dependent signalling is required for resistance exercise mediated increases in protein translation.

AAs and whole proteins are also known to stimulate MPS/FSR. Early studies utilised intravenous AA infusions to study these effects, finding that these infusions can raise mixed muscle FSR by up to 100% (20). Later research then suggested that AA-stimulated increases in FSR were associated with increased mTORC1 signalling (157, 166, 223, 225-229). Furthermore, this enhancement of FSR is often seen to occur dose-dependently (12, 163) before arriving at a ‘breakpoint’ where FSR is maximally stimulated and greater protein intake will have no effect (230). This point has recently been coined the ‘muscle-full’ effect, as time at which muscle cells reach their maximum capacity of intracellular

AAs (160, 231). Of the 20 proteinogenic AAs found in eukaryotes, the nine EAAs are believed to be the main drivers of MPS (232). Indeed, ingestion of a combination of only EAAs stimulates increases in both mTORC1 signalling and MPS/FSR (159, 162, 206, 208, 232-234). In particular, the BCAAs, leucine, isoleucine and valine, seem to contribute considerably to the effect of EAAs on muscle protein translation (208, 235). Leucine itself has been suggested as the most potently anabolic BCAA and, as previously discussed, can increase mTORC1 related signalling when ingested alone (162, 208). These effects translate to FSR measurements where leucine ingestion alone significantly elevates rates of MPS (162, 208). However, these elevations are of lower magnitude compared to those reported with essential AA or whole protein ingestion (162, 208). This led to the hypothesis that leucine acts to stimulate FSR/MPS, however, the other EAAs are needed to act as substrates in this process (114). To this end, the addition of leucine to lower amounts of whey protein rescues FSR to a similar level to that seen when higher amounts of whey protein are ingested (114). Rapamycin ingestion alongside EAAs blocked increases in FSR/MPS (24), reaffirming the notion that MPS responses to anabolic stimuli are governed by mTORC1.

Interestingly, the combination of these two anabolic stimuli are often observed to elevate rates of FSR over and above those witnessed when either practice is completed in isolation (12, 20, 220, 221, 236). This synergistic effect of mechanical loading and protein ingestion is in fact the only method by which NPB can become positive, as the large rise in MPS exceeds the exercise-induced rise in MPB (20). When these practices are completed regularly over a sustained period of time, skeletal muscle hypertrophy occurs to a greater extent than when resistance exercise is completed without protein supplementation (170, 171, 237-239). In addition to this synergism, a single bout of

resistance exercise seems to 'sensitise' the muscle to AA intake for up to 48h post-exercise (224). Here any protein ingestion enhances rates of FSR to a greater extent than when ingested at rest, although the extent to which rates are stimulated decrease progressively throughout this timeframe (224). The molecular basis for these synergistic effects is most likely through the divergent mechanisms by which each stimuli can activate mTORC1 kinase activity. As previously discussed, mechanical load/resistance exercise elicits an increase in the phosphorylation of TSC2 within its RxRxxS*/T* consensus motif, removing it from its association with Rheb/the lysosome and allowing Rheb to become GTP-loaded (90, 182). Production of PA also increases through enhanced activity of DGKζ, and possibly PLD1, and associates with the lysosome (89, 102, 103). The simultaneous ingestion of AAs/protein elevates intralysosomal/intracellular AAs, activating the v-ATPase/Ragulator/Rag complex, recruiting mTORC1 to the lysosome (29, 97, 98). mTORC1 is therefore in closer proximity to its direct activators and an abundance of AAs, the substrates for the process of MPS, becomes active and stimulates protein translation. Completion of either activity alone will only initiate portions of the process and therefore the stimulation of protein translation will be lower. Thus, it can be concluded that anabolic stimuli enhance protein translation in human skeletal muscle and this happens in an mTORC1-dependent manner, most likely through the downstream mechanisms discussed.

1.5.2 Repression of autophagy

1.5.2.1 ULK1 & Atg13

Recent advances in the field of mTORC1 signalling have discovered that mTORC1 is able to directly inhibit autophagy. The first complex which mTORC1 is known to affect in this process is the Unc-like kinase 1 (ULK1) complex, comprised of the kinase ULK1

bound to 3 regulatory subunits, Autophagy related protein 13 (Atg13), Atg101 and focal adhesion kinase family interacting protein of 200 kD (FIP200) (240). The action of this complex when active is to phosphorylate downstream targets, in particular Beclin1 (240, 241), which in turn cause the nucleation of newly formed phagophores (241). mTORC1 is believed to phosphorylate two components of the ULK1 complex, ULK1 itself at serine residues 637 and 757 (242, 243) and the regulatory subunit Atg13 at Ser258 (244). The addition of phosphate groups to these particular residues on ULK1 elicits a conformational change to this protein such that it can no longer associate with AMPK (243), which normally phosphorylates and activates ULK1 under nutrient-deplete conditions (242). Indeed, under nutrient sufficient conditions the phosphorylation of $ULK1^{Ser757}$ is observed to rapidly increase, whereas the phosphorylation of this kinase at AMPK-specific sites is markedly reduced (242). The removal of this potential activation reduces ULK1s ability to phosphorylate Beclin1 and initiate phagophore nucleation.

The second substrate of mTORC1 in the ULK1 complex, Atg13, is also phosphorylated following serum-stimulation in MEF cells (244). This phosphorylation event reduces ULK1 kinase activity and is regulated in an mTORC1-dependent manner (244). The precise mechanism by which $Atg13^{Ser258}$ phosphorylation is believed to inhibit ULK1 kinase activity is through a reduction in ULK1s translocation to locations of need (244). Thus, ULK1 is no longer able to move to the cellular locations where its downstream substrates reside. Interestingly, the phosphatase PP2A has been shown to dephosphorylate ULK1 at its mTORC1-specific site (245), relieving this inhibition during starvation and allowing ULK1 to associate with AMPK. As previously mentioned, when nutrient levels are high, PP2A is phosphorylated by mTORC1 causing its inhibition. Therefore, it seems mTORC1 also represses autophagy induction by a dual mechanism whereby the

phosphorylation of ULK1 is directly affected and its dephosphorylation is indirectly inhibited, similar to the mechanism noted in the stimulation of protein translation. In summary, mTORC1 acts to reduce the kinase activity of the ULK1 complex which impacts autophagy induction through a reduction in phagophore nucleation.

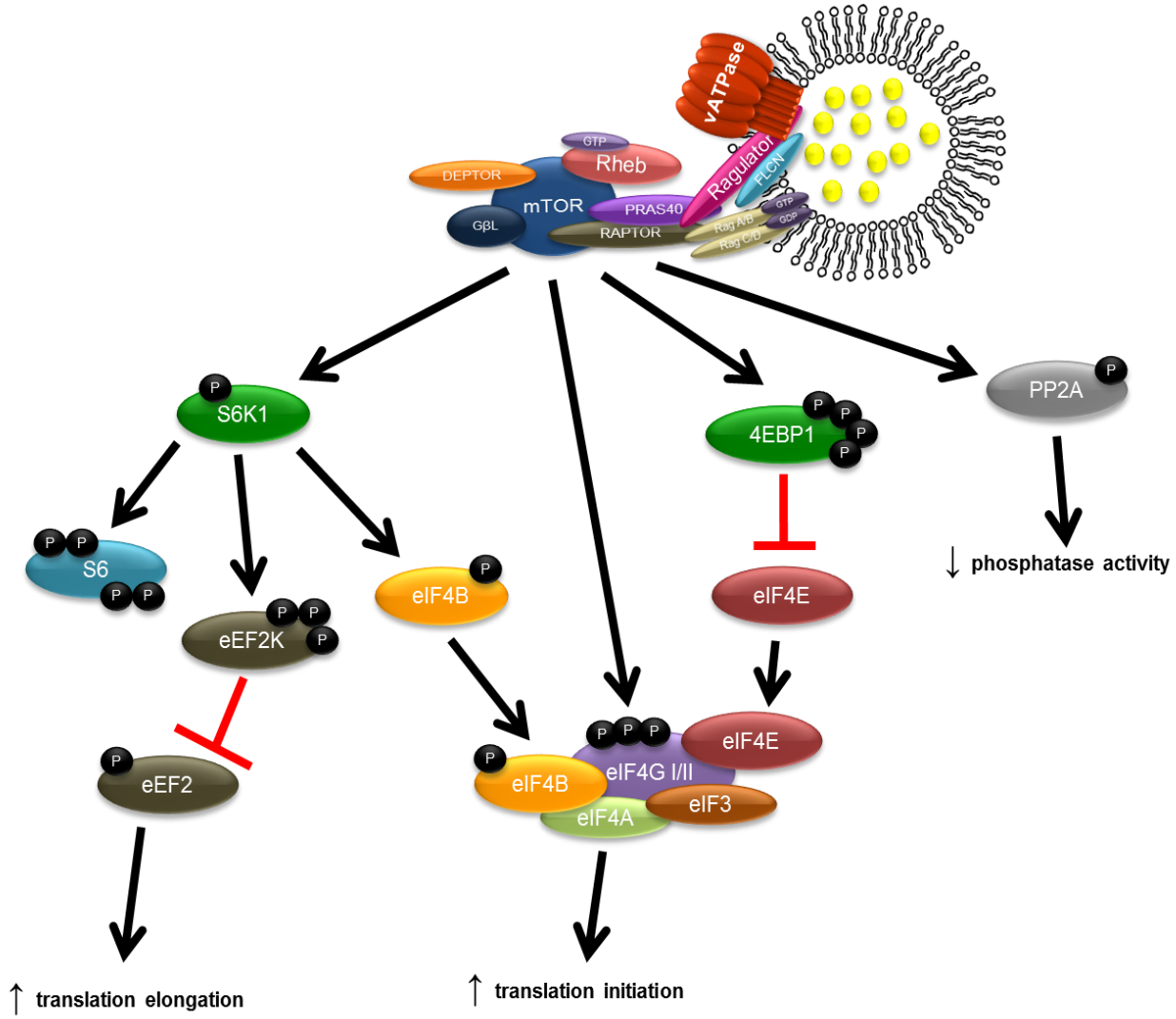

Figure 1.3. mTORC1-dependent regulation of protein translation

When mTORC1 is activated, this kinase complex phosphorylates several downstream targets to elevate protein translation. mTORC1 directly phosphorylates S6K1 at Thr389, activating this kinase. S6K1 then phosphorylates 3 downstream targets, the first of which is ribosomal protein S6 which becomes phosphorylated at Ser235/236 and Ser240/244, although the function of this phosphorylation remains unknown. S6K1 also phosphorylates eIF4B at Ser422, allowing this initiation factor to elevate eIF4A's helicase activity to elevate the translation of mRNA strands with a secondary structure. The final target of S6K1 is eEF2 kinase which becomes inhibited, alleviating its inhibition of eEF2 and enhancing translation elongation. The second target of mTORC1 is 4EBP1 which is phosphorylated, and inhibited, at 4 sites. This removes 4BP1's inhibition of eIF4E and allowing the recruitment of the 40S ribosomal subunit to mRNA strand. mTORC1 can also phosphorylate eIF4G, a component of the pre-initiation complex, at 3 sites which allows the more efficient construction of this complex. Finally, mTORC1 phosphorylates the phosphatase PP2A, reducing its activity toward other mTORC1 targets.

1.5.2.2 Atg14

The second autophagic complex which is directly inhibited by mTORC1 kinase activity is the Vps34 complex, which is comprised of 4 proteins, Vps34, Vps15, Beclin1 and Atg14 (246). Vps34 is a PI3Kinase which directly phosphorylates phosphatidylinositol to generate phosphatidylinositol-3-phosphate (PI3P) (247), a compound which can control vesicle trafficking (248). Further research into the function of PI3P has implicated it as an essential component in autophagy induction (246). This PI3Kinase has been implicated in several differing, and in some cases opposing, cellular processes and as such it is now believed that the other components of each Vps34 complex direct it activity toward differing pathways (249). In the autophagic complex, Atg14 seems to be of specific importance to Vps34-dependent autophagy regulation (249). A regulatory role of mTORC1 toward the complex was first revealed by the finding that exposing MEF cells to rapamycin elevated the activity of this specific Vps34 complex (250). Results from several *in vitro* assays suggested that mTORC1 was in fact regulating Atg14 specifically. Mass spectrometry analysis then confirmed this notion, finding 5 residues on the Atg14 peptide chain which were directly phosphorylated by mTORC1 (250). When these sites were mutated to block phosphorylation mTORC1 could no longer inhibit Vps34-dependent PI3P production *in vitro*. HEK293 cells expressing phospho-deficient Atg14 had greater autophagic flux under nutrient-rich conditions (250), confirming the inhibitory role mTORC1 would normally exert at these times. Thus mTORC1 can directly affect another site of autophagy induction, alongside its inhibitory role toward the ULK1 complex. Interestingly, as the ULK1 complex can also phosphorylate Beclin1 (241), a component of the autophagic Vps34 complex, it seems mTORC1 also exerts an indirect inhibitory effect toward this complex via ULK1.

1.5.2.3 TFEB

The final mechanism by which mTORC1 can inhibit autophagy is at the gene transcription level. Transcription factor EB (TFEB) is a transcription factor which regulates the expression of a variety of mRNAs involved in both autophagy and lysosomal biogenesis (251). It was recently displayed that mTORC1 can directly phosphorylate this transcription factor at Ser211 (252, 253). The action of this phosphorylation event is inhibitory, sequestering TFEB to the cytoplasm via an association with 14-3-3 binding proteins (252). Here TFEB is then unable to associate with DNA and elevate gene transcription. Upon nutrient withdrawal, TFEB is rapidly dephosphorylated and localises to the nucleus (252). Intriguingly, TFEB also controls the transcription of several proteins implicated in mTORC1 activation including RagD and certain components of the v-ATPase (254, 255). This suggests a potential feedback mechanism which reduces the transcription of mTORC1-related genes when this kinase complex's activity is elevated. Nevertheless this is a third intricate mechanism by which mTORC1 inhibits autophagy, whilst simultaneously elevating protein translation.

1.5.2.4 mTORC1-dependent autophagy inhibition in human skeletal muscle

The effect of anabolic stimuli on autophagy regulation in skeletal muscle has begun to become a more popular tropic of research in recent times. Several investigations have studied this paradigm, however results remain equivocal. Initial research from the Rasmussen laboratory (256) reported that an acute bout of resistance exercise increased autophagy for at least 24 hours post-exercise. Specifically, the conversion of microtubule-associated proteins 1A/1B light chain 3bI (LC3bI) to LC3bII, indicative of autophagy induction, was reduced for this entire period which is suggestive of reduced

autophagasome formation (256). Another study found that LC3bI protein expression was reduced at 1 and 4h following a resistance exercise bout, depending on protein supplementation (34). The levels of LC3bII protein were not reported in this study making conclusions about autophagy initiation challenging. Interestingly, this seems to be the only investigation to study mTORC1-dependent ULK1^{Ser757} phosphorylation in human skeletal muscle. Here, phosphorylation at this site was increased at 1h following exercise only when protein was ingested following the bout (34). This suggests there may be a threshold of mTORC1 activation needed before ULK1 phosphorylation occurs.

A combination of resistance and endurance exercise has also been observed to elicit changes in some markers of autophagy in human skeletal muscle. Specifically, this concurrent exercise bout, followed by protein ingestion caused attenuation in LC3bII protein levels at 4 hours post-exercise, whilst LC3bI expression remained similar suggesting blunted autophagy induction (257). Again, this may have been partly driven by an increase in mTORC1-associated autophagy inhibition as mTORC1 activation is known to increase in response to such exercise bouts (213). Moreover, the amount of nuclear TFEB protein expression was decreased 8h following this exercise bout (257), suggesting possible cytoplasmic sequestration of this protein due to mTORC1-driven phosphorylation at TFEB^{Ser211}. This notion was not reflected in cytoplasmic TFEB levels which were largely unchanged and even decreased slightly at the same time point (257). Therefore, as little research has been conducted into this area, and results are contradictory, it is difficult to draw full conclusions at this time.

1.6 - Novel Regulators of mTORC1

The mechanisms by which mTORC1 is activated by anabolic stimuli, and how mTORC1 affects its downstream targets, have been well characterised, principally *in vitro*.

However, there still remain certain gaps in the knowledge of this field. In particular, the mechanisms by which both AAs and mechanical loading are 'sensed' and their signals propagated to mTORC1 have not yet been elucidated, especially in skeletal muscle (30, 31, 90, 92, 182, 258). The next section of this thesis will discuss three potential novel regulators of mTORC1 activation which are beginning to garner attention. Furthermore the investigation of these mechanisms in human skeletal muscle will be the focus of this thesis hereafter.

1.6.1 Lysosomal Trafficking

As discussed, several cell and rodent-based investigations have suggested that the recruitment of mTORC1 to the lysosome is a critical factor in mTORC1 activation in response to elevated AA availability (97, 98, 104, 105). However, more recently, a role for the translocation of mTORC1-lysosome complexes has been proposed as an additional layer of mTORC1 activation (33). Korolchuk et al. (33) first proposed this hypothesis following the observation that physiologically relevant AA deprivation, milder in nature to that previously utilised (29, 97), did not result in mTOR disassociation from the lysosome yet reduced mTORC1 activity in HeLa cells. This notable observation suggested that under physiological nutrient-deprived conditions, i.e. the post-absorptive period in human skeletal muscle when autophagy and protein breakdown increase to maintain intracellular AA concentrations (38, 259), mTORC1 activity may not be governed by lysosomal association. Following this, the authors reported an association between the number of cells with predominantly peripheral lysosomes, measured through immunofluorescence microscopy, and the extent of mTORC1 activation following AA stimulation. This notion was then tested more directly through the use of nocodazole, a drug which depolymerises microtubules and prevents lysosomal movement. When this

drug was administered to cells, the response of mTORC1 activity to nutrient reintroduction following deprivation was abolished (33). Further investigation through the use of siRNA targeting a protein implicated in lysosomal movement, ADP-ribosylation factor-like protein 8B (ARL8B), confirmed these observations. The knock down of this protein fixed lysosomes close to the nucleus of HeLa cells, and prevented $S6K1^{Thr389}$ phosphorylation irrespective of intracellular AA levels (33). Moreover, when this protein was overexpressed in HeLa cells, the amount of peripheral lysosomes were raised by 350% but mTORC1 activity was only elevated when AA concentrations were sufficient (33).

This data has been replicated in the osteosarcoma cell line U2OS (260) expressing an increased activity of the transcription factor E2F1. Upon activation of E2F1, lysosomal associated membrane protein 2 (LAMP2)-positive structures (lysosomes) were seen to translocate to the cell membrane and once again this movement coincided with an increase in mTORC1-dependent S6K1 phosphorylation. Furthermore, this movement was displayed to be a result of a v-ATPase-dependent mechanism as the use of siRNA targeting V0 subunit C of ATP-ase resulted in a reduction in mTORC1 activity and peripheral lysosome content. These data, taken together, suggest that the cellular localisation of mTOR-lysosome complexes, rather than the trafficking of mTOR to the lysosome, could be a fundamental regulator of mTORC1 activation. We believe these differing findings are a result of the divergent nutrient-deprivation models utilised (complete vs. milder and more 'physiologically relevant'). Therefore, this potential mechanism of mTORC1 activation is more likely to relate to the physiological processes occurring in human skeletal muscle, and consequently deserved further investigation.

Recently, our laboratory has made significant advances in this field of research by investigating the translocation of mTOR/lysosomal compartments within human skeletal muscle following resistance exercise in the presence or absence of protein-carbohydrate feeding (261). Similar to the findings of Korolchuk et al. (33), and contrary to complete starvation protocols (29, 97, 98, 113), our results display no changes in co-localisation of mTOR and the lysosome between the initial post-absorptive period and any post-exercise/feeding time point, reinforcing the notion that physiological states of nutrient deprivation do not appear to alter mTOR localisation at the lysosome (33). In further agreement with Korolchuk et al. (33) we also displayed an increase in mTOR and LAMP2 localisation with a marker of the muscle plasma membrane (WGA) following resistance exercise with or without feeding (261). This translocation of mTOR/LAMP2-positive structures was accompanied by a significant increase in S6K1 activity in both subject cohorts, with a greater increase apparent in subjects consuming a protein-carbohydrate beverage post-exercise, a finding consistent with previous data in the field (224, 262). An increase in S6K1 activity is suggestive of a greater phosphorylation status in response to mTOR activation and results from these kinase assays are proposed to be comparable to immunoblotting techniques targeted toward mTOR activity (S6K1-Thr389) (162). Therefore, together with the findings discussed previously (33), our data reinforces the notion that mTORC1/lysosomal translocation is a principal event mediating mTORC1 activation following both resistance exercise and nutrient availability. However, as this area of research in human skeletal muscle is still in its infancy, more investigations into mTOR-lysosomal trafficking are need to fully characterise this mechanism.

1.6.2 LAT1

It is well established that skeletal muscle cells require an abundance of AAs in order for anabolism to be stimulated (11). One mechanism by which AAs are provided to cells is through AA transport, a vital cellular function (263). This process is catalysed by AA transporters, whose action is to move AAs across plasma membranes (264). As alterations in the activity of these transporters could affect mTORC1 activation, and subsequent skeletal muscle anabolism, these proteins have garnered greater attention in recent times. In particular, the L-Type AA transporter 1 (LAT1) is purported to be central in skeletal muscle as it controls the transport of leucine (265), and other BCAAs, the most potently anabolic AA (113). LAT1 is a permease which associates with the glycoprotein CD98, forming a heterodimer at the cell membrane (266). The formation of this heterodimer is integral to the activity of this transporter, as without it LAT1 cannot associate with the cell membrane and transport AAs (266). The transport of leucine by LAT1 occurs via bi-directional transport and requires a second AA transporter, the sodium-coupled neutral AA transporter 2 (SNAT2). SNAT2 transports glutamine into mammalian cells in a process which also involved the influx of sodium ions (267). It is this intracellular glutamine that is needed in the bi-directional transport system catalysed by LAT1. Glutamine is transported from the cell whilst leucine, or other BCAAs, are simultaneously transported into the cell by LAT1 (265). As such, the blockage/deletion of SNAT2 which reduces intracellular glutamine, also reduces intracellular BCAAs due to LAT1 inhibition (268). Moreover, when the activity of this bi-directional transport system is pharmalogically attenuated in, mTORC1-dependent phosphorylation is vastly reduced in response to EAAs (269), suggesting this system plays a pivotal role mTORC1 activation.

In skeletal muscle, the role of LAT1 has been investigated via the creation of a muscle-specific LAT1 knockout mouse strain (LAT1 mKO) (270). These mice exhibited reduced AA transport into muscle in response to feeding (270), displaying that this system is the primary method of essential AA transport. Furthermore, no change is S6K1^{Thr389} phosphorylation was observed in response to intraperitoneal leucine injections in LAT1 mKO muscles whereas wild-type (WT) muscles exhibited a 100% increase (270), suggestive of an inability to activate mTORC1. Furthermore, when these mice were fed a high protein diet (30%), mKO muscles were again unable to activate mTORC1. These data implicate LAT1 in the regulation of mTORC1 activity in response to AAs in skeletal muscle. Investigations into LAT1 in human skeletal muscle have focussed mainly on the protein and gene expression of this transporter. Bolus protein ingestion (159), acute resistance exercise (271), and a combination of these anabolic stimuli (234), have been reported to enhance both the mRNA and protein expression of LAT1, however these alterations occurred at varying time points in each investigation (159, 234, 271). Additionally, as these effects were measured following anabolic stimuli, at the same time as mTORC1 activity is raised, these changes are unlikely to have affected mTORC1 activity itself. Conclusions from these data are therefore hard to infer. This difficulty may be due to the methods by which LAT1 was measured. As a transporter, LAT1 needs to be associated with a membrane in order to be active. Thus, the cellular location of LAT1 in basal skeletal muscle, and in response to various anabolic stimuli could provide a vital insight into how AA transport, and mTORC1 activity, is regulated. One such study has begun to tackle this research question through the preparation of skeletal muscle membrane fractions (272). Immunoblotting targeting LAT1 in these fractions suggested a greater amount of this transporter associated with the membrane following resistance

exercise and protein feeding (272). This method can require fairly large amounts of muscle tissue (at least 30mg) and as such the identification, optimisation and validation of methods which can be used to determine LAT1 cellular location in a smaller tissue sample are needed. Subsequently, this method can then be utilised to ascertain any alterations to LAT1 cellular location in response to anabolic stimuli. Recently a new potential role for LAT1 has also been purported, that of a 'transceptor'. This theory suggests that LAT1 may be able to initiate intracellular signalling cascades in response to elevated extracellular AA levels (273-275). As this is only a hypothesis based on *in vitro* data, further research is needed to confirm this.

1.6.3 Vps34

As previously mentioned Vps34 is a class III PI3Kinase which acts to phosphorylate phosphatidylinositol to generate phosphatidylinositol-3-phosphate (PI3P) (247), and exists in several different complexes (249). One of these complexes has recently been particularly scrutinised due to its sensitivity to nutrient provision. *In vitro,* the kinase activity of non-autophagic Vps34 complexes is elevated when nutrient levels are high and reduced with nutrient starvation (119). A potential role for Vps34 as a nutrient sensor was first hypothesised following the discovery that the overexpression of Vps34 in HEK293 cells elicited a significant rise in S6K1 kinase activity similar to that induced by insulin administration (276). Moreover, the effect of insulin on $S6K1^{Thr389}$ phosphorylation could be completed abolished if Vps34 expression was inhibited with siRNA (276). This effect extended to AA-induced mTORC1 activation, where shRNA targeting Vps34 also completely blocked $S6K1^{Thr389}$ hyper-phosphorylation following AA administration (119). Collectively, these data suggest that Vps34 is required for mTORC1 activation in

response to nutrients, a mechanism which may translate to skeletal muscle, a highly nutrient-sensitive tissue.

Very little research into the nutrient-sensing role of Vps34 in skeletal muscle has been conducted. Gran and Cameron-Smith (147) studied the effects of leucine and insulin incubation of varying lengths on anabolic signalling and Vps34 protein expression in human primary myotubes. Here, incubation with 5mM leucine, 100nM insulin or a combination of both significantly elevated Vps34 protein expression no matter whether incubation occurred for 30 minutes, 3 hours or 24 hours (147). The majority of these elevations were accompanied by corresponding changes in mTORC1 activity, suggesting a possible relationship between these two measures. However, from this data it is impossible to conclude whether increases in Vps34 protein expression were affecting mTORC1 activity or *vice versa*, or in fact whether these measures were truly associated at all. In addition, no functional measures of Vps34 were conducted and as such we do not know if the experimental conditions affected the kinase activity of Vps34.

A more in-depth investigation was conducted by MacKenzie et al. (203) who investigated the effects of muscular contractions on Vps34 kinase activity in rodent skeletal muscle. Forced contraction of the lower limbs of rodents elicited large increases in Vps34 kinase activity, predominantly in muscle which underwent eccentric contractions (203). These increases were most apparent at 3h, but remained elevated at 6 hours post-contraction (203). Concomitant rises in S6K1 kinase activity were also noted at these time points, however a more substantial increase in this mTORC1-activity readout was observed 30 minutes following contraction (203). Nevertheless the change in S6K1 activity at 3h post contraction in each muscle analysed (tibialis anterior, soleus & plantaris) was positively associated with the corresponding change in Vps34 kinase activity. Thus, it seems that

the regulation of Vps34 and S6K1 kinase activity may be tightly regulated. Given its purported role as a leucine sensor, the authors then hypothesised that the increase in Vps34 activity noted with contraction would be related to alterations in intramuscular leucine concentrations. To confirm this they measured intramuscular leucine following contractions and added this amount of leucine to C2C12 myotubes. Following supplementation with leucine, Vps34 activity rose 3-4 fold compared to control conditions (203), suggesting Vps34 may be able to sense alterations in intracellular leucine which in turn may enhance its intrinsic activity. As there currently is no data regarding the regulation of Vps34 in response to protein-carbohydrate feeding, alone or in combination with resistance exercise, the true nature of this mechanism within human skeletal cannot yet be determined.

1.7 – Specific Aims of this Thesis

The overarching aim of this thesis is to investigate novel mechanisms of mTORC1 activation and muscle protein synthesis in human skeletal muscle.

Chapter 2 describes the general methodology and analytical techniques used throughout the thesis.

Chapter 3 describes the effect of acute resistance exercise and/or protein-carbohydrate feeding on the cellular localisation and activity of mTOR complexes and lysosomal structures.

Chapter 4 describes the validation and optimisation of immunofluorescent staining approaches to characterise LAT1 localisation and distribution in human skeletal muscle.

Chapter 5 describes *in vitro* and *in vivo* examination as to the role of Vps34 as a nutrient sensor in mouse and human skeletal muscle.

Chapter 6 describes the effect of acute resistance exercise and/or protein-carbohydrate feeding on LAT1 cellular location, novel sites of Vps34 regulation and mTORC1 activity in human skeletal muscle.

Chapter 7 then provides a general discussion focussing on the results gleaned from this thesis and the implications of these findings on the field of skeletal muscle physiology and mTORC1 research.

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Chapter 2

GENERAL METHODS

2.1 – Ethical Approval

The study presented in Chapter 3 was conducted in the Department of Kinesiology at McMaster University (Hamilton, ON, Canada) and analysed at the University of Birmingham (U.K.). This study was approved by the Hamilton Integrated Research Ethics Board (REB 14-736). The data presented in Chapter 4 were collected at The University of Birmingham, McMaster University and The University of Guelph, under approval of the NHS West Midlands Black Country Research Ethics Committee (14/WM/0088 and 15/WM/0003), the Hamilton Health Sciences Research Ethics Board (12-631), and the University of Guelph Research Ethics Board (120C018) respectively. Ethical approval for the study presented in Chapter 5 was obtained from the NHS West Midlands Black Country Research Ethics Committee (15/WM/0003). Finally, the study presented in Chapter 6 was approved by NHS West Midlands Black Country Research Ethics Committee (17/WM/0068). For each experiment written informed consent was obtained from all participants prior to commencement in the study. All studies conformed to the standards presented by the Declaration of Helsinki (seventh version).

2.2 – Sample Collection

All human muscle samples were obtained from the *vastus lateralis* using the Bergstrom percutaneous needle biopsy technique, modified for suction (1), under local anaesthesia (1% lidocaine). Briefly, local anaesthesia was administered following which a small incision (1cm) was made in the skin and fascia above the *vastus lateralis* muscle. The Bergstrom biopsy needle was then inserted and suction applied to obtain muscle samples. Each separate muscle biopsy sample (~100mg) was obtained from a different incision site. Following sample collection, samples were blotted free of blood and any apparent

adipose or connective tissue was dissected. Specific details of experimental designs of each study are included in each individual chapter.

2.3 – Sample Processing

2.3.1 Standard processing

For all analysis other than histology, muscle samples were snap-frozen in liquid nitrogen immediately following sample collection. These samples were then stored at -80ºC until analysis was completed.

2.3.2 Processing for histology

For histological analysis, a small piece of skeletal muscle (~20mg) was covered in Tissue-Tek OCT and frozen in liquid nitrogen-cooled isopentane. Samples were then stored at -80ºC until further analysis. Muscle samples were sectioned in a cryostat (Bright 5040, Bright Instrument Company limited, Huntingdon, England)) maintained at -25ºC. Here, samples were fixed to cylinders by Tissue-Tek OCT and positioned in front of the microtome blade in the correct orientation to produce cross-sectional muscle sections. 5µm muscle sections were then cut and transferred to room-temperature glass slides (Corning, NY, USA.) and allowed to dry for ~30 minutes. The integrity and orientation of sections were visualised under an optical microscope with 10X magnification and damaged sections discarded. Slides were stored at -80ºC until staining.

2.4 – Histology

2.4.1 Fixation, Blocking and Antibody Incubation

Muscle sections were fixed in a 3:1 solution of acetone and ethanol for 5 minutes, following which they were blocked in 5% normal goat serum (NGS), diluted in phosphate buffered saline (PBS). NGS was chosen to prevent non-specific secondary antibody binding.

2.4.2 Overall Staining Procedure

Slides were removed from the freezer and left to thaw for 45 minutes before being fixed as previously described. Sections were then outlined with a PAP pen to prevent liquid leaving sections during incubations and washed for 3 x 5 minutes in 1XPBS (supplemented with 0.2% Tween20 (PBST)). Primary antibodies were then diluted in 5% NGS (diluted in PBS) to the required predetermined concentration. For each new antibody used, preliminary testing was conducted to determine an optimal staining dilution. If multiple protein targets were being stained for then an antibody cocktail was made in 5% NGS allowing multiple proteins to be simultaneously stained for. At this point, primary antibodies were administered to muscle sections and incubated for 2 hours at room temperature (RT). Following primary incubation, excess primary antibody was removed through 3 x 5 minute PBST washed. Secondary antibodies, conjugated to Alexa fluorophores (1:200 dilution in PBS for all secondary antibodies) were then applied to the muscle sections and left to incubate for 1 hour at RT. Again excess antibody was removed via 3 x 5 minute PBST washes. If needed, sections were then incubated for 30 minutes in Wheat Germ Agglutinin (WGA, conjugated to 350nm fluorophore) in order to mark the sarcolemmal membrane. After this, a final wash in PBST and then 1 x 5 minute wash in PBS was conducted. Slides were left to air dry in a dark cabinet before being mounted in Mowiol to protect fluorophores. A glass coverslip was subsequently applied and slides

left at RT overnight to dry. At this point, slides were then frozen until imaging commenced.

2.4.3 Antibody Validation

Antibodies targeting RAPTOR (Abcam no. ab40768) and RICTOR (CST no.53A2) were validated using muscle-specific knockout (mKO) tissue for the target protein kindly donated by Prof. Markus Ruegg. The antibody used to target LAT1 (SLC7A5 – Abcam no. ab85226) was validated through the use of mKO tissue (2) and a peptide competition assay.

2.4.3.1 mKO Tissue

For RAPTOR and RICTOR antibody validation using mKO tissue, slides were prepared with 5µm sections of mKO gastrocnemius muscle (n=2) alongside wild-type (WT) littermate controls (n=2). Staining for each protein was then conducted as described above with a subset of samples stained with primary antibodies omitted (CON). This was conducted to examine any background staining from the secondary antibody alone which may have occurred in mKO tissue. If valid, the intensity of the stain produced in mKO tissue should be equivalent to that apparent in CON samples. All sections were imaged in identical conditions in order to allow accurate comparisons to be made.

For LAT1 (SLC7A5) antibody validation slides were prepared with 5µm sections of mKO extensor digitorum longus (EDL) muscle (n=2). Separate slides were prepared containing WT littermate control EDL sections. Again, staining was conducted as described above and image capture protocols were identical for each sample analysed. The staining intensity of each section was visualised to determine specificity of the antibody.

2.4.3.2 Peptide Competition Assay

For peptide competition assays, 5µm muscle sections from the *vastus lateralis* of one young, healthy male were loaded onto slides. A subset of sections were stained for the protein of interest as described previously. For the second subset of sections, primary antibodies were pre-incubated with its corresponding peptide (20x concentration of antibody) for 1 hour at RT on an orbital shaker. This cocktail was then added to the sections. If the antibody is specific to its target protein, it should bind to its corresponding peptide and be fully quenched. Therefore, the staining intensity on these samples should be greatly reduced compared to normal staining procedures.

2.4.4 Image Capture

Prepared slides were observed under a Nikon E600 widefield microscope using a 40×0.75 numerical aperture objective. Images per area were captured under three colour filters achieved by a SPOT RT KE colour three shot CCD camera (Diagnostic Instruments Inc., MI, USA), illuminated by a 170 W Xenon light source. For image capture, DAPI UV (340–380 nm) filter was used to view WGA-350 (blue) signals and proteins tagged with Alexa 488 fluorophores (green) were visualised under the FITC (465–495nm) excitation filter. The Texas-Red (540–580 nm) excitation filter was used to capture signals of proteins conjugated to Alexa Fluor 594 fluorophores. All images were taken were taken using a 40X magnification objective (0.75NA). Filter box switching was conducted with a semi-automated filterwheel (10B 10 Position Filterwheel, Sutter, USA). On average, 6 images were captured per human section, and each image contained ~8 muscle fibres. All sections were imaged in duplicate and therefore approximately 100 fibres per subject were used for analysis. All image capture factors factors i.e. exposure time and gain, were

kept constant between all images on each individual slide, Image processing and analysis was undertaken on ImagePro Plus 5.1 (Media Cybernetics, MD., USA.) and all factors i.e. exposure time and gain, were kept constant between all images on each individual slide. All images underwent flattening and de-speckling prior to quantification of co-localization to limit the contribution of background, non-specific fluorescence. Image signals generated by WGA or dystrophin were used to estimate cell membrane borders, and MHC1 staining was utilised to identify type I fibres. The intensity of LAT1 staining in these groups of fibres was used to assess any differences in fibre type abundance of the LAT1 protein.

2.4.5 Image Analysis

2.4.5.1 Fluorescence Intensity

For fluorescence intensity analysis of antibody validity, FIJI (ImageJ) software was utilised. Images were analysed for mean pixel intensity across the entire image and the mean of these intensities was calculated across all images from a particular section. These values were then compared between WT, mKO and, where applicable, CON samples to suggest antibody specificity. For fibre-type specific fluorescence intensity, type I muscle fibres were identified using a MHCI antibody (DSHB, A4.480) and the membranes of fibres were identified via dystrophin (DSHB, MANDYS1 3B7) or WGA-350 (ThermoFisher, ##11263). Regions of interest (ROIs) were then produced around these fibres and the fluorescence intensity of LAT1-positive signals were measured. MHCI-negative fibres (type II fibres) underwent an identical process and these values were then compared to understand any fibre-type preferential staining patterns.

2.4.5.2 Colocalisation

Image processing and analysis for colocalisation was undertaken on ImagePro Plus 5.1 (Media Cybernetics, MD., USA.). All images underwent flattening and despeckling to limit the contribution of background, non-specific fluorescence to quantification. Image signals generated by WGA or dystrophin were used to estimate cell membrane borders. Signals from each individual filter were then overlaid and the Pearson's correlation coefficients produced to estimate colocalisation of proteins in each image. This method of assessing colocalisation was chosen as it measures colocalisation between two channels on pixel-by-pixel basis and is relatively free of user bias (3). In cases where muscle tissue did not cover the entire image, or there was extensive background/non-specific staining in certain areas, then a ROI was manually drawn around the area to be used for analysis and Pearson's correlation coefficient measured in this area only.

2.5 – Immunoblotting

2.5.1 Tissue Homogenisation and Protein Content Analysis

For western blot analysis of human tissue, snap-frozen muscle tissue was powdered using a Cellcrusher tissue pulverizer (Cellcrusher, Co. Cork, Ireland) on dry ice. Subseuently, ~20mg of powdered muscle was homogenised in ice-cold sucrose lysis buffer (50 mM Tris, 1mM EDTA, 1mM EGTA, 50mM NaF, 5mM $Na_4P_2O_7$-$10H_2O$, 270mM sucrose, 1M Triton-X, 25mM β-glycerophosphate, 1μM Trichostatin A, 10mM Nicotinamide, 1mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2; Sigma, 1% Sigma Phosphatase Inhibitor Cocktail 2; Sigma, 4.8% cOmplete Mini Protease Inhibitor Cocktail; Roche) using a hand-held Polytron homogeniser. The subsequent solution was briefly vortexed before centrifugation at 8000g for 10 minutes at 4ºC to remove insoluble

material. For western blot analysis of C2C12 and human primary myotubes, cells were collected in ice-cold sucrose lysis buffer and underwent ultrasonic homogenisation for 3 x 15 seconds at 50% maximal wattage. This cell lysate was then briefly vortexed and centrifuged at 8000g for 10 minutes at 4ºC to remove insoluble material. Protein content was determined by the DC protein assay (Bio-Rad, Hercules, California, USA) and samples were diluted to the desired protein concentration in sucrose lysis buffer and 1 x Laemmli Sample Buffer (LSB). Western blot samples were then boiled for 5 minutes at 95ºC to denature proteins and stored at -80ºC until further analysis.

2.5.2 Gel Preparation and Electrophoresis

8-15% polyacrylamide separating gels were prepared using relative amounts of 30% acrylamide (BioRad), Tris-SDS (pH 8.8) and doubly-distilled water (ddH_2O). Small amounts of 10% ammonium persulfate (APS, 100µL) and TEMED (12-16µL) were then added to catalyse polymerisation. When separating gels were set, 5% polyacrylamide stacking gels were prepared as described above and added on top of the separating gel. 10- or 15-well combs were then added depending on number of samples loaded. Once set, equal volumes of protein were loaded to gels and separated by SDS-PAGE at a constant current of 23mA per gel. Proteins were allowed to run to the bottom of gels (~60mins) before transfer to membranes was undertaken.

2.5.3 Transfer and Blocking

Proteins were transferred on to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, Florida, USA) via wet transfer at 100 V for 1 h. Membranes were stained with Ponceau S (Sigma-Aldrich, Gillingham, UK) and imaged to check for even

loading. Membranes were then blocked in 3% dry-milk in tris-buffered saline with tween (TBST) for 1 hour to minimise non-protein specific binding.

2.5.4 Antibodies

2.5.4.1 Primary Antibody

Following blocking, membranes were washed for 5 minutes in 1X TBST to remove excess milk. Membranes were then incubated in primary antibodies (diluted in TBST) overnight at 4ºC.

2.5.4.2 Secondary Antibody

Following primary antibody incubation, membranes were again washed for 3 x 5 minutes in 1X TBST to remove excess antibody solution. Membranes were the incubated in the appropriate horse radish peroxidase-conjugated secondary antibody at room temperature for 1 hour. Subsequently membranes were again washed 3 x 5 minutes in 1X TBST before imaging commenced.

2.5.5 Imaging and Analysis

Antibody binding was detected via the use of enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK). Images were acquired using a G:Box Chemi-XR5 (Syngene, Cambridge, UK) and band quantification was undertaken on the corresponding GeneSys software (Syngene, Cambridge, UK). Background corrections were also completed to remove any effects of background antibody binding.

2.6 – Kinase Assays

2.6.1 S6K1 and AKT Kinase Assays

200µg of protein from muscle lysates was used for the S6K1 kinase assay. The target protein was then immunoprecipitated using 4µg of anti-S6K1 antibody (santa cruz biotech, no.2708) and 2.5µL of Protein G Sepharose beads for 2 hours at 4ºC. Immunoprecipitates were then washed in high-salt buffer (50mM Tris·HCl pH 7.5, 0.1mM EGTA, 1mM EDTA, 0.5M NaCl, 1% (vol/vol) Triton X-100, 50mM NaF, 5mM NaPPi, 0.27M sucrose, 0.1% -mercaptoethanol, 1mM $Na_3(OV)_4$, and 1 Complete (Roche) protease inhibitor tablet per 10mL) and once in assay buffer (50mM Tris·HCl pH 7.4, 0.03% Brij35, and 0.1% β-mercaptoethanol). The immunoprecipitates were then resuspended in 10µL assay buffer and activity assays commenced every 20 seconds. This occurred through the addition of a hot assay mix, which consisted of assay buffer, ATP-$MgCl_2$ (100µM ATP +10mM $MgCl_2$), 32γ-ATP and a synthetic peptide substrate (S6tide - KRRRLASLR at 30µM). Reactions ceased at 20 second intervals by spotting onto chromatography paper (Whatman; GE Healthcare, UK) and immersing in 75 mM phosphoric acid. Chromatography papers were washed 3 x 5 minutes in 75 mM phosphoric acid and 1 x 5 minutes in acetone and then dried. Following immersion in Gold Star LT Quanta scintillation fluid (Meridian Biotechnologies, Chesterfield, UK), spots were counted in a Packard 2200CA TriCarb scintillation counter (United Technologies). Assays were quantified in quantified in $fmol \cdot min^{-1} \cdot mg^{-1}$. For AKT kinase assays, AKT was immunoprecipitated (santa cruz biotech, no.4691) from the same lysates used for S6K1 immunoprecipitates, a method previously validated (4). Kinase assays were then completed in an identical manner, with the only alteration being the synthetic peptide substrate used (Crosstide - GRPRTSSFAEG at 30µM). Assays occurred for 45 minutes for S6K1 and 20 minutes for AKT. This methodology has previously been completely validated (4).

2.6.2 Vps34 Kinase Assay

Target proteins were immunoprecipitated from lysates overnight at 4ºC in the presence of 2µg anti-Vps34 antibody (sheep antibody produced by Dr. James Murray, Trinity Biomedical Science Institute, Trinity Collage Dublin) and immobilised on Protein G sepharose beads for 1hour. Immunoprecipitates were then washed 3 times in Cantley lysis buffer, once in Tris-LiCl (10mM Tris, pH 7.5, 5mM LiCl, 0.1mM Na_2VO_4) and 2 times in TNE (10mM Tris, pH 7.5, 150mM NaCl, 1mM EDTA, 0.1mM Na_2VO_4), before resuspension in 60µL TNE+ (TNE, 0.5mM EGTA, pH 8.0, 1:1000 2-mercaptoethanol). This suspension was then incubated with 20µg of Vps34 antigen peptide for 10 minutes at RT. Substrates for this assay were 10µL of 30mM MnCl2 and 10µL of 2mg/ml^{-1} phosphotidylinositol (PI, sonicated for 5 min in 10mM Tris, pH 7.5–1mM EGTA prior to addition) (bovine liver, Avani Polar Lipids), added to each sample. Reactions were performed at 30ºC on a shaker. Reactions were initiated by the addition of 5µL of ATP mix (400µM unlabelled ATP, 12.5µCi of 32γ ATP, 4.3µL water) and occurred for 10 minutes. Termination of the reaction was achieved by the addition of 20µL of 8M HCl and phase separated with 160µL 1:1 chloroform:methanol and centrifugation for 1 minute at 16000g. The lower phase of this suspension was then spotted onto an aluminium-backed 60A silica ˚ TLC plate (Merck, Damstadt, Germany) and run in a TLC chamber solvent system (60mL chloroform, 47mL methanol, 11.2mL water and 2mL ammonium hydroxide). The plates were then left to develop and air dry before quantification by densiometry. Again this method has been previously optimised and validated (5).

2.7 – Blood analyses

2.7.1 – Serum Insulin Concentrations

Serum insulin concentrations were determined using an enzyme-linked immunosorbent assay (ELISA, IBL International, Hamburg, Germany) following manufacturers instructions. Briefly, 25µL serum samples, and provided standards, were combined with 25µL enzyme conjugate in a microtiter plate, mixed thoroughly and incubated for 30 mins at RT. 50µL of provided enzyme complex was then added to each well and incubated for 30 mins at RT. Following 3 washes with the provided wash solution, 50µL of substrate solution was added to each well and reactions were allowed to occur for 15 minutes at RT. The provided stop solution was then added to terminate reactions and absorbance at 450nm was read on a FluoStar Omega plate reader (BMG Labtech, Aylesbury, UK). A standard curve was then created using known standard concentrations, allowing serum insulin concentrations to be estimated.

2.7.2 – Plasma AA concentrations

Plasma concentrations of leucine, phenylalanine and threonine were determined using gas chromatography-mass spectrometry (GCMS;model 5973; Hewlett Packard, Palo Alto, CA, USA). Once thawed, known concentrations of stable isotope-labelled internal standards corresponding to each AA of interest were added to the plasma samples (100µL per 1mL plasma). Samples were then diluted 1:1 with acetic acid and purified on acidified cation-exchange columns (Dowex50W-X8-200; Sigma-Aldrich, Poole, UK). AAs were collected into glass tubes through the addition of ammonium hydroxide to columns and subsequently dried under N_2. AAs were then converted to their N-tert-butyldimethyl-silyl-N-methyltrifluoracetamide (MTBSTFA) derivative through the addition of acetonitrile and MTBSFTA (1:1 ratio) to the dried AAs before heating at 70°C for 1h. Samples were then run on GCMS and AA concentrations (peaks on ions of interest) were calculated based on the known concentration of each internal standard.

2.8 – *In vitro* experimental studies

2.8.1 C2C12s

C2C12 myoblasts were purchased from American Type Culture Collection (Manassas, VA). C2C12 myoblasts were initially cultured on 150mm culture plates in high glucose Dulbecco's minimum essential medium (DMEM, ThermoFisher Scientific, Waltham, MA, USA.), supplemented with 10% Foetal bovine serum (FBS, Hyclone, VWR, Lutterworth, UK.) and 1% penicillin-streptomycin (PS, ThermoFisher Scientific, Waltham, MA, USA.). When 80% confluent, cells were trypsinised (ThermoFisher Scientific, Waltham, MA, USA.) and transferred to 6-well plates at a concentration of 2 x 10^5 cells per well. Myoblasts were then cultured until 90% confluency (~36 hours) when media was changed to elicit differentiation into myotubes (high glucose DMEM supplemented with 2% horse serum (HS, Hyclone, VWR, Lutterworth, UK.) and 1% PS). Differentiation was allowed to occur for 5 days, until fully-formed myotubes were visualised, before experimental procedures were began. Specific details of experimental procedures will be described in specific chapters. Upon completion of experimental procedures, myotubes were briefly washed twice in ice-cold PBS (ThermoFisher Scientific, Waltham, MA, USA.) before being collected via scraping into ice-cold sucrose lysis buffer (formulation described above, 150µL per well). These cell lysates were then snap-frozen in liquid nitrogen and placed at -80ºC until further analysis.

2.8.2 Human Primaries

Human primary myoblasts from 4 patients (age – 60.8±6.4yrs, BMI – 28.7±0.65kg/m^2, Mean±SEM) were obtained from the laboratory of Dr. Simon Jones (Queen Elizabeth Hospital, Institute of Inflammation and Ageing, Birmingham, U.K.). Fresh muscle

samples were minced with scissors for 5 minutes and the sample transferred to a universal tube. 5mL of 1x trypsin-EDTA was added to the sample and vortexed to mix. This was then placed at 37ºC for 15 minutes whilst rotating. Following this, 5mL of Hams F-10 media (ThermoFisher Scientific, Waltham, MA, USA, supplemented with 20% FBS and 1% PS) was added to inactivate the trypsin. This suspension was then passed through a filter (70µm, Corning, NY, USA.) to remove residual connective tissue etc. The cell suspension was then centrifuged for 5 minutes at 1700rpm and the supernatant subsequently discarded. The cell pellet was then resuspended in 10mL of Hams F10 media (20%FBS, 1%PS) and added to an uncoated 100mm culture plate. The plate was then incubated for 30 minutes at 37ºC to allow fibroblasts to adhere to the plate, yet maintain myoblasts in suspension. The residual cell suspension (myoblasts) was then removed and added to a 0.2% gelatin (Sigma Aldrich, Dorset, UK.) coated 10mm culture plate. This plate was then incubated for 48-72 hours before the first media change to allow myoblasts to fully adhere to the plate. A second 'pre-plate' procedure was then conducted at first passage to remove further fibroblast cells. This isolation method has previously been shown to consistently remove a large proportion of fibroblasts from muscle samples (6). Thereafter media was changed every 48 hours until 60% confluent.

Cells were passaged at 60% confluency to prevent spontaneous fusion of myoblasts to myotubes. At passage 3, cells were trypsinised and seeded onto 6-well plates at a concentration of 5 x 10^4 cells per well. These cells were then cultured to 80-90% confluency at which point media was changed to elicit differentiation (Hams F10 supplemented with 2% HS and 1% PS). Experiments were then conducted when fully formed myotubes could be visualised, which occurred after 6-10 days. Specific details of experimental conditions will be described in later chapters. Upon completion of

experimental procedures, myotubes were briefly washed twice in ice-cold PBS before being collected via scraping into ice-cold sucrose lysis buffer (formulation described above, 100μL per well). Cell lysates were then snap-frozen in liquid nitrogen and placed at -80ºC until further analysis.

2.9 – Statistics

Statistical analysis was completed using SPSS (version 22 for Windows; SPSS, Chicago, IL) with *post-hoc* analysis completed in Microsoft Excel (version 14 for Windows, Microsoft). Specific details of the statistical tests used are described in each individual chapter.

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Chapter 3

DIFFERENTIAL LOCALIZATION AND ANABOLIC RESPONSIVENESS OF MTOR COMPLEXES IN HUMAN SKELETAL MUSCLE IN RESPONSE TO FEEDING AND EXERCISE

3 DIFFERENTIAL LOCALISATION AND ANABOLIC RESPONSIVENESS OF MTOR COMPLEXES IN HUMAN SKELETAL MUSCLE IN RESPONSE TO FEEDING AND EXERCISE

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Author Contributions:

Human testing and sample collection was undertaken at McMaster University by **Dr. Chris McGlory** and **Sara Oikawa**, under the supervision of **Prof. Stuart Phillips**. Analysis plans were conceived by **Nathan Hodson, Zhe Song** and **Dr. Andy Philp**. Raptor and Rictor knockout tissue was provided by **Prof. Markus Ruegg**. All immunohistochemical analysis was conducted by **Nathan Hodson**. S6K1 and AKT kinase activity assays were conducted by **Dr. Stewart Jeromson** and **Dr. D Lee Hamilton**. Statistical analysis and interpretation of results were completed by **Nathan Hodson**.

3.1 – Abstract

Mechanistic target of rapamycin (mTOR) resides as two complexes within skeletal muscle. mTOR complex 1 (mTORC1–Raptor positive) regulates skeletal muscle growth, whereas mTORC2 (Rictor positive) regulates insulin sensitivity. To examine the regulation of these complexes in human skeletal muscle, we utilised immunohistochemical analysis to study the localisation of mTOR complexes prior to and following protein-carbohydrate feeding (FED) and resistance exercise plus protein-carbohydrate feeding (EXFED) in a unilateral exercise model. In basal samples, mTOR and the lysosomal marker LAMP2 were highly co-localized and remained so throughout. In the FED and EXFED states, mTOR/LAMP2 complexes were redistributed to the cell periphery (WGA positive staining) (time effect; p=.025), with 39% (FED) and 26% (EXFED) increases in mTOR/WGA association observed 1h post-feeding/exercise. mTOR/WGA colocalisation continued to increase in EXFED at 3h (48% above baseline) whereas colocalisation decreased in FED (21% above baseline). A significant effect of condition (p=.05) was noted suggesting mTOR/WGA co-localization was greater during EXFED. This pattern was replicated in Raptor/WGA association, where a significant difference between EXFED and FED was noted at 3h post-exercise/feeding (p=.014). Rictor/WGA colocalization remained unaltered throughout the trial. Alterations in mTORC1 cellular location coincided with elevated S6K1 kinase activity, which rose to a greater extent in EXFED compared to FED at 1h post-exercise/feeding (p<.001), and only remained elevated in EXFED at the 3h time point (p=.037). Collectively these data suggest that mTORC1 redistribution within the cell is a fundamental response to resistance exercise and feeding, whereas mTORC2 is predominantly situated at the sarcolemma and does not alter localisation.

3.2 – Introduction

Resistance exercise and protein ingestion are potent anabolic stimuli, elevating muscle protein synthesis (MPS) (1, 2) resulting in a positive net protein balance (NPB) (2). Such elevations in MPS are underpinned by the activation of the conserved serine/threonine kinase, mechanistic target of rapamycin (mTOR). This kinase can both augment MPS (3) and offset muscle protein breakdown (MPB) (4). In skeletal muscle, mTOR resides in two distinct complexes distinguishable by the composition of proteins within each. For example, complex 1 (mTORC1) contains mTOR, RAPTOR, GβL, PRAS40 and DEPTOR (5), believed to activate protein synthetic machinery (6), whereas complex 2 (mTORC2) is comprised of mTOR, RICTOR, DEPTOR, GβL, Sin1 and Protor, and is implicated in insulin sensitivity and actin cytoskeleton dynamics (5). Due to the critical role mTORC1 plays in regulating protein synthesis, this complex has received the most detailed examination in relation to resistance exercise and protein feeding. Acute resistance exercise, protein ingestion, or combinations of such stimuli are consistently reported to elevate mTORC1 activity (1, 2), with effects maintained for up to 24 hours (7). Furthermore, the acute inhibition of mTORC1 with rapamycin administration ablates any effect of anabolic stimuli on MPS (8, 9).

As mTORC1 activity seems to be directly implicated in the stimulation of MPS, research has focussed on understanding the mechanism by which mTORC1 is activated. Sancak et al. (10), identified the interaction of mTORC1 with the lysosome to be of particular importance to the activation of the kinase complex *in vitro*. A similar mechanism has also been reported in rodent skeletal muscle, where eccentric contractions of the tibialis anterior muscle induce mTOR-lysosome colocalisation (11) in parallel to increases in mTORC1 activity (inferred by the phosphorylation of $S6K1^{Thr389}$). Together these data

infer an importance of mTOR-lysosome colocalisation in the activation of molecular pathways implicated in protein synthesis. Recently, however, Korolchuk et al. (12) reported that the cellular localisation of these mTOR/lysosomal complexes play a pivotal role in mTOR activation. In support of this hypothesis, we recently reported that a single bout of resistance exercise initiated mTOR/lysosome translocation to the cell periphery, and occurred in parallel to an increase in mTOR activity and interaction between mTOR and proteins involved in translation initiation (13).

Whilst the use of immunofluorescence approaches allowed us to study the cellular localisation of mTOR, this approach did not enable us to distinguish between mTOR complexes. Consequently, we were unable to conclude whether the movement of mTOR following anabolic stimuli was mTORC1 or mTORC2 specific. Further, given the parallel group design we employed(13), we were unable to assess whether mTOR translocation was amplified by feeding. Therefore, the aim of the current study was to evaluate whether mTOR translocation following resistance exercise and/or protein-carbohydrate feeding is specific to mTORC1. In addition, we utilised a within-subject design to evaluate whether a synergistic effect of exercise and feeding exists. We hypothesised that exercise plus protein-carbohydrate feeding would elicit a greater mTOR/LAMP2 translocation to the cell periphery compared to feeding alone. Further, we hypothesised this translocation would be specific to mTORC1.

3.3 – Methods

3.3.1 Subjects

Eight young, healthy, recreationally active males (age=22.5±3.1y, BMI=24.6±2.2kg/m^2, body fat=17.6±4.8%) volunteered to partake in the study. Potential participants were

informed about all experimental procedures to be undertaken and any risks involved before written informed consent was obtained. The study was approved by the Hamilton Integrated Research Ethics Board (REB 14-736) and adhered to the ethical standards outlined by the Canadian tri-council policy statement regarding the use of human participants in research as well as the principles according to the Declaration of Helsinki as revised in 2008.

3.3.2 Experimental design

Following initial assessment for 1 repetition maximum (1-RM) on leg extension 7 d previously, participants reported to the laboratory at ~7.00am after a 10-h overnight fast. Participants then rested in a semi-supine position on a bed and an initial skeletal muscle biopsy was taken from the *vastus lateralis* using a modified bergstrom needle. Following this biopsy, participants performed 4 sets of unilateral leg extension (Atlantis, Laval, QC, Canada) at 70% 1RM until volitional failure interspersed by 2 min recovery. Immediately following the cessation of the final set of leg extension all participants consumed a commercially available beverage (Gatorade Recover®, Chicago, IL, USA) that provided 20, 44, and 1g of protein, carbohydrate, and fat respectively. Subsequent bilateral skeletal muscle biopsies were obtained from the *vastus lateralis* at 1h and 3h after beverage ingestion to examine mTORC1-related signalling and associated localisation.

3.3.3 Skeletal muscle immunohistochemistry

Skeletal muscle immunohistochemical preparation and staining was conducted as described previously in Chapter 2 (section 2.4). All samples from each subject were sectioned onto the same slide, in duplicate, to ensure accurate comparisons between time points could be made.

3.3.4 Antibodies

The mouse mono-clonal anti-mTOR (#05-1592) antibody was purchased from Merck Chemical Ltd. (Nottingham, UK). The corresponding conjugated secondary antibody to this was Goat anti-mouse IgGγ1 Alexa®594 (#R37121, ThermoFisher, UK). Antibodies targeting LAMP2 (#AP1824d, Abgent, USA), Rictor (CST#53A2, Cell Signalling Technologies, USA) and Raptor (#ab40768, Abcam, Cambridge, UK) were visualised using Goat anti-rabbit IgG(H+L) Alexa®488 secondary antibodies (#A11008, ThermoFisher, UK). Finally, wheat germ agglutinin (WGA-350, #11263, ThermoFisher, UK) was used to identify the sarcolemmal membrane of muscle fibres.

3.3.5 Antibody Validation

The specificity of Rictor (CST#53A2) and Raptor (Abcam#ab40768) primary antibodies were tested utilising skeletal muscle samples from the gastrocnemius of muscle-specific knockout (mKO) mice for each protein respectively (14). Wild-type, littermate muscle samples for each mouse model were used as controls. Primary antibodies were also omitted from a subset of samples on slides to examine any background staining from the secondary antibody utilised. The fluorescence intensity of each image was then calculated using ImageJ software (Version 1.51 for Windows).

3.3.6 Image capture

Prepared slides were imaged as described previously in section 2.4.5 of this thesis. DAPI UV (340–380 nm) filter was used to view WGA-350 (blue) signals and mTOR proteins tagged with Alexa Fluor 594 fluorophores (red) were visualised under the Texas red (540–580 nm) excitation filter. The FITC (465–495nm) excitation filter was used to capture signals of mTOR-complex proteins and LAMP2, which were conjugated with Alexa

Fluor 488 fluorophores. On average, 8 images were captured per section, and each image contained ~8 muscle fibres such that around 120 fibres per time point (per subject) were used for analysis. Image processing and analysis was undertaken on ImagePro Plus 5.1 (Media Cybernetics, MD., USA.) and all factors i.e. exposure time and de-speckling, were kept constant between all images on each individual slide. Image signals generated by WGA were used to estimate cell membrane borders, which were merged with the corresponding target protein images to identify the association between the protein of interest and the plasma membrane. Pearson's correlation coefficient (Image-Pro software) was used to quantify colocalization with the plasma membrane and mTOR-associated proteins. This process was also completed to quantify the localization of mTOR with complex-associated proteins (Raptor & Rictor) and a marker of the lysosomal membrane (LAMP2).

3.3.7 AKT and S6K1 Kinase Activity Assays

At each time point during the experimental trial, a separate piece of muscle tissue was blotted and freed from any visible adipose or connective tissue. The tissue was then frozen in liquid nitrogen and stored at -80ºC. The kinase activity of AKT and S6K1 was determined via [-γ-32P] ATP kinase assays following immuno-precipitation of the target protein, as previously described in Chapter 2 (Section 2.6.1).

3.3.8 Statistical Analysis

All statistical analysis was conducted on SPSS version 22 for Windows (SPSS Inc., Chicago, IL, USA). Differences in staining intensity between mKO, wild type (WT) and primary omitted (CON) muscle sections were analysed using a one-way analysis of variance (ANOVA). Differences in kinase activity, fluorescence intensity and staining

colocalisation were analysed using a two-factor mixed-model ANOVA with two within subject factors (time; three levels – PRE.vs.1h.vs.3h and condition; two levels – FEDvs.EXFED), with Bonferonni correction for multiple comparisons. Pairwise comparisons were conducted when a significant main/interaction effect was found. Significance for all variables analysed was set at $p \leq .05$. Data are presented as means±SEM unless otherwise stated.

3.4 – Results

3.4.1 Rictor and Raptor antibodies are specific to their target proteins

Rictor protein staining intensity in Rictor mKO tissue was significantly lower than that in littermate WT controls ($p < .001$, Fig. 3.1b). Furthermore, the staining intensity in this tissue was comparable to when the primary antibody was omitted in both mKO and WT tissue ($p > .999$, Fig. 3.1b). Raptor protein staining intensity in Raptor mKO tissue was also significantly lower than that noted in littermate WT controls ($p < .001$, Fig. 3.1c), with this staining intensity again similar to when the primary antibody was omitted in either tissue ($p > .999$, Fig. 3.1c). Therefore, we take this as evidence that the Rictor (CST#53A2) and Raptor (Abcam#ab40768) antibodies are specific to their target protein.

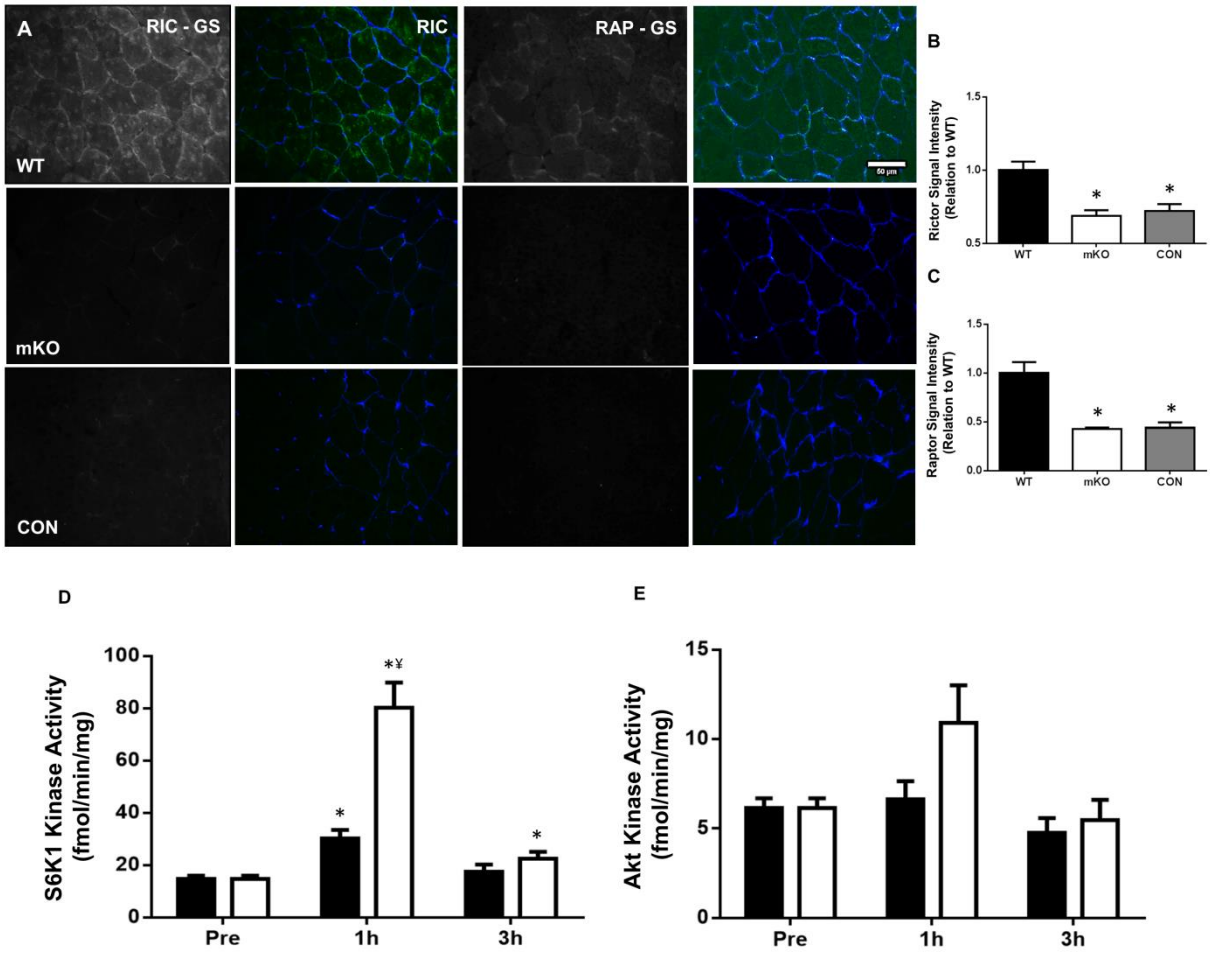

Figure 3.1. Rictor and Raptor antibody validation and S6K1 and AKT kinase activity. Immuno-fluorescent staining of each protein was performed in mKO and littermate WT samples, in addition to staining of each sample with primary antibodies omitted (CON). Rictor/Raptor is displayed in green and WGA (cell membrane) is stained in blue. Representative images of staining in each condition are displayed (A) alongside the corresponding quantifications for Rictor (B) and Raptor (C). Scale bars are 50µm. Data presented as mean±SEM. *Significantly different WT ($p<.001$). S6K1 (D) and AKT (E) kinase activity following unilateral resistance exercise and/or protein-carbohydrate feeding. Black bars denote FED condition and open bars denote EXFED condition. Data presented as Mean±SEM. *Significantly different to baseline ($p<.05$), ¥significant difference between conditions at this time point ($p<.001$).

3.4.2 S6K1 and AKT kinase activity

A significant condition by time effect was observed for S6K1 activity (p<.001). S6K1 activity rose above baseline in both conditions at 1h post-exercise/feeding (FED-p=.015, EXFED-p<.001), and kinase activity at this time point was 165% greater in the EXFED condition (p<.001, Fig. 3.1d). At 3h post-exercise/feeding, kinase activity only remained above baseline values in the EXFED condition (52.8% greater than baseline, p=.037, Fig. 3.1d). A significant main effect for time was noted for AKT kinase activity (p=.023, Fig. 3.1e). Pairwise comparisons displayed a trend toward an increase in AKT kinase activity 1h post-intervention, when conditions were combined, compared to 3h post-intervention (p=.073).

3.4.3 Lysosomal content and colocalisation with mTOR

LAMP2 fluorescence intensity was unchanged from baseline in either condition, however a significantly greater intensity was noted in the EXFED condition, compared to FED, at 3h post-exercise/feeding (p=0.41, Fig. 3.2b). A significant condition × time effect was observed for mTOR-Lamp2 colocalisation (p=.004). Consistent with our previous work (13), mTOR and LAMP2 were highly localised in basal skeletal muscle (Fig. 3.2c). The colocalisation of these two proteins did not change from baseline in either condition over the 3h post-exercise/feeding period. However, at the 3h time point, the colocalisation of the proteins was greater in the FED condition compared to the EXFED condition (0.51(FED)vs.0.47(EXFED), p=.011, Fig. 3.2c).

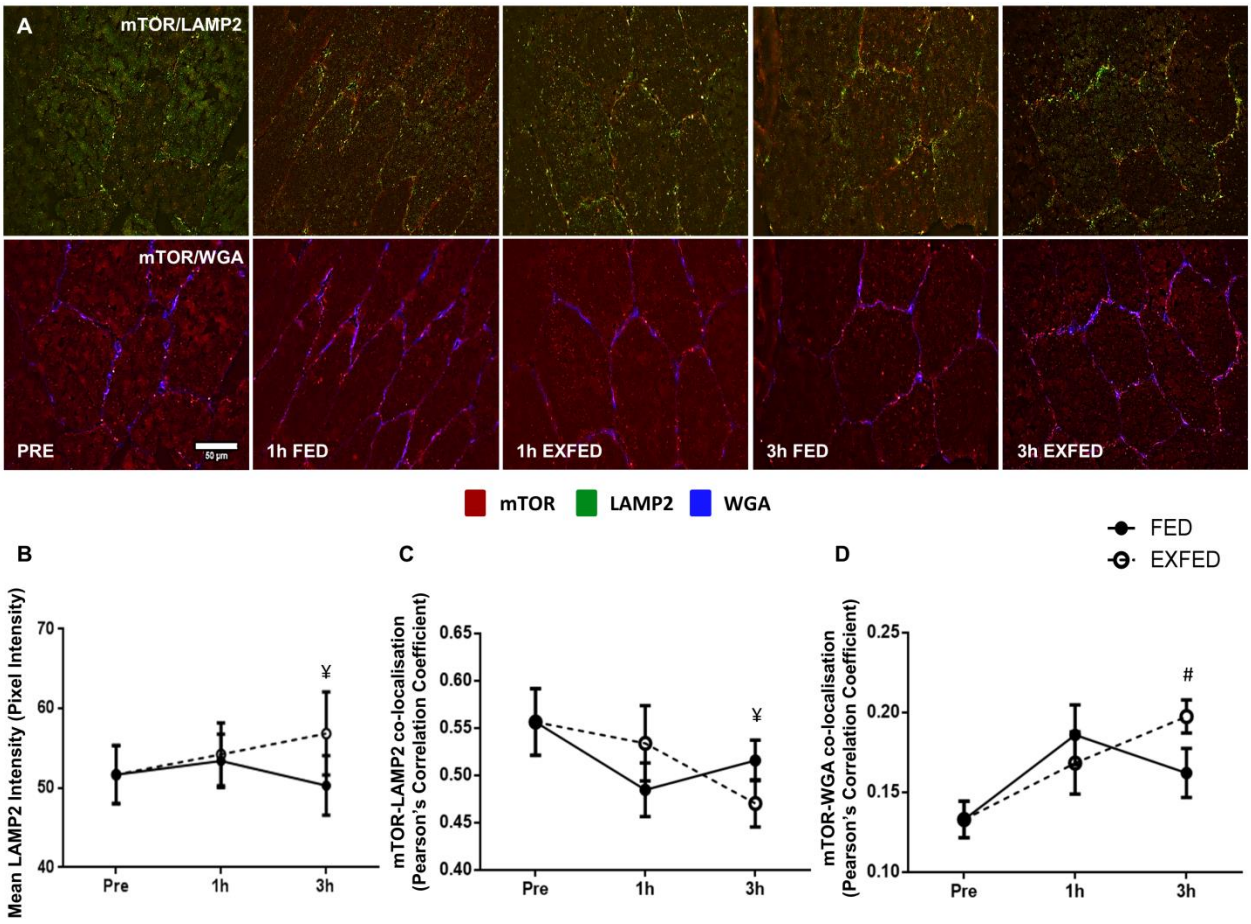

Figure 3.2. The effect of resistance exercise and/or protein carbohydrate feeding on mTOR-LAMP2 and mTOR-WGA colocalisation. Representative images of mTOR-LAMP2 and mTOR-WGA co-localisation at rest, and following resistance exercise and/or protein-carbohydrate feeding (A). Orange/yellow regions denote areas of mTOR localisation with the marker of the lysosome in images on the top row. mTOR-positive staining is shown in red, LAMP2-positive in green and WGA-positive in blue. Quantification of LAMP2 fluorescence intensity (B), mTOR-LAMP2 colocalisation (C) and mTOR-WGA (D) co-localisation at each time point. Scale bars are 50µm. Data presented as Mean±SEM. ¥Significant difference between conditions at this time point ($p<.05$), #significantly difference compared to baseline when conditions combined ($p=.008$).

3.4.4 mTOR/lysosome translocation to the cell membrane

Significant main effects of condition (p=.05) and time (p=.025) were observed for mTOR colocalisation with the cell membrane (WGA positive staining). The significant main effect of condition suggests that, when all time points are combined, mTOR-WGA was greater in the EXFED condition compared to the FED condition. Subsequent pairwise comparisons also display that when both conditions were combined, mTOR colocalisation with the cell membrane was greater at 3h post-exercise/feeding compared to baseline values (p=.008, Fig. 3.2c). Further comparisons also displayed a trend toward a difference between mTOR-WGA colocalisation between conditions at the 3h time point (0.16(FED) vs. 0.19(EXFED), p=.085). This pattern of colocalisation was mirrored when analysing LAMP2-WGA colocalisation (main effect of time, p=.031, data not shown.), reiterating the constant colocalisation of mTOR and the lysosome.

3.4.5 Rictor colocalisation with mTOR and WGA

Significant main effects of group (p=.046) and time (p=.035) were noted for Rictor colocalisation with mTOR proteins (Fig.3B). Overall, there was a greater colocalisation of these two proteins in the EXFED condition compared to the FED condition. Following pairwise comparisons, there was no difference in the colocalisation between Rictor and mTOR between any time points (p>.05, Fig. 3.3b). Furthermore, Rictor colocalisation with WGA did not change from baseline at any time point in either condition (Fig. 3.3c), suggesting post exercise translocation is specific to mTORC1.

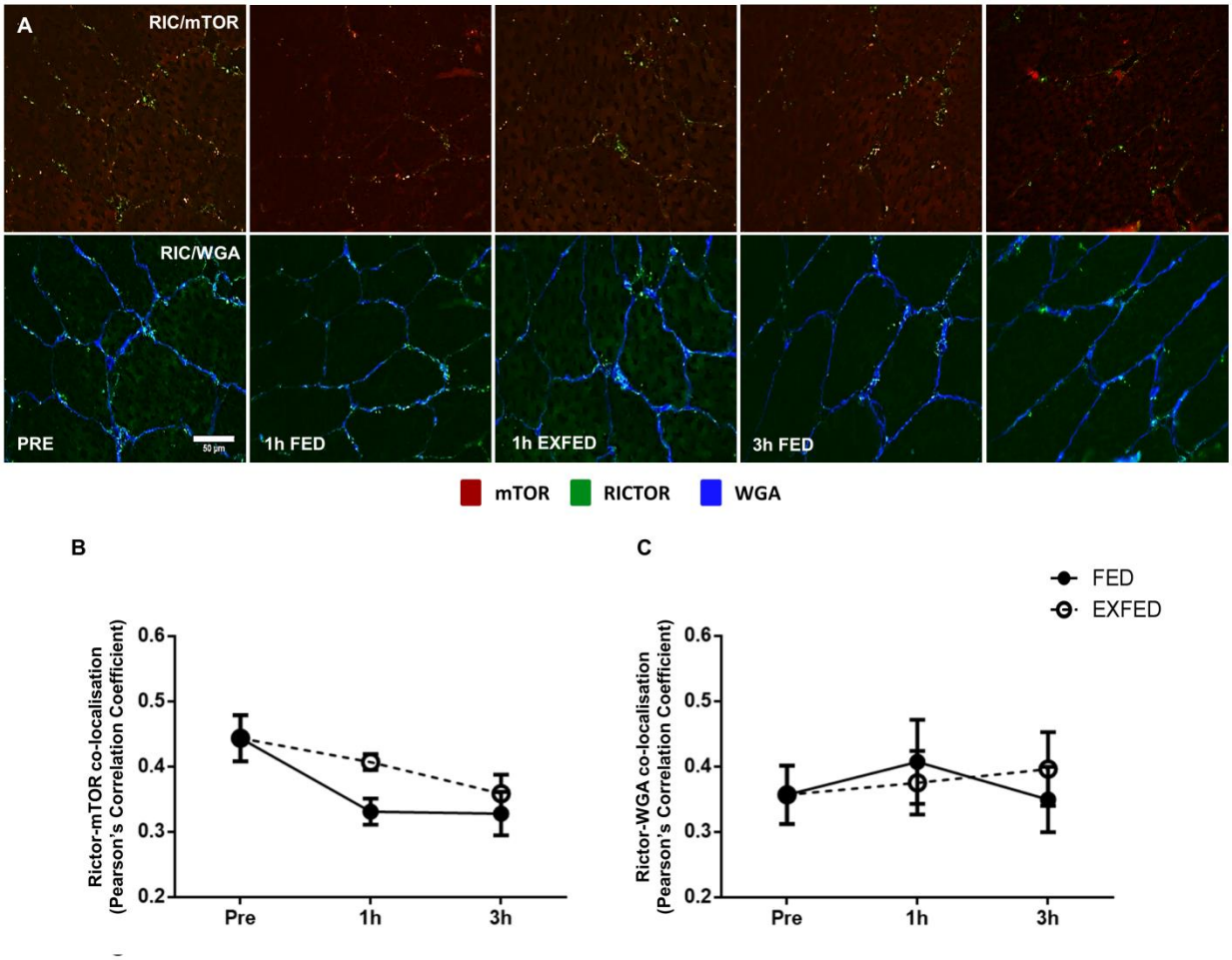

Figure 3.3. The effect of resistance exercise and/or protein carbohydrate feeding on Rictor-mTOR and Rictor-WGA colocalisation. Representative images of Rictor-mTOR and Rictor-WGA co-localisation at rest, and following resistance exercise and/or protein-carbohydrate feeding (A). Orange/yellow regions denote areas of Rictor localisation with mTOR on top row. mTOR-positive staining is shown in red, Rictor-positive in green and WGA-positive in blue Quantification of Rictor-mTOR (B) and Rictor-WGA (C) co-localisation at each time point. Scale bars are 50µm. Data presented as Mean±SEM.

3.4.6 Raptor colocalisation with mTOR and WGA

The colocalisation of Raptor and mTOR proteins did not change in either group, at any time point, suggesting any alterations in sub-cellular location of either protein occurred concurrently (Fig. 3.4b). A significant condition x time effect was observed for Raptor colocalisation with WGA (p=.029). Here, Raptor colocalisation with WGA rose to a similar extent to the previously reported increase in mTOR-WGA colocalisation at 1h post-exercise/feeding in both conditions. At the 3h time point, Raptor-WGA colocalisation in the FED group dropped below baseline and 1h post-ex/feeding levels (p=.007, Fig. 3.4c), and colocalisation at this time point was greater in the EXFED condition (0.12(FED) vs. 0.17(EXFED), p=.014, Fig. 3.4c).

3.5 – Discussion

Utilising a within-subject design, we report that a combination of unilateral resistance exercise and protein-carbohydrate feeding elicits a greater mTOR translocation toward the cell membrane than feeding alone. This observation is consistent with previous findings from our laboratory in which we reported that mTOR associates with the lysosome in basal skeletal muscle, with mTOR/lysosomal complexes translocating to the cell periphery following mTOR activation(13). Utilising immunofluorescent approaches to distinguish between mTORC1 and mTORC2, the present study extends this observation, suggesting that mTORC1 seems to be the predominant mTOR complex translocating in human skeletal muscle following anabolic stimuli, with mTORC2 in constant association with the cell membrane.

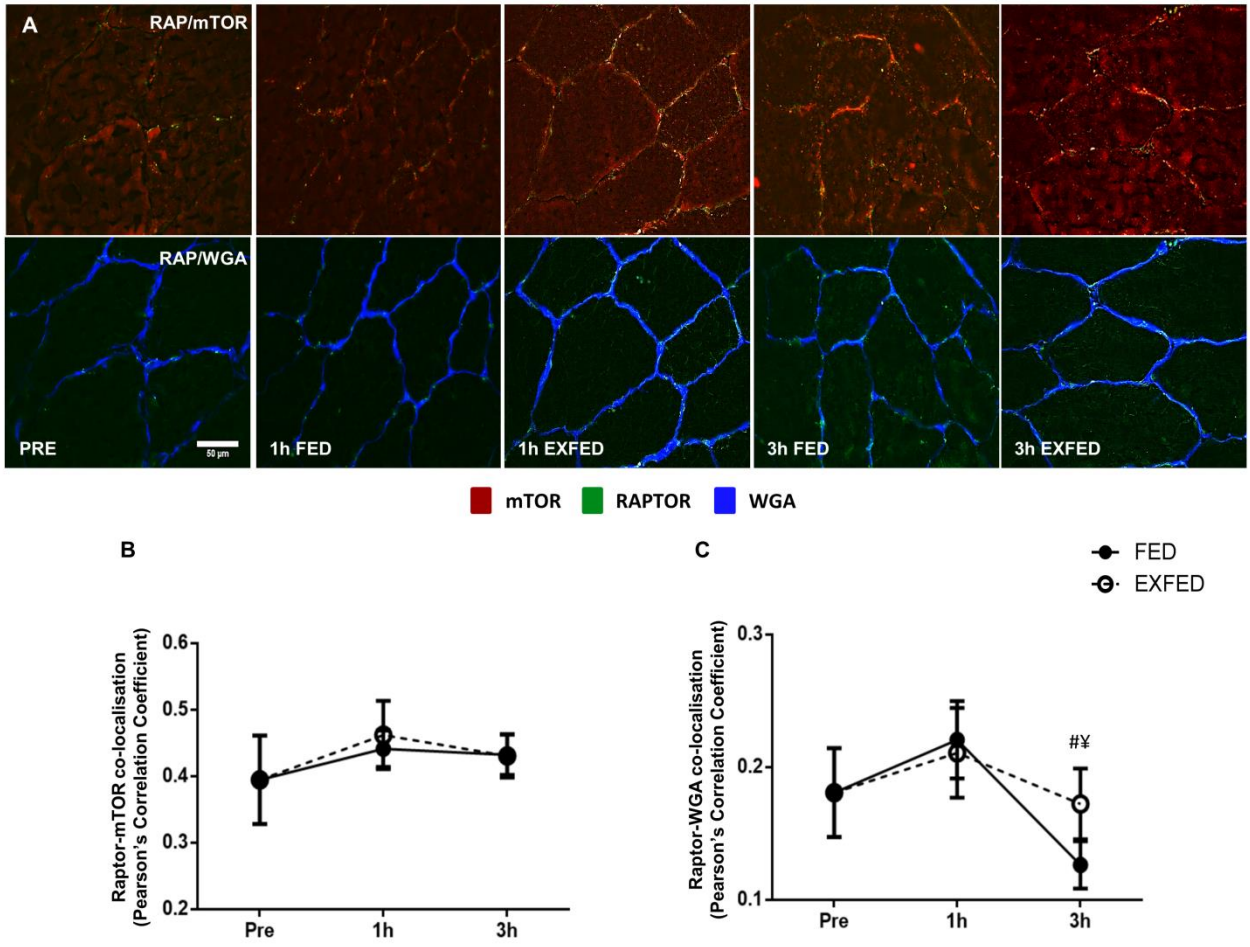

Figure 3.4. The effect of resistance exercise and/or protein carbohydrate feeding on Raptor-mTOR and Raptor-WGA colocalisation. Representative images of Raptor-mTOR and Raptor-WGA co-localisation at rest, and following resistance exercise and/or protein-carbohydrate feeding (A). Orange/yellow regions denote areas of Raptor localisation with mTOR. mTOR-positive staining is shown in red, Raptor-positive in green and WGA-positive in blue Quantification of Raptor-mTOR (B) and Raptor-WGA (C) co-localisation at each time point. Scale bar is 50µm. Data presented as Mean±SEM. ¥Significant difference between conditions at this time point (p=014), #significantly difference compared to baseline when conditions combined (p=.007).

In addition to mTORC1 translocation to the cell periphery, we report a greater colocalisation of mTOR and LAMP2 in the FED condition, compared to the EXFED

condition, at the 3h time point. This finding was unexpected and contrasted our previous research using a parallel group design (13). The greater association of mTORC1 with lysosomes in the FED condition would infer greater mTORC1 activity in this leg(10, 11); however, this was not apparent in our S6K1 kinase activity data. A possible explanation for this difference is the increased lysosomal content (LAMP2 fluorescence intensity) noted in the EXFED condition at this time point (Fig. 3.2b). It is possible that the acute resistance exercise bout may have elicited an increase in chaperone assisted selective autophagy as a stress response to the strenuous exercise, as previously reported(15). This may have increased the free-lysosomal pool(15) and altered the ratio of mTOR-LAMP2 association. As this is only a proxy measure of lysosomal content, further research directed towards lysosomal biogenesis in response to physiological stimuli would be needed to address this mechanism.

Previous research from our laboratory has shown an elevation in mTOR association with the cell membrane in response to resistance exercise, in both the fed and fasted state (13). This association coincided with an increase in S6K1 kinase activity, suggesting that mTOR trafficking is associated with an increase in intrinsic mTOR activity. Consistent with this hypothesis, here we report that mTORC1-cell membrane association increased 1h post-intervention, in both FED and EXFED conditions, and the increment was similar to that noted in our previous work(13). However, in contrast to our previous results, mTOR-WGA colocalisation in the FED condition returned close to baseline values at 3h and colocalisation in the EXFED condition displayed a continued elevation. In addition to the main effects of time ($p=.025$) and condition ($p=.05$) apparent here, a trend toward greater colocalisation in the EXFED condition ($p=.085$) was noted at the 3h time point. This greater colocalisation is suggestive of retention of mTOR at the cell periphery when

resistance exercise is followed with protein/carbohydrate ingestion, inferring a synergistic effect of resistance exercise and protein-carbohydrate feeding, an observation previously reported for MPS(2).

The mTORC1 and mTORC2 protein complexes are involved in varying metabolic signalling processes in skeletal muscle, and as such are suggested to reside in distinct cellular locations(5). As mTOR-lysosome translocation has been previously associated with mTORC1 activation in response to amino-acids *in vitro*(12), we sought to determine whether mTORC1 is the principal mTOR complex translocating in human skeletal muscle as we have previously reported(13).The colocalisation of Raptor with WGA increased at 1h post-intervention in both conditions, and to a similar extent to that noted in mTOR-WGA colocalisation, suggesting that mTORC1 is a spatially regulated mTOR complex in human skeletal muscle. Further to this notion, a disparity between conditions became apparent at the 3h time point, with Raptor-WGA colocalisation enhanced in the EXFED condition ($p=.014$). This is in agreement with the data regarding mTOR-WGA colocalisation where a trend toward EXFED eliciting greater membrane colocalisation compared to FED is reported. Raptor colocalisation with mTOR itself was not altered at any time point, or between conditions, however we did observe a reduction in raptor association with WGA at 3h in both FED and EXFED. We are currently unable to explain this result, however, it could be due to an increase in free Raptor content(16) or increased Raptor degradation(17), both potential mechanisms proposed to regulate mTORC1 activity. In contrast, co-staining of Rictor with WGA, suggested that mTORC2 localises with the cell membrane in basal tissue, with this colocalisation unaffected by resistance exercise or protein/carbohydrate ingestion. This finding has also been replicated using *in*

vitro models, where a large proportion of mTORC2 activity was noted at the plasma membrane of HEK293 cells(18).

Our data are congruent with both *in vitro*(12) and *in vivo*(13) studies suggesting that mTORC1 cellular colocalisation is linked to mTORC1 activity. Whilst this observation is in contrast to previous *in vitro* studies(10, 19), where mTOR translocation to the lysosome is deemed essential, we believe the increase in autophagy/MPB in post-absorptive skeletal muscle prevents the disassociation of mTOR and the lysosome noted in previous *in vitro* studies, where a complete amino acid withdrawal protocol is utilised. Further, many physiological mechanisms occur at the cell periphery suggesting the redistribution of mTORC1/lysosomal complexes to the cell periphery is physiologically relevant. mTORC1 is known to stimulate MPS which, through the use of the SUnSET technique(20) and immunohistochemical staining methods, is purported to occur primarily in peripheral regions of muscle fibres(21). Consistent with this, we previously identified mTOR to interact with Rheb, eIF3F and the microvasculature at the cell periphery following resistance exercise in the fed state(13). Collectively this data therefore suggests that both upstream regulators and downstream substrates of mTORC1 are membrane-associated in skeletal muscle(21). Further, given we observed that mTORC1 association with the cell periphery was prolonged with feeding [a scenario of heightened MPS], we propose that maintaining mTORC1 at the cell periphery may provide an mechanistic explanation as to why exercise in the fed state results in prolonged increases in MPS in human skeletal muscle compared to exercise or feeding in isolation(7).

3.6 – Conclusions

In summary, our data show that mTOR-lysosome translocation in response to resistance exercise and feeding is driven primarily by mTORC1, and occurs in parallel to increases in S6K1 activity. Further, we report that resistance exercise combined with protein-carbohydrate feeding sustains this response, compared to feeding alone, suggesting a synergistic effect of these two stimuli. Collectively, these data add further support to the importance of spatial regulation of mTORC1 in response to anabolic stimulation. Further research should now examine the relevance of mTORC1 colocalisation in clinical scenarios, i.e ageing(22) or obesity(23). Finally, the tools described herein to study mTORC2 localisation could be used to examine the regulation of skeletal muscle glucose uptake and insulin sensitivity, factors thought to be under the direct control of mTORC2(24).

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Chapter 4

CHARACTERISATION OF L-TYPE AMINO ACID TRANSPORTER 1 (LAT1) EXPRESSION IN HUMAN SKELETAL MUSCLE BY IMMUNOFLUORESCENT MICROSCOPY

4 CHARACTERISATION OF L-TYPE AMINO ACID TRANSPORTER 1 (LAT1) EXPRESSION IN HUMAN SKELETAL MUSCLE BY IMMUNOFLUORESCENT MICROSCOPY

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Author Contributions:

Human *vastus lateralis* samples were collected by **Dr. Leigh Breen**. Analysis plan was conceived by **Nathan Hodson, Dr. Sophie Joanisse** and **Dr. Andy Philp**. LAT1 knockout tissue was provided by **Dr. Nick Aguirre** and **Prof. Keith Baar.** Immunohistochemical analysis presented in Figure 4.1 and 4.2A&B was conducted by **Thomas Brown**. All other immunohistochemical analysis was conducted by **Nathan Hodson**. Data presented in Figure 4.2D was provided by **Dr. Daniel West** and **Dr. Daniel Moore**. Statistical analysis and interpretation of results were conducted by **Dr. Nathan Hodson**.

4.1 – Abstract

The branched chain amino acid leucine is a potent stimulator of protein synthesis in skeletal muscle. Leucine rapidly enters the cell via the L-Type Amino Acid Transporter 1 (LAT1); however, little is known regarding the localisation and distribution of this transporter in human skeletal muscle. Therefore, we applied immunofluorescence staining approaches to visualise LAT1 in wild type (WT) and LAT1 muscle-specific knockout (mKO) mice, in addition to basal human skeletal muscle samples. LAT1 positive staining was visually greater in WT muscles compared to mKO muscle. In human skeletal muscle, positive LAT1 staining was noted close to the sarcolemmal membrane (dystrophin positive staining), with a greater staining intensity for LAT1 observed in the sarcoplasmic regions of type II fibres (those not stained positively for myosin heavy-chain 1, Type II—25.07 ± 5.93, Type I—13.71 ± 1.98, $p < 0.01$), suggesting a greater abundance of this protein in these fibres. Finally, we observed association with LAT1 and endothelial nitric oxide synthase (eNOS), suggesting LAT1 association close to the microvasculature. This is the first study to visualise the distribution and localisation of LAT1 in human skeletal muscle. As such, this approach provides a validated experimental platform to study the role and regulation of LAT1 in human skeletal muscle in response to various physiological and pathophysiological models.

4.2 - Introduction

Amino acid transport is an essential component in the survival of all cells(1), providing substrates for processes such as protein synthesis(2, 3) and cell division(4). Amino acid transport into cells is of particular importance within skeletal muscle to maintain positive protein balance(2, 3). Some evidence suggests that amino acid transporter proteins may also act as receptors, converting extracellular amino acid abundance into intracellular signals(5-7), a role termed 'transceptor'(8).

Of the amino acid transporters identified in skeletal muscle, the L-Type amino acid transporter 1 (LAT1) also known as the solute carrier family 7 member 5 (SLC7A5), has received significant attention(9-15) due to its role in the ingress of amino acids, vital for optimal stimulation of muscle protein synthesis (MPS)(16-18). LAT1 transports leucine (or other branched chain amino acids (BCAAs)) into the cell via a bi-transport system which simultaneously exports glutamine(19). The increase in intracellular leucine/BCAAs subsequently activates the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) resulting in an elevation in MPS(20). Leucine's reported potency toward mTORC1 in skeletal muscle(16) is modulated primarily through LAT1, as this is the main transporter by which leucine moves into cells(9). Other L-type amino acid transporters are expressed in skeletal muscle, including LAT2 and LAT4(21, 22), however it seems LAT1 is the primary BCAA transporter. In LAT1 muscle-specific knockout (mKO) mice, a compensatory increase in SLC7A8/LAT2 mRNA was reported(14), yet a significant reduction in amino acid transport into muscle was apparent, suggesting LAT1 is integral to this process. Furthermore, LAT4 is only weakly expressed in skeletal muscle(21) and is believed to be primarily required in placenta as this

transporter functions to deliver amino acids from the mother to the foetus(23). Due to its apparent importance to amino acid transport in skeletal muscle, understanding the role and regulation of LAT1 is essential for studies aimed at maximising the delivery of dietary protein and amino acids in the context of post-exercise recovery.

Gene expression and protein content of LAT1 is positively associated with mTORC1 activity; however, increases in LAT1 expression are abolished by the administration of rapamycin, suggesting that mTORC1 may also regulate LAT1 activity(24). In human skeletal muscle, essential AA (EAA) consumption elicits an early increase in LAT1 gene expression followed by elevations in protein content(12). Furthermore these transient effects have also been reported in response to resistance exercise alone (11), and in combination with EAA consumption(10). Such data suggest that anabolic stimuli may initiate signalling pathways which up-regulate both LAT1 gene expression and protein levels in human skeletal muscle, possibly translating to greater leucine/BCAA transport and a superior protein synthetic response(2, 3, 16, 25). However, it is difficult to reconcile the physiological relevance of changes in LAT1 gene and protein content with LAT1 activity since LAT1 is only active when at the plasma membrane(9, 26). Therefore, any measure of protein or gene expression in whole muscle homogenates(10-12) may not allow reliable conclusions to be drawn. To date, the localisation of LAT1 in human muscle is unknown and requires investigation.

Therefore, the aim of the present study was develop and validate immunofluorescent approaches to study LAT1 cellular distribution and localisation in human skeletal muscle. We hypothesised that in basal human skeletal muscle, LAT1 would be located at the plasma membrane and would be ubiquitously expressed across fibre types.

4.3 – Materials and Methods

4.3.1 Mouse skeletal muscle sample collection and preparation

LAT1 mKOmice were provided as a gift by Dr. Peter Taylor of the University of Dundee. Methods of generation of this mouse strain has been previously described(14). Mice (n=2 mKO, n=2 wild type (WT)) were housed in a climate-controlled facility at the University of California Davis, in a standard 12h light/dark cycle and fed standard chow *ad libitum.* All procedures were approved by the UC Davis Institutional Animal Care and Use Committee (IACUC) and performed under protocol number 19244. At 3 months of age, mice were fasted for 5h before the extensor digitorum longus (EDL) muscle was surgically removed under anaesthetic (2.5% isofluorane), pinned to cork and frozen in liquid nitrogen-cooled isopentane. Muscles were subsequently stored at -80°C until sectioned. Prior to sectioning, the belly of the EDL was blocked in Tissue-Tek Optimal Cutting Temperature (OCT) Compound (VWR International, Leicestershire, UK.) and frozen in liquid nitrogen-cooled isopentane at which point the sample was ready for cryo-sectioning. During staining protocols, both WT and mKO samples were processed and stained simultaneously, and image capture paradigms were also identical.

4.3.2 Human skeletal muscle sample collection and preparation

Ethical approval for the collection of muscle samples was granted by the NHS West Midlands Black Country Research Ethics Committee (14/WM/0088 & 15/WM/0003), the Hamilton Health Sciences Research Ethics Board (12-631), and the University of Guelph Research Ethics Board (120C018). The study conformed to the standards presented by the Declaration of Helsinki (seventh version). Written informed consent was obtained from all participants before samples were collected. Muscle samples for

immunofluorescence were obtained from the vastus lateralis muscle of 6 young, healthy males (mean age - 22±2yrs), who attended the laboratory after an overnight (10h) fast, using the Bergstrom percutaneous needle technique. Samples were blotted free of excess blood and dissected free of any fat or connective tissue before being placed in OCT compound and frozen in liquid nitrogen-cooled isopentane. Muscle samples were then stored at -80°C until further analysis. Muscle samples for Western blot were collected after an overnight fast and 15min after an acute bout of exercise, as previously described (27). Samples (200-300mg) were immediately processed for sarcolemmal vesicles and cytosolic fractions, as previously described(28), before being stored at -80°C until further analysis.

Primary Antibody	Source	Dilution	Secondary Antibody	Dilution
Rabbit polyclonal anti-SLC7A5 antibody isotype IgG	Abcam, ab85226	1:100	Goat anti-rabbit IgG(H+L) Alexa®488	1:200
SLC7A5 peptide	Abcam, ab192836	1:10	N/A	N/A
Mouse monoclonal anti-Dystrophin antibody, isotype IgG2a	DSHB, MANDYS1 3B7	1:200	Goat anti-mouse IgG2a Alexa®594	1:200
Mouse monoclonal anti-MHC1 antibody, isotype IgM	DSHB, A4.480	1:500	Goat anti-mouse IgM Alexa®594	1:200
Mouse monoclonal anti-eNOS antibody, isotype IgG1	BD Transduction, #610297	1:200	Goat anti-mouse IgG1 Alexa®594	1:200
Wheat Germ Agglutinin-350	W11263, Invitrogen	1:20	Alexa Fluor® 350 Conjugated	N/A

Table 4.1. Summary of Antibodies used.

4.3.3 Immunofluorescent Staining

Embedded muscle samples were fixed in front of the microtome blade (Bright 5040, Bright Instrument Company limited, Huntingdon, England) and cryo-sections (5µm)

collected onto room temperature uncoated glass slides (VWR international, UK). Sections were left to air dry at room temperature for 10min to remove excess crystallized water inside sections under storage. Sections were fixed in acetone and ethanol (3:1) solution (Fisher, UK) for 5 min and then washed for 3 x 5min in phosphate buffered saline (PBS) supplemented with 0.2% Tween (PBS-T) to remove fixation reagent. Sections were then incubated in primary antibody solution diluted with 5% normal goat serum (Invitrogen, UK) for 2hr at room temperature. For blocking peptide experiments, the anti-SLC7A5 antibody was pre-incubated with its corresponding peptide (20 times greater than primary antibody concentration) on an orbital shaker for 1 hour at room temperature. Following incubation, sections were washed for 3 x 5min in PBST and incubated in the appropriate secondary antibody for 1h at room temperature. If needed, sections were finally incubated with Wheat Germ Agglutinin (WGA-350) for 30min at room temperature to mark the sarcolemmal membrane. After a final wash in PBS, slides were left to air dry until the visual water stains evaporated: 1-2 min at room temperature. Sections were mounted with 20µL Mowiol® 4-88 (Sigma-Aldrich, UK) and sealed by glass coverslips to protect the muscle sections and to preserve fluorescence signals. Slides were left overnight in a dark room before observation. Primary antibodies, blocking peptides and corresponding secondary antibodies and working dilutions are listed in Table 1. All secondary antibodies were sourced from ThermoFisher Scientific Inc. (Waltham, MA, USA).

4.3.4 Image Capture

Prepared slides were observed under a Nikon E600 widefield microscope using a 40×0.75 numerical aperture objective. Images per area were captured under three colour filters achieved by a SPOT RT KE colour three shot CCD camera (Diagnostic Instruments Inc.,

MI, USA), illuminated by a 170 W Xenon light source. For image capture, DAPI UV (340–380 nm) filter was used to view WGA-350 (blue) signals and LAT1 stains tagged with Alexa 488 fluorophores (green) were visualised under the FITC (465–495nm) excitation filter. The Texas-Red (540–580 nm) excitation filter was used to capture signals of eNOS (blood vessels), dystrophin (sarcolemma) or Myosin heavy chain 1 (Type I Fibres), which were conjugated with Alexa Fluor 594 fluorophores. On average, 8 images were captured per section, and each image contained ~8 muscle fibres. As 2 sections per participant were imaged, approximately 120 fibres per subject were used for analysis. Image processing and analysis was undertaken on ImagePro Plus 5.1 (Media Cybernetics, MD., USA.) and all factors i.e. exposure time and gain, were kept constant between all images on each individual slide. All images underwent flattening and de-speckling prior to quantification of co-localization to limit the contribution of background, non-specific fluorescence. Image signals generated by WGA or dystrophin were used to estimate cell membrane borders, and MHC1 staining was utilised to identify type I fibres. The intensity of LAT1 staining in these groups of fibres was used to assess any differences in fibre type abundance of the LAT1 protein.

4.3.5 Immunoblotting

As a qualitative methodological comparator with immunofluorescence for the spatial distribution of LAT1, giant sarcolemmal vesicles were isolated from the vastus lateralis of a subset healthy males (N=2, ~21 years of age) as previously described(29). The protein pellets that remained were resuspended and homogenized in a buffer containing MOPS (10mM), KCl (140mM), EDTA (10mM), and protease inhibitors. Samples were centrifuged at 800*g* × 10min at 4°C to remove insoluble proteins. Protein concentrations

in cytosolic and sarcolemmal vesicle fractions were determined by BCA assay and prepared at equal concentrations in 4X Laemmli buffer. Three µg of protein was loaded on a precast gel (BioRad 5671094), run at 200V for 45 min, transferred (100V for 1h) to nitrocellulose membrane, stained with Ponceau S solution, blocked in 5% skim milk, and probed overnight (1:1000 in TBST) using the same anti-LAT1 antibody used for immuno-fluorescent staining (see table 1). The membrane was incubated in goat anti-rabbit IgG HRP conjugated secondary (Thermo Fisher,cat. 31460; 1:10 000 in TBST) for 1h at RT before washing in TBST and detection by chemiluminescence. Images were captured using a Fluorochem E Imaging system (Protein Simple; Alpha Innotech, Santa Clara, CA).

4.3.6 Statistical Analysis

All statistical analysis was conducted on SPSS version 22 for Windows (SPSS Inc., Chicago, IL, USA). Differences between the staining intensity in type I and type II fibres was analysed by a paired-samples t-test. Significance threshold was set at $p \leq .05$. Data are presented as Mean±SE unless otherwise stated.**3.**

4.4 – Results

4.4.1 Characterisation and validation of LAT1 immunofluorescence labelling approaches

In WT skeletal muscle samples, positive LAT1 signals were noted at the periphery of many fibres, in addition to displaying a stronger sarcoplasmic staining than seen in mKO samples (Fig 4.1a). In mKO samples, positive LAT1 staining was almost completely abolished (Fig 4.1b). We take this data to suggest that the anti-LAT1 antibody utilised here is specific to the LAT1 protein in skeletal muscle. This was further confirmed as the

pattern of staining noted in mKO samples was similar to that seen when the primary antibody was omitted from staining protocols.

Next, we applied the same staining technique to human skeletal muscle (Figure 4.2a & 4.2b). Here a similar staining pattern was noted, with positive-LAT1 puncta close to the sarcolemmal membrane (dystrophin positive staining) as well as within the cytosol in some fibres. Additionally, a possible fibre-type difference in LAT1 content was apparent in human tissue. Finally, when the anti-LAT1 antibody was incubated with a LAT1-specific blocking peptide, the immunofluorescent signal and staining pattern was greatly reduced (Figure 4.2c), further confirming the antibody's specificity to the LAT1 protein. The immunoblot data also demonstrate the presence of LAT1 in the sarcolemma and cytosolic protein fractions (Fig 4.2d).

4.4.2 LAT1 protein content is greater in fast-twitch skeletal muscle fibres

To examine fibre type distribution, LAT1 was co-stained with myosin heavy-chain 1 (MHC1) to identify type I fibres. This allowed us to quantify LAT1 positive staining in type I and II fibres. Representative images of this stain (Figs 4.3a & 4.3b) displayed a difference in LAT1 staining between fibre types with a greater staining intensity seemingly apparent in type II fibres. Quantification of the staining intensity in each fibre type (Fig4.3c) confirmed this as an 82.7% greater staining intensity was noted in type II fibres compared to type I fibres (25.07±5.93 vs. 13.71±1.98, $p<.01$, Figure 3C). This results suggests a greater amount of LAT1 is expressed in more glycolytic fibres, which do not express MHC1; however, it seems the difference predominantly occurs in the sarcoplasm as positive LAT1 staining is still apparent at the sarcolemma of type I fibres (Fig 4.3a & 4.3b).

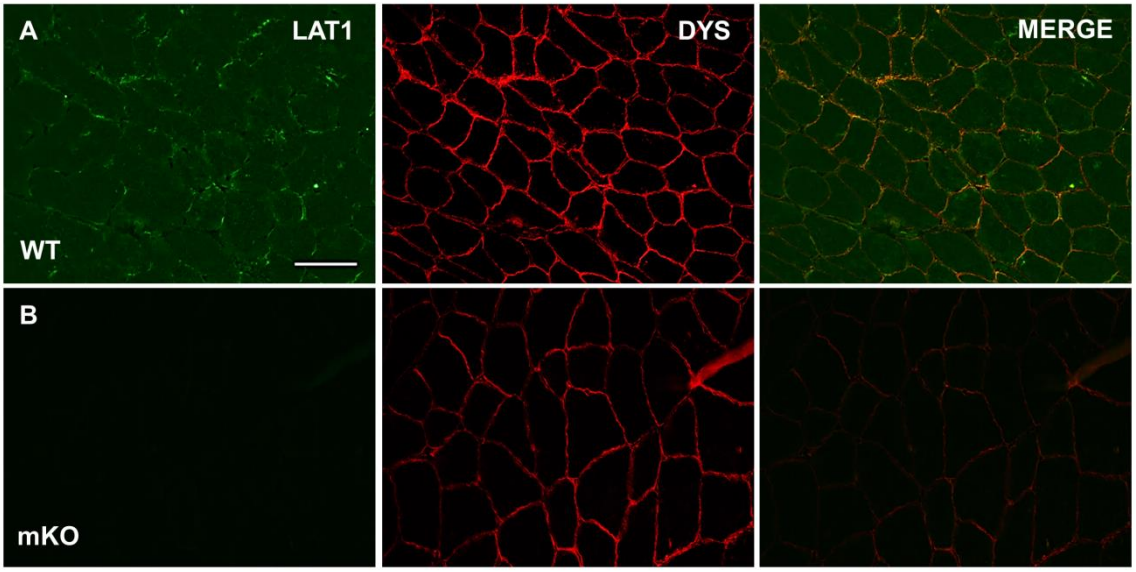

Figure 4.1. Immunofluorescent detection of LAT1 in wild type (WT) and LAT1 muscle-specific knockout (mKO) mouse extensor digitorum longus (EDL) muscle. Sections were stained with anti-LAT1 antibody (far left panels, green) and then co-stained with dystrophin (middle panels, red) for the identification of the sarcolemma. Representative images for both WT (A) and mKO (B) are shown. Scale bar in top left panel is 50μm and all images were captured at the same magnification.

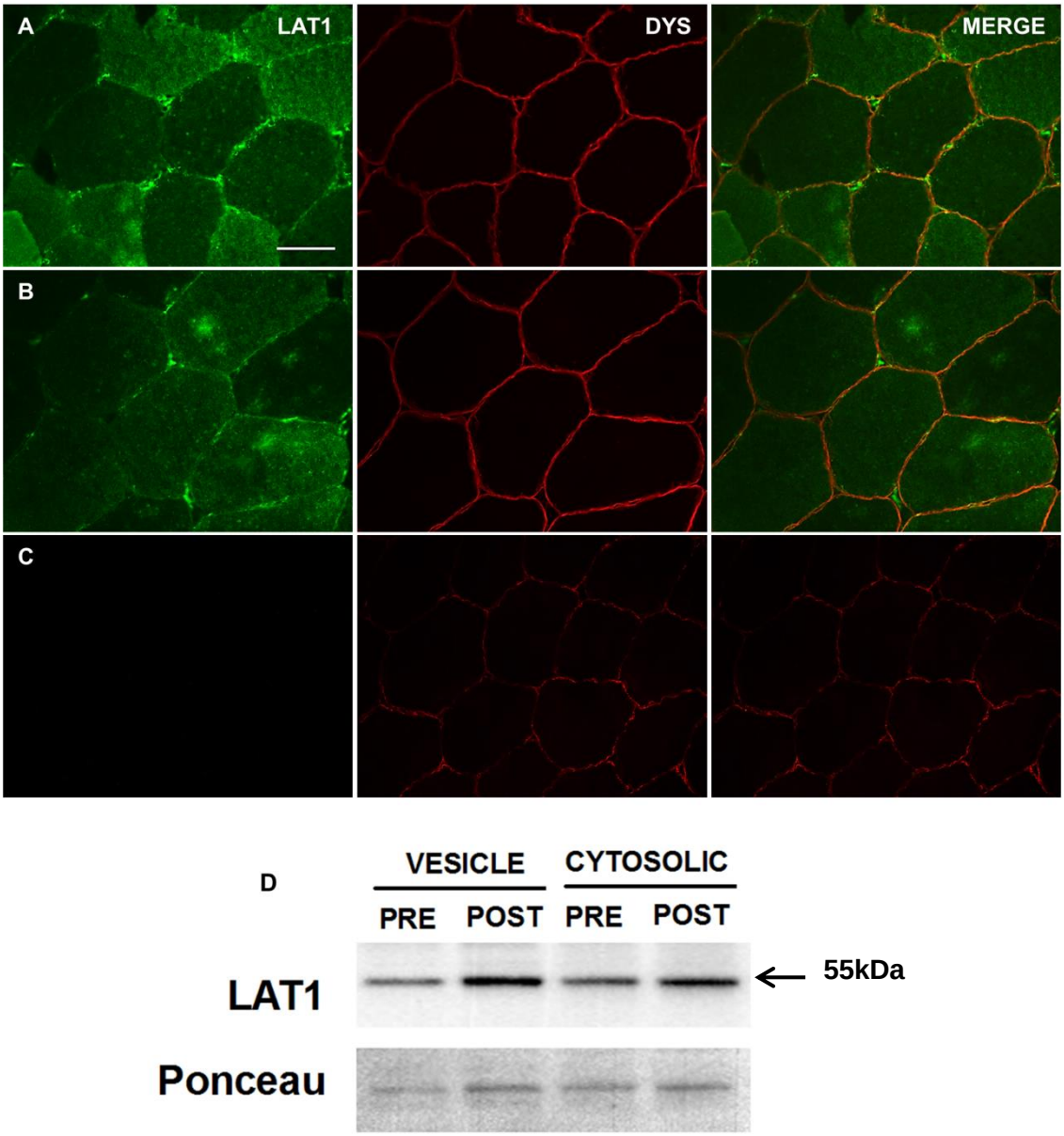

Figure 4.2. Immunofluorescent detection of LAT1 in human *vastus lateralis.* muscle. Sections were stained with anti-LAT1 antibody (far left panels, green) and then co-stained with dystrophin (middle panels, red) for the identification of the sarcolemma. Representative images from two participants are displayed (A & B). Use of a peptide competition assay reduced the signal intensity of LAT1 staining (C). A strong positive signal was noted close to the sarcolemmal membrane and possible sites of blood vessels. In addition a possible fibre type difference in LAT1 staining was noted. Immunoblots for the LAT1 protein displayed bands to be apparent in both giant sarcolemmal vesicles and cytosolic fractions before (PRE) and after (POST) an acute bout of resistance exercise (D) (N=2). Scale bar in top left panel is 50µm and all images were captured at the same magnification.

4.4.3 LAT1 localises close to the microvasculature

To assess LAT1 interaction with the microvasculature, LAT1 was co-stained with anti-eNOS antibody, to identify blood vessels. This antibody has previously been utilised in human skeletal muscle to positively stain capillaries, and other micro-vessels (30). Here, it was apparent that positive LAT1 staining occurred close to positive staining of the microvasculature (eNOS), as shown in the merged and zoomed images (Figure 4, right panels), suggesting LAT1 localises close to skeletal muscle vasculature at rest.

4.5 – Discussion

LAT1/SLC7A5 is a transmembrane amino-acid transporter, which imports leucine and other BCAAs into cells in exchange for glutamine. This places LAT1 as a central regulator of skeletal muscle protein dynamics given the importance of leucine in activating mTORC1 and initiating MPS after feeding(31). Previous studies investigating LAT1 in human skeletal muscle have focussed on gene expression and protein levels of this transporter in whole muscle homogenates, measures that do not provide much information about LAT1 physiological function as the LAT1 protein is only 'active' when associated with cellular membranes(9). Therefore, in this study, we aimed to validate and optimise an immunofluorescence staining method to visualise LAT1 in human skeletal muscle. Using this approach we observed LAT1 to be in two pools within in basal human skeletal muscle: within the sarcoplasm and associated with the sarcolemma. Furthermore, we report novel information showing LAT1 to be expressed in a fibre-specific manner and located in close proximity to the microvasculature.

Staining for LAT1 in fasted human skeletal muscle tissue displayed a strong immunoreactivity close to the sarcolemmal membrane (Fig. 4.2), which was confirmed by its presence within a giant sarcolemmal vesicle preparation (Fig. 4.2d). This fraction, which is primarily sarcolemmal membrane but may also contain recycling endosomal membranes(32), has been previously shown to contain intracellular fat (e.g. FAT/CD36) and glucose transporters (e.g. GLUT4) in rodent and human muscle(28, 33). This primary location of LAT1-positive staining along the sarcolemmal membrane is in congruence with its purported role as a transceptor(34). As the primary transporter of BCAAs into muscle cells(19), fully functioning LAT1 would need to associate with the sarcolemmal membrane in order to convey free amino acids into muscle fibres(35). Structurally, LAT1, a permease, can associate with a glycoprotein, CD98 (SLC3A2)(35, 36), to form a heterodimer at a membrane. This association tethers LAT1 to the membrane, allowing the permease to transport amino acids across the membrane(36, 37). Furthermore, any role as a sensor of extracellular amino acids would also require LAT1 to be associated with the sarcolemma. Therefore, as either a transporter or a signalling molecule LAT1 would have to associate with the membrane(8, 34). Although our data is the first study to utilise immunofluorescence staining techniques to detect LAT1 in human skeletal muscle, this protein has previously been visualised in other tissues. In human placenta, LAT1 was visualised predominantly at the microvillous membrane of terminal villi and was essential for leucine uptake into placenta cells(38). Further, LAT1 is located at plasma membranes of Laryngeal Squamous tumours(39), where amino acid uptake is elevated to provide substrates for tumour growth(40). Several other tissues also express LAT1 principally in membranous structures including foetal intestines(41), non-small cell lung carcinomas(42) and ovarian tumours(43). This consistent visualisation of LAT1 at

membranes of tissues where protein synthesis/cell division is high consolidates our finding in human skeletal muscle, a tissue in which a regular supply of EAAs is imperative for optimal cell function and turnover(2).

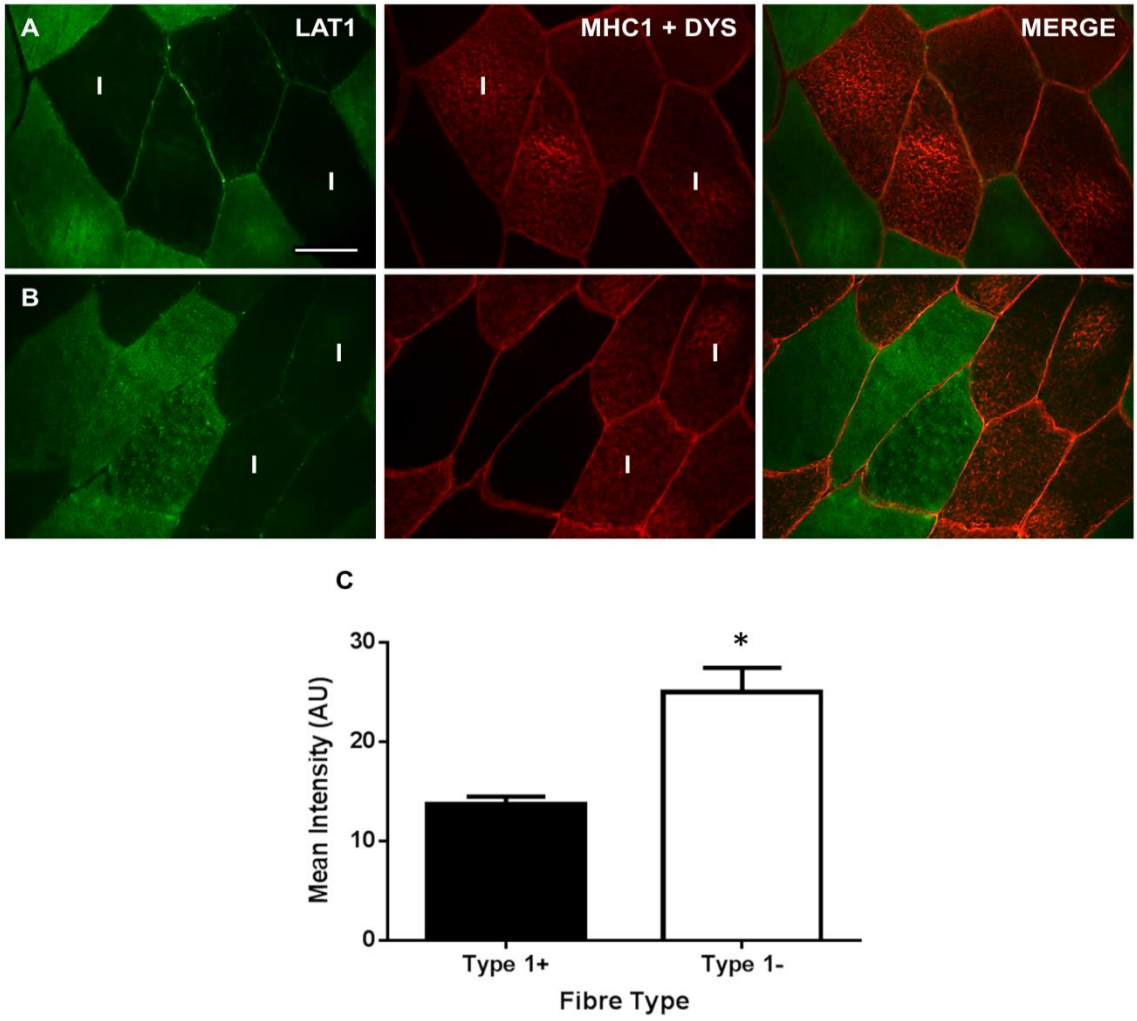

Figure 4.3. Immunofluorescent detection of LAT1 in human *vastus lateralis.* muscle co-stained with myosin heavy chain 1 (MHC1) and dystrophin (DYS). Sections were stained with anti-LAT1 antibody (far left panels, green) and then co-stained with dystrophin (middle panels, red) for the identification of the sarcolemma and MHC1 (middle panels, red sarcoplasmic staining, marked with I) for the identification of type I muscle fibres. Representative images from two participants are displayed (A & B). Quantification of the mean immunofluorescent staining in type I and type II fibres displayed a greater staining in type II fibres (C). On average 103±17 fibres were quantified per participant. Values are Mean±SE. *Significantly different to type I ($p<.01$). Scale bar in top left panel is 50µm and all images were captured at the same magnification.

Skeletal muscle is perfused by an intricate network of capillaries, and other microvessels, providing the working tissue with adequate supplies of oxygen and substrates whilst simultaneously removing waste products. Essential amino acids are transported to the muscle via this network, entering the bloodstream via absorption in the small intestine after food is consumed(44). As this is the most ready supply of essential amino acids for skeletal muscle (other than intracellular amino acid recycling from protein breakdown), we investigated whether LAT1 would localise close to blood vessels. We display strong LAT1 immunoreactivity with eNOS. Such close association between an area of high supply (bloodstream) and one of high demand (skeletal muscle) suggests a high efficiency of amino acid transport in human skeletal muscle. Similar localisations have been displayed in other tissues with high LAT1 expression noted at blood-brain barriers in rodents(45, 46) and chickens(47). Additionally, other amino acid transporters are also seen in these regions (47) further implying a high efficiency of amino acid transport at blood-tissue junctions.

When staining for the LAT1 protein in skeletal muscle, a difference in the intensity of LAT1 staining between some fibres was apparent. We hypothesised this may be specific to a particular fibre type and confirmed this by co-staining LAT1 with an anti-MHC1 antibody to identify type I (oxidative) fibres (Fig 4.3). LAT1 expression was greater in type II fibres and this seemed to be driven by a greater staining intensity within the sarcoplasm of these fibres, suggesting a greater dynamic pool in these fibres. This was confirmed via immunoblotting of mixed human skeletal muscle giant sarcolemmal vesicles and cytoplasmic fractions (Fig 4.2d), the latter of which may include membranes associated with sorting endosomes, lysosomes, and intracellular organelles (e.g.

endoplasmic reticulum, Golgi apparatus) that would be distinct from those extracted in the giant sarcolemmal vesicles(32, 48). Immunoblotting displayed expression of the LAT1 protein in both fractions adding further validation to our observations of greater LAT1 staining intensity in the sarcoplasm of type II fibres. Several mechanisms within skeletal muscle may explain this apparent increase in sarcoplasmic LAT1 within more glycolytic fibres. Firstly, increases in LAT1 protein could result from mTORC1 activation. In response to essential amino acid ingestion(12), resistance exercise(11), or both in combination(10, 49), stimuli which have been consistently reported to transiently activate mTORC1, LAT1 gene expression and protein levels are elevated in young and old males. In addition, alterations in LAT1 gene expression and protein levels following platelet-derived growth factor incubation are abolished by co-incubation with rapamycin(24), an mTORC1 inhibitor. As type II fibres show a greater elevation in mTORC1 activity in response to exercise(50), it is possible that this drives greater transcription/translation of LAT1 in these fibres, which remains apparent in fasted tissue. It may also be possible that, due to the greater levels of mTORC1 activity experienced in these fibres, LAT1 is required on the membrane of intracellular lysosomes as shown in some *in vitro* models (51). Here, LAT1 could maintain intralysosomal amino acid concentrations which are believed to then contribute to mTORC1 activation(52). It is also possible that sarcoplasmic LAT1 may translocate toward the sarcolemmal membrane in response to stimuli where an increase in amino acid transport occurs (i.e., mechanical stimulation or feeding(20)). In fact, the translocation capacity of LAT1 has been previously reported in BeWo Chariocarcinoma cells after exposure to IGF-1, where the transporter translocated from perinuclear regions toward the cell membrane(53). An increase in membrane-associated LAT1 protein will then increase the amount of amino

acids which can be transported into the cell for protein synthesis/repair. Since we show a greater intracellular concentration of LAT1 in type II fibres, if LAT1 does translocate this would suggest that type II fibres would have the greatest anabolic potential. It is important that this fibre-type difference in LAT1 expression is further investigated to fully understand the underpinning mechanisms and how signalling/metabolism in these fibres are affected.

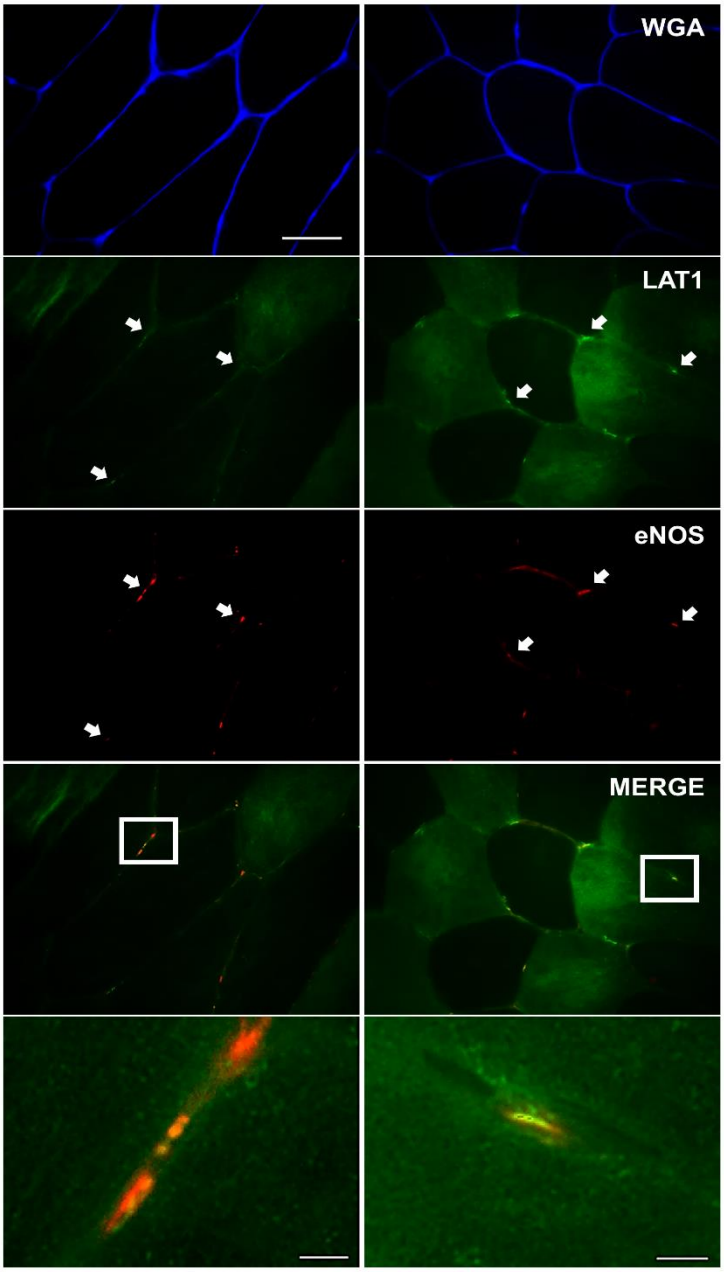

Figure 4.4. Immunofluorescent detection of LAT1 in human *vastus lateralis.* muscle co-stained with endothelial nitric oxide synthase (eNOS) and Wheat Germ Agglutinin (WGA). Sections were stained with anti-LAT1 antibody (green) and then co-stained with eNOS (red) for the identification of blood vessels. WGA (blue) was used to identify membrane borders. Representative images from two participants are displayed. Merged images display positive LAT1 staining localising close to positive eNOS staining, shown in greater detail in zoomed images (bottom panels). Scale bar in top left panel is 50µm and all images (except zoomed images) were captured at the same magnification. Scale bar in bottom panels is 5µm.

4.6 – Conclusions

To conclude, we have validated and optimised a method by which LAT1 can be visualised in human skeletal muscle. As this amino acid transporter is only active when associated with cellular membranes, this novel method allows a greater depth of investigation into the dynamics of LAT1 in human muscle in response to anabolic stimuli. For the first time, we have identified LAT1 to be located close to the plasma membrane of all fibres and in close proximity to the microvasculature. Furthermore, we note a greater immunoreactivity of this protein in the sarcoplasm of type II fibres, potentially supporting the greater anabolic potential in these fibres. We believe this technique may provide a valuable insight into the role of LAT1 in skeletal muscle amino acid sensing, intracellular signalling, and subsequently adaptation. It is important to acknowledge however, that the use of immunofluorescence microscopy as discussed here can only infer localization of target proteins. To gain greater insight into whether the LAT1 protein is directly localized to the sarcolemmal membrane, microvasculature or actin myosin bundles, immune-electron microscopy could be utilized and/or isolation of giant sarcolemmal vesicles. However, the present immunofluorescence technique requires less tissue (i.e. ~20mg vs. >200mg) than isolated giant sarcolemmal vesicles and can be characterized from frozen as compared to fresh samples, which represent significant advantages when muscle tissue samples are limited. Future research should now focus on identifying the possible underlying mechanisms, and consequences, of the fibre type differences reported here. Moreover, investigations into how factors such as ageing, exercise and feeding regulate LAT1 cellular location should also be undertaken. As LAT1 is the principal mechanism by which leucine enters the cell, approaches to increase LAT1 content or activity in

skeletal muscle could have substantial relevance to post-exercise recovery strategies in which dietary protein or amino acids are of direct importance.

4.7 – References

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Chapter 5

INVESTIGATING THE ROLE OF VPS34 AS A NUTRIENT SENSOR IN HUMAN SKELETAL MUSCLE

5 INVESTIGATING THE ROLE OF VPS34 AS A NUTRIENT SENSOR IN HUMAN SKELETAL MUSCLE

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5.1 – Abstract

The mechanistic target of rapamycin (mTOR) complex 1 (mTORC1) regulates cell size and growth in response to nutrients, however the full mechanisms by which alterations in nutrient levels are 'sensed' to mTORC1 are yet to be fully elucidated. Recent *in vitro* evidence has identified the Class III PI3Kinase Vps34 as a potential nutrient sensor, as its activity seems to be essential to mTORC1 activation in response to nutrients. As skeletal muscle size is principally regulated by nutrient levels, in addition to mechanical loading, we sought to investigate the role of Vps34 as a nutrient sensor both *in vitro* and *in vivo* in skeletal muscle. To understand the importance of Vps34 kinase activity to mTORC1 activation *in vitro*, we administered the Vps34-specific inhibitor SAR405 to C2C12 and human primary myotubes and investigated how this inhibitor affected mTORC1 signalling response to serum withdrawal and recovery. In addition, we then investigated the effects of protein-carbohydrate feeding on Vps34 kinase activity, cellular location and protein-protein interactions in human skeletal muscle (n=8, age – 21.1±0.5yrs, Mean±SEM**).** *In vitro,* SAR405 had no effect on the mTORC1-related signalling response to serum withdrawal/recovery in either C2C12 or human primary myotubes ($p=0.069$). Furthermore, Vps34 kinase activity was found to be unresponsive to protein-carbohydrate feeding in human skeletal muscle ($p>0.05$). This feeding protocol however did initiate the translocation of both mTOR and Vps34 to the cell periphery 1h following feeding. These proteins' colocalisation was also noted to increase at this time point, and these processes occurred in parallel to elevations in mTORC1 activity (S6K1 kinase activity). This data therefore suggests that it may be changes in Vps34 cellular location, rather than kinase activity, that contributes to Vps34s purported role as a nutrient sensor in skeletal muscle.

5.2 – Introduction

Amino acids (AAs) are critical to skeletal muscle plasticity, acting as substrates to stimulate muscle protein synthesis (MPS). In parallel, these nutrients can also initiate signalling pathways which stimulate MPS(1), and inhibit muscle protein breakdown (MPB)(2). Net protein balance (NPB) can only become positive following resistance exercise if AAs are also ingested, as acute resistance exercise alone elevates MPS and MPB in parallel(2). As AA ingestion and resistance exercise exhibit synergistic effects on MPS, it is hypothesised that each of these anabolic stimuli activate MPS via divergent signalling pathways. In skeletal muscle, it is believed that increases in MPS are governed primarily by the activation of the mechanistic target of rapamycin complex 1 (mTORC1)(3, 4), an evolutionarily conserved serine/threonine kinase complex which stimulates translation initiation and elongation(5-7).

AAs are thought to stimulate mTORC1 activation by increasing the localisation of Rag proteins with lysosomes (8) where they can recruit mTORC1 to the surface of this organelle (8, 9). We (10, 11), and others (12), have recently challenged this paradigm in human skeletal muscle finding no change in mTOR-lysosome association following resistance exercise and/or protein-carbohydrate feeding, events which both potently stimulate mTORC1 signalling. What is currently unknown is to how AA stimulate mTORC1 in skeletal muscle and whether this process results in mTORC1 translocation to the cell periphery. A potential candidate for AA action on mTORC1 is the vacuolar protein sorting 34 (Vps34), a class III PI3Kinase, whose action is to produce phosphatidylinositol 3-phosphate (PI(3)P) through the phosphorylation of phosphatidylinositol (13). This product is then able to recruit various proteins to phospholipid bilayers i.e. plasma and lysosomal membranes (14).

Vps34 implication in nutrient sensing was first proposed by Byfield et al.(15), who reported that overexpression of Vps34 in HEK293 cells elicited a 2-fold increase in S6K1 activity, a similar elevation to that induced by insulin. Furthermore, in the presence of siRNA targeting Vps34, insulin-stimulated S6K1^{Thr389} phosphorylation was abolished and the removal of AAs reduced endogenous Vps34 activity even in the presence of insulin(15). A similar study by Nobukuni et al.(16) reiterated these findings, displaying that siRNA-mediated reductions in Vps34 expression, in HEK293 cells, dramatically attenuated mTORC1 activation in response to both AA and insulin stimulation. Interestingly, recent evidence has suggested that activated Vps34 colocalises with mTOR, close to cell membranes, following insulin stimulation(17). Moreover, when Vps34 kinase activity was inhibited, mTOR recruitment to the plasma membrane and the subsequent phosphorylation of S6K1^{Thr389} was significantly attenuated (17). This data implicates Vps34 in the recruitment of mTOR toward the cell periphery, a process we have recently identified in human skeletal muscle (10).

In human primary myotubes, evidence for the involvement of Vps34 in nutrient-sensing pathways has also been reported. In response to leucine (5mM) and insulin (100nM) stimulation, Vps34 protein abundance was elevated by 20-50% at 0.5h, 3h, and 24h (18). This increase in Vps34 was mirrored by rises in mTOR^{Ser2448} and S6K1^{Thr389} phosphorylation (18), however, it was not clear from this data as to whether there was an interaction between Vps34 and mTORC1 activation, or whether the co-ordinated induction was coincidental. In addition to AA/insulin stimulation, contractile loading is also known to increase Vps34 kinase activity in rodent skeletal muscle (19). In an attempt to elucidate a mechanism behind this elevation of Vps34 activity, the level of intramuscular leucine noted following contraction was then added to C2C12 muscle cells

for 3h. This resulted in a ~5-fold increase Vps34 kinase activity, yet incubation with physiological levels of all AAs did not affect kinase activity (19). Overall, such data implicates a possible role for Vps34 in AA sensing within skeletal muscle; however a more detailed investigation of Vps34 in human tissue is still required to understand the mechanism by which Vps34 may 'sense' nutrient levels particularly in response to anabolic stimuli other than mechanical loading i.e. feeding.

To further elucidate a role for Vps34 in human skeletal muscle nutrient sensing, this chapter examined cell responses to the Vps34 inhibitor, SAR405(20, 21), determining the importance of Vps34 kinase activity for mTORC1 activation in C2C12 and human primary skeletal muscle myotubes. Following this, we then aimed to investigate how AA/carbohydrate feeding affected Vps34 activity and cellular localisation in human skeletal muscle. We hypothesised that inhibition of Vps34 would impair serum/leucine activation of mTORC1. In addition, we hypothesised Vps34 activity would increase in response to AA/carbohydrate feeding in human skeletal muscle in parallel to increases in mTORC1 signaling.

5.3 – Methods

5.3.1 In vitro experimental design

C2C12 and human primary myoblasts were cultured and subsequently differentiated into myotubes as described in Chapter 2 (Sections 2.8.1 and 2.8.2). When full myotubes had formed, cells were nutrient deprived for ~14h in Earl's Balanced Salt Solution (EBSS), supplemented with PenStrep (1%). For human primary samples (characteristics described in Section 2.8.2 of this thesis), a subset of myotubes from each individual was placed in F-10 Hams media (supplemented with 20% FBS and 1% Pencillin-Streptomycin) for this

period and used as a baseline condition. For C2C12 cells, a subset of myotubes were cultured in normal differentiation media for ~14h to use as a baseline condition. Another set of cells were collected following this deprivation to determine the effects of this protocol on mTORC1 signalling. The remaining myotubes were split into 2 conditions; serum recovery, and serum recovery + Vps34 inhibition. In human primary cells, the serum recovery condition consisted of 30 minutes of incubation in F-10 Hams media (supplemented with 20% FBS and 1% Pencillin-Streptomycin) following the nutrient deprivation protocol. In C2C12 myotubes, serum recovery consisted of incubation in normal differentiation media, for 30 minutes, following nutrient deprivation. Vps34 inhibition conditions were identical to the corresponding serum recovery conditions apart from a 60 minute pre-incubation with 10μM SAR405. Following each treatment, cells were washed twice in PBS (Fisher Scientific, Loughborough, UK) and scraped into ice-cold sucrose lysis buffer (see Chapter 2 - 2.5.1). Lysates were frozen in liquid nitrogen and stored at -80ºC until further analysis. To initiate analysis, samples were thawed, sonicated for 3 x 15 seconds at 50% maximum wattage, centrifuged at 8000g, for 10 minutes at 4ºC and the supernatant used for analysis. Protein concentration was determined using the DC protein assay as described in Chapter 2 (Section 2.5.1). Experiments in human primary myotubes were run in triplicate for each donor and experiments in C2C12 myotubes were run in triplicate and repeated on 3 separate days to enhance the reliability of our measurements.

5.3.2 Immunoblotting

Identical amounts of protein were separated on 8-12.5% polyacrylamide gels by SDS-PAGE as described in Chapter 2 (Section 2.5.2). Proteins were then transferred to BioTrace NT nitro-cellulose membranes (Pall Life Sciences, UK) at 100V for 1h, blocked

in 3% milk/TBST and incubated overnight in the desirable primary antibodies (all 1:1000 dilution in TBST). The following day, membranes were washed in TBST and incubated in corresponding secondary antibodies (diluted 1:10000 in TBST) at room temperature for 1h. Chemiluminescence horseradish peroxidase reagent kit (Merck-Millipore) was used to quantify protein content following IgG binding. Images were captured with a G:Box Chemi-XR5 (Syngene (A Division of Synoptics Ltd.), Cambridge, UK) imaging system and blot bands were quantified using GeneTools software (Syngene). Each protein target was expressed in relation to a loading control (Ponceau) and each phosphorylation site is presented in relation to its corresponding total protein abundance.

5.3.3 Antibodies

For immunoblotting analysis all primary antibodies were purchased from Cell Signaling Technologies (CST) and diluted at 1:1000 in TBST unless stated otherwise. Antibodies utilised were: p70 ribosomal S6 kinase 1 (S6K1, #2708), p-S6K1^{Thr389} (#9205), ribosomal protein S6 (S6, #2217), p-S6$^{Ser235/236}$ (#4858), p-S6$^{Ser240/244}$ (#5364), eukaryotic translation initiation factor 4E-binding protein 1 (4EBP1, #9452, 1:500), p-4EBP1$^{Thr37/46}$ (#9459), AKT (#4697), p-AKT^{Thr308} (#2965), p-AKT^{Ser473} (#4060) and eukaryotic elongation factor 2(eEF2, #2332). All primary antibodies were produced in rabbits and as such anti-rabbit IgG HRP-linked secondary antibody (CST#7074) was utilised. Antibody binding was detected via the use of enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK).

For enzyme kinase activity assays, antibodies utilised were as follows: S6K1 (sc-2708, Santa Cruz Biotech., Dallas, TX, USA), AKT (sc-4691, Santa Cruz Biotech.) and Vps34

(anti-sheep antibody produced by Dr. James Murray, Trinity Biomedical Science Institute, Trinity College, Dublin, IE).

For immunohistochemical analysis, antibodies utilised were as follows: anti-mTOR (#05-1592, Merck Chemical Ltd., Nottingham, UK), anti-LAMP2 (#AP1824d, Abgent, USA), anti-Vps34 (CST#3358) and Wheat Germ Agglutinin (WGA-350, #11263, ThermoFisher, UK). Corresponding secondary antibodies were goat anti-mouse IgGγ1 Alexa®594 (#R37121, ThermoFisher, UK, targeting mTOR) and goat anti-rabbit IgG(H+L) Alexa®488 secondary antibodies (#A11008, ThermoFisher, UK, targeting LAMP2/Vps34).

5.3.4 Human Trial Experimental Design

Eight young, healthy men (age – 21.1±0.5yrs, Mean±SEM) volunteered to partake in the study. Participants were healthy (as assessed by a general health questionnaire), recreationally active and non-smokers. All participants provided written informed consent prior to partaking in the study.

On the day of the trial, participants reported to the laboratory at the School of Sport, Exercise and Rehabilitation Sciences building at 08.00h, following an overnight fast (~10h) and having refrained from strenuous exercise and alcohol or caffeine consumption for 48h prior to the trial. Participants were placed in a supine position for ~20 minutes to allow for reliable resting samples to be taken. A 21G cannula was then inserted into the antecubital vein of one arm of the participant and a resting blood sample (10mL) was drawn. Subsequent blood samples were obtained every 20 minutes throughout the experimental trial. Each blood sample was split (5mL) between serum separator and EDTA-coated (plasma) vacutainers (BD Biosciences, Oxford, UK.). EDTA-coated

vacutainers were placed immediately on ice and serum separator tubes remained at room temperature for 30 minutes to coagulate before being placed on ice. Following the trial blood samples were centrifuged for 10 mins at 3000g at 4°C and plasma and serum were transferred to eppendorfs at stored at -80°C until further analysis. A muscle biopsy sample was then taken from the *vastus lateralis* using the bergstrom percutaneous needle technique, modified for suction. Participants then consumed a commercially available protein-carbohydrate beverage (Gatorade Recover®, Chicago, IL, USA.) providing 20/44/1g of protein, carbohydrate and fat respectively. Subsequent muscle biopsies were taken 1h and 3h post-consumption of this beverage. Muscle samples were blotted free of blood and dissected free of any excess adipose or connective tissue. A piece of each muscle samples was immediately frozen in liquid nitrogen and kept at -80ºC until analysed. A separate piece was placed in OCT compound (VWR, Lutterworth, UK.) and frozen in liquid nitrogen-cooled isopentane before storage at -80ºC. The experimental design is depicted in Figure 5.1.

5.3.5 Blood analyses

Serum insulin and plasma AA concentrations were determined by ELISA and GC-MS respectively as described in sections 2.7.1 ad 2.7.2 of this thesis.

5.3.6 Kinase Activity Assays

Kinase activity assays were conducted targeting S6K1, AKT and Vps34 as described in Chapter 2 (Section 2.6.1 for S6K1 and AKT, section 2.6.2 for Vps34) utilising 32γ-ATP and synthetic peptide substrates for S6K1 and AKT or phosphatidylinositol for Vps34.

5.3.7 Skeletal Muscle Immunohistochemistry

Skeletal muscle samples were processed for immunohistochemical analysis as described in Chapter 2 (Sections 2.3 and 2.4). In this chapter, protein targets of interest were mTOR(594nm)-LAMP2(488nm)-WGA(350nm) and mTOR(594nm)-Vps34(488nm)-WGA(350nm), each combination visualised as described in section 2.4.5 of this thesis. Image analysis for colocalisation of these targets was conducted using ImagePro software as described in section 2.4.6.2.

5.3.8 Statistics

Changes in phosphorylation status of proteins in human primary myotubes were analysed using a repeated measures ANOVA with one within-subject factor (treatment). Changes in phosphorylation status of proteins in C2C12 myotubes were analysed using a one-way ANOVA with one between-subject factor (treatment). Kinase activity and protein-protein colocalisation were analysed using a repeated measures analysis of variance (ANOVA) with one within-subject factor (time). When a significant main effect was found, *post-hoc* tests were completed with the Holm-Bonferroni correction for multiple comparisons. Significance for all variables analysed was set at $p \leq .05$. Data are presented as mean±SEM unless otherwise stated.

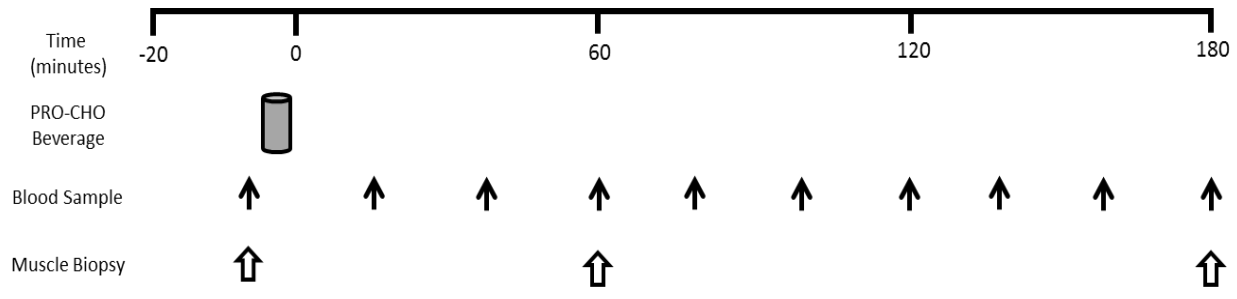

Figure 5.1. Human trial experimental design.

5.4 – Results

5.4.1 In Vitro Study

5.4.1.1 Effects of SAR405 on serum-induced mTORC1 activation in C2C12 myotubes

A significant treatment effect was noted for the phosphorylation of S6K1^{Thr389} following serum withdrawal and recovery in C2C12 myotubes ($p<0.0001$, Fig. 5.2a). Serum withdrawal decreased S6K1^{Thr389} phosphorylation (~35% reduction, $p<0.001$) and subsequent serum recovery for 30mins, irrespective of SAR405 administration, elevated S6K1^{Thr389} phosphorylation above both baseline and serum withdrawal levels (SR – 65% above baseline, SR+SAR405 – 50% above baseline, $p<0.0001$, Fig. 5.2a). No difference in S6K1^{Thr389} phosphorylation was observed between SR or SR+SAR405 treatments ($p=0.26$). A treatment effect was also observed for 4EBP1$^{Thr37/46}$ phosphorylation ($p<0.0001$, Fig. 5.2b). Serum withdrawal elicited a 32% reduction in 4EBP1$^{Thr37/46}$ phosphorylation ($p<0.001$, Fig. 5.2b). Subsequent serum recovery, both in the presence and absence of SAR405, significantly elevated phosphorylation above serum withdrawal levels (62% and 65% elevation respectively, $p<0.0001$, Fig. 5.2b) with no difference between SR and SR+SAR405 apparent ($p=0.80$).

A treatment effect was noted for S6$^{Ser235/236}$ phosphorylation ($p<0.0001$, Fig. 5.2c). Similarly, serum withdrawal reduced S6 phosphorylation at this site (35% reduction, $p<0.001$) compared to baseline. Serum stimulation elevated S6$^{Ser235/236}$ phosphorylation above baseline and serum withdrawal values (33% above baseline, $p=0.038$ vs. baseline, $p<0.001$ vs. SW, Fig. 5.2c). Again, SAR405 had no effect on these responses (30% above baseline, $p=0.05$ vs. baseline, $p<0.001$ vs. SW, Fig. 5.2c) nor was there any difference between SR and SR+SAR405 treatments ($p=0.83$, Fig. 5.2c). A treatment effect was also

noted for S6 phosphorylation at Ser240/244 ($p<0.0001$, Fig. 5.2d). Here, serum withdrawal elicited an 18% reduction in $S6^{Ser240/244}$ phosphorylation ($p<0.001$), with subsequent serum recovery elevating phosphorylation above serum withdrawal levels irrespective of SAR405 (SR – 27.7% above SW, $p<0.001$; SR+SAR405 – 26.1% above SW, $p<0.001$, Fig. 5.2d). No difference between the responses in SR and SR+SAR405 were observed ($p=0.80$).

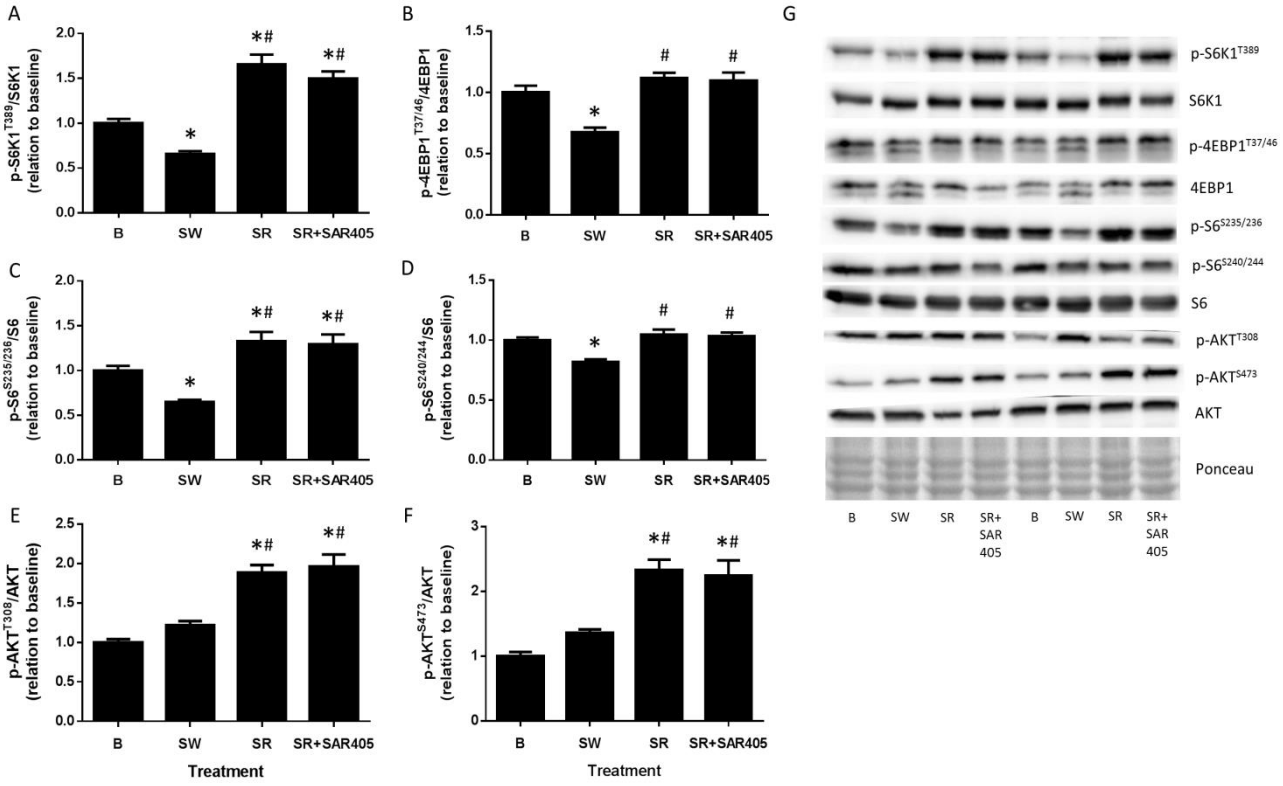

Figure 5.2. The effects of overnight serum withdrawal and subsequent serum recovery (30 minutes), with or without SAR405), on anabolic signalling in C2C12 myotubes (n=9/group). p-$S6K1^{Thr389}$ (A), p-$4EBP1^{Thr37/46}$ (B), p-$S6^{Ser235/236}$ (C), p-$S6^{Ser240/244}$ (D), p-AKT^{Thr308} (E) and p-AKT^{Ser473} (F) phosphorylation was quantified in relation to their total proteins and ponceau staining was used as a loading control. Representative images are also provided (G). Data is presented as Mean±SEM. *Significantly different to B ($p<0.05$). #Significantly different to SW ($p<0.05$).

A treatment effect was also observed for AKT ^{Thr308} and AKT^{Ser473} phosphorylation (both $p<0.0001$). Serum withdrawal elevated AKT^{Thr308} phosphorylation by 22% ($p=0.007$), and subsequent serum recovery increased phosphorylation at this site above both baseline and serum withdrawal levels (88.2% above baseline, $p<0.001$, Fig. 5.2e). Serum recovery in the presence of SAR405 also elevated AKT^{Thr308} phosphorylation above both baseline and serum withdrawal levels (95.7% above baseline, $p<0.0001$, Fig. 5.2e), with no difference when compared to SR ($p=0.69$). AKT^{Ser473} was affected in a similar manner, with serum recovery causing a significant rise in phosphorylation above baseline ($p<0.0001$) and serum withdrawal ($p<0.001$) values irrespective of inhibition treatment (SRvs.SR+SAR405 $p=0.76$, Fig. 5.2f). Representative images for all immunoblots described are displayed in Fig. 5.2g.

5.4.1.2 Effects of SAR405 on serum-induced mTORC1 activation in Human Primary myotubes

A significant treatment effect was noted for S6K1 ^{Thr389} phosphorylation following serum withdrawal and recovery in Human primary myotubes ($p<0.0001$). Here, serum withdrawal significantly reduced S6K1^{Thr389} phosphorylation by ~70% ($p=0.02$, Fig. 5.3a). Serum recovery, with or without SAR405 administration, elevated S6K1^{Thr389} phosphorylation above both baseline and SW (SR – 92% above baseline, SR+SAR405 – 54% above baseline, all $p<0.05$, Fig. 5.3a), with no difference between SR and SR+SAR405 conditions ($p=0.069$, Fig. 5.3a) A treatment effect was also noted for 4EBP1$^{Thr37/46}$ phosphorylation ($p=0.004$). Here, serum withdrawal elicited a 63% reduction in 4EBP1$^{Thr37/46}$ phosphorylation (Fig. 5.3b). Further serum recovery, with or without SAR405, elevated phosphorylation 40% and 26% above baseline respectively.

However, no comparisons were significant following *post-hoc tests* and no difference was observed between the effects of SR and SR+SAR405 (p=0.20, Fig. 3b).

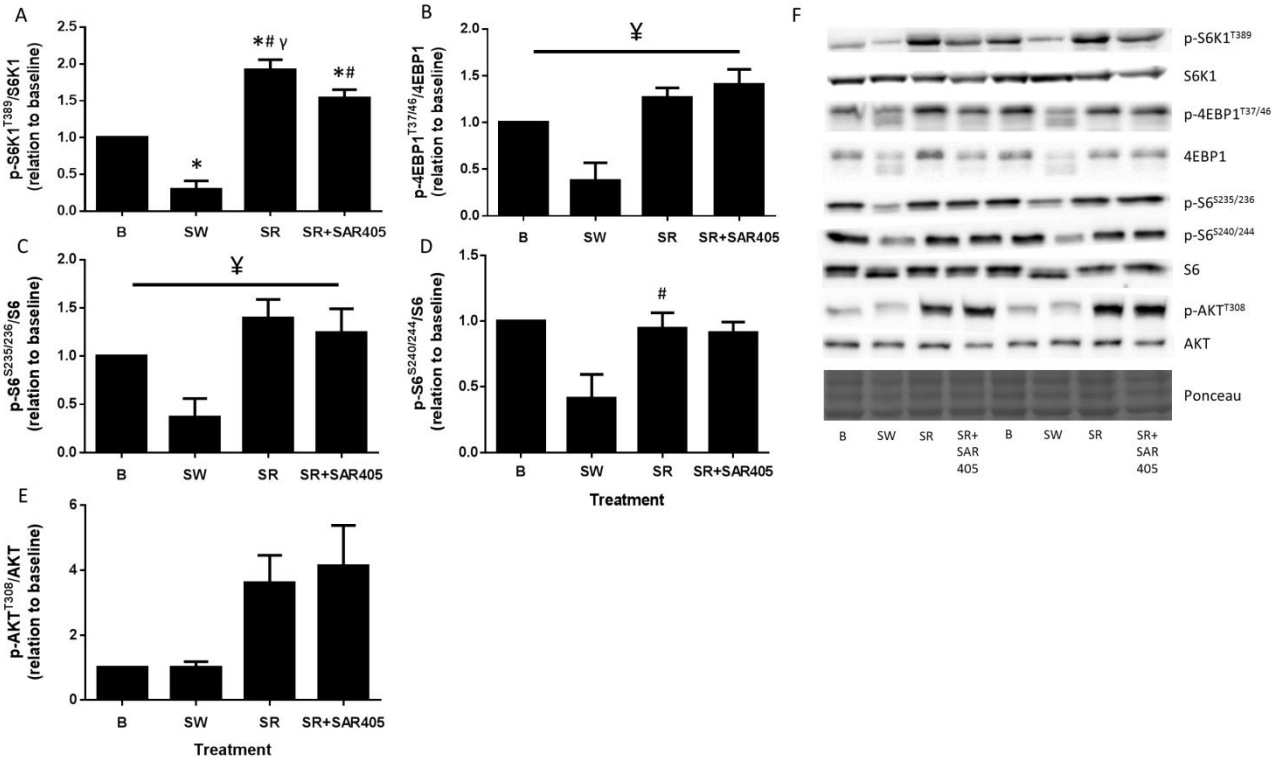

Figure 5.3. The effects of overnight serum withdrawal and subsequent serum recovery (30 minutes), with or without SAR405), on anabolic signalling in Human Primary Myotubes (n=4/group). p-S6K1^{Thr389} (A), p-4EBP1$^{Thr37/46}$ (B), p-S6$^{Ser235/236}$ (C), p-S6$^{Ser240/244}$ (D) and p-AKT^{Thr308} (E) phosphorylation was quantified in relation to their total proteins and ponceau staining was used as a loading control. Representative images are also provided (F). Data is presented as Mean±SEM. *Significantly different to B ($p<0.05$). $^{\#}$Significantly different to SW ($p<0.05$). $^{\gamma}$Trend toward different to SR+SAR405 ($p<05$). $^{¥}$Significant Treatment effect ($p<0.05$).

S6$^{Ser235/236}$ phosphorylation displayed a significant treatment effect (p=0.01, Fig. 5.3c). Serum withdrawal elicited a 64% reduction in S6$^{Ser235/236}$ phosphorylation, whereas serum recovery elevated S6$^{Ser235/236}$ phosphorylation 3.8 fold above SW levels. No difference was observed between SR and SR+SAR405 groups (p=0.45). A significant treatment

effect was also noted for $S6^{Ser240/244}$ phosphorylation at (p=0.003), where serum recovery elevated $S6^{Ser235/236}$ phosphorylation ~3 fold above SW levels. No difference was observed between SR and SR+SAR405 groups. A trend toward a treatment effect was apparent for AKT^{Thr308} phosphorylation (p=0.083, Fig. 5.3e). Here, no comparisons were significant following *post-hoc* analysis, most likely due to the large variability in individual responses noted in this measure. Representative images for all immunoblots described are displayed in Fig. 5.3f.

5.4.2 Human Study

5.4.2.1 Serum Insulin Concentrations

Serum insulin concentrations were significantly elevated above baseline levels at 40 and 80 minutes post-drink ingestion (5- and 2.9-fold increase respectively, p<0.005, overall time effect – p<0.0001, Fig. 5.4a). Trends toward elevated serum insulin concentrations were also noted at 20 (7.1-fold), 60 (4.2-fold), 100 (2.75-fold) and 120 (2.59-fold) minutes post-feeding (all p<0.081, Fig. 5.4a). Serum insulin returned to basal values at 140 minutes post-ingestion (p=0.12, Fig. 5.4a).

5.4.2.2 Plasma AA Concentrations

A significant time effect was noted for the fold change, from baseline, in plasma leucine concentrations (p=0.0001, Fig. 5.4b). Here, plasma leucine concentrations rose above baseline at 20 minutes post-drink ingestion (1.7 fold increase, p<0.03) and remained elevated (all time points p<0.01) before returning to basal values at 160 min (p=0.40, Fig. 5.5.4b). The plasma concentrations of threonine and phenylalanine followed a similar pattern to this, rising above baseline at 20 minutes post-ingestion (22% and 34.8%

elevation respectively, $p<0.01$) and remaining above baseline until 140mins following ingestion ($p<0.05$, Fig. 5.5.4c & d).

5.4.2.3 Kinase Activity Assays

A significant time effect was noted for S6K1 kinase activity ($p=0.001$). There was a ~9 fold increase in S6K1 kinase activity 1 hour following ingestion of the protein-carbohydrate beverage (10.8±1.02fmol/min/mg vs. 92.57±13.8fmol/min/mg, $p=0.002$, Fig. 3a), before returning to baseline at 3 hours post-ingestion (10.8±1.02fmol/min/mg vs. 15.06±2.08fmol/min/mg, $p=0.11$, Fig. 5.5a). S6K1 kinase activity at 1h post-ingestion was also significantly greater than that noted at 3h post-ingestion ($p=0.001$). A significant time effect was also noted for AKT kinase activity ($p=0.004$). AKT kinase activity was significantly elevated above baseline at 1 hour post-ingestion (28.04±5.37fmol/min/mg vs. 44.01±2.14fmol/min/mg, $p=0.03$, Fig. 5.5b) and returned to baseline values by 3 hours post-ingestion (28.04±5.37fmol/min/mg vs. 28.56±3.57fmol/min/mg, $p=0.89$, Fig. 5.5b). Similarly, AKT kinase activity at 1 hour following consumption was significantly greater than 3 hours post-ingestion ($p=0.02$). Finally, no significant differences at any time point were noted in VPS34 kinase activity ($p>0.05$, Fig. 5.5c).

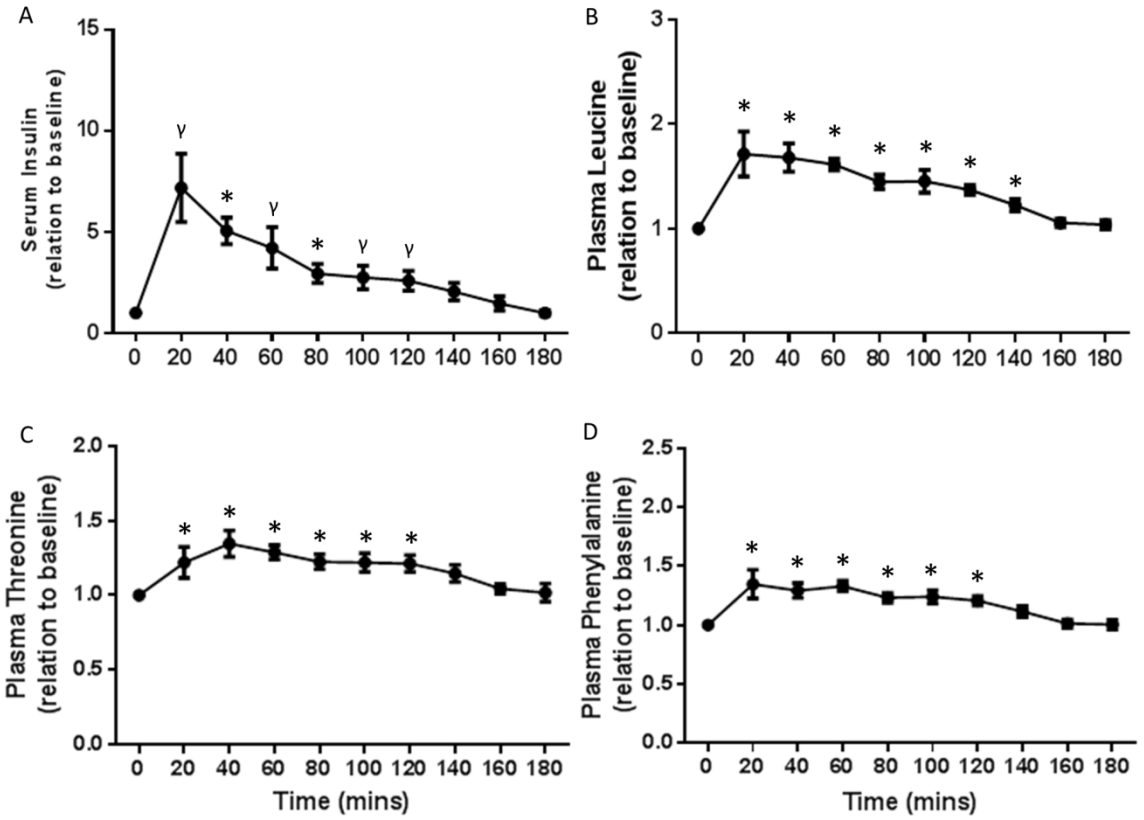

Figure 5.4. The effects protein-carbohydrate ingestion on serum insulin (A) and plasma leucine (B), threonine (C) and phenylalanine (D) concentrations (n=8). All values are in relation to individual baseline values. Data presented as Mean±SEM. *Significantly different from baseline ($p<0.05$). ᵞTrend toward different to baseline ($p<0.081$).

5.4.2.4 mTOR colocalisation with LAMP2 and WGA

No significant differences were noted at any time points in the colocalisation of mTOR and LAMP2 (lysosomal marker) suggesting these two proteins are constantly localised ($p=0.347$, Fig. 5.6b). However, a significant time effect for the colocalisation of mTOR and WGA was observed ($p=0.026$). mTOR-WGA colocalisation increased from baseline values by ~17% 1h post-feeding, before returning close to baseline values at 3 hours following ingestion (Fig. 5.6c), however these differences were not significant following *post-hoc* analysis.

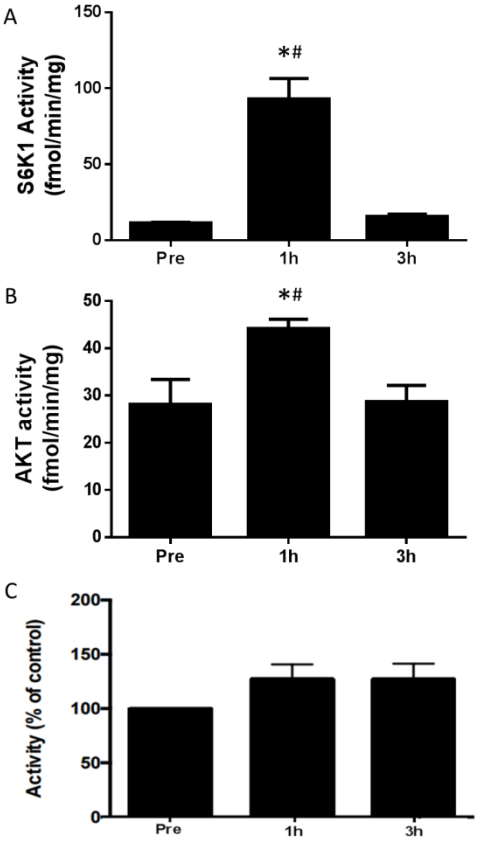

Figure 5.5. The effects of protein-carbohydrate ingestion on S6K1 (A), AKT (B) and Vps34 (C) kinase activity (n=8). Data presented as Mean±SEM. *Significantly different to Pre values ($p<0.05$). #Significantly different to 3h values ($p<0.05$).

5.4.2.5 VPS34 colocalisation with mTOR and WGA

A significant time effect was noted for mTOR-VPS34 colocalisation ($p=0.045$), with mTOR-VPS34 colocalisation increased by ~11% 1 hour post-ingestion before returning to baseline 3 hours post-ingestion (Fig. 5.7b). A trend toward a greater mTOR-VPS34 colocalisation was apparent at 1 hour post-ingestion compared to 3 hours post-ingestion ($p=0.067$, Fig. 5.7b). A trend toward a significant time effect was also found when studying VPS34 colocalisation with WGA ($p=0.053$). Here, VPS34-WGA colocalization rose at 1 hour following drink consumption (~27% increase from baseline, Fig. 5.7c), which then returned to baseline at 3 hours post-ingestion.

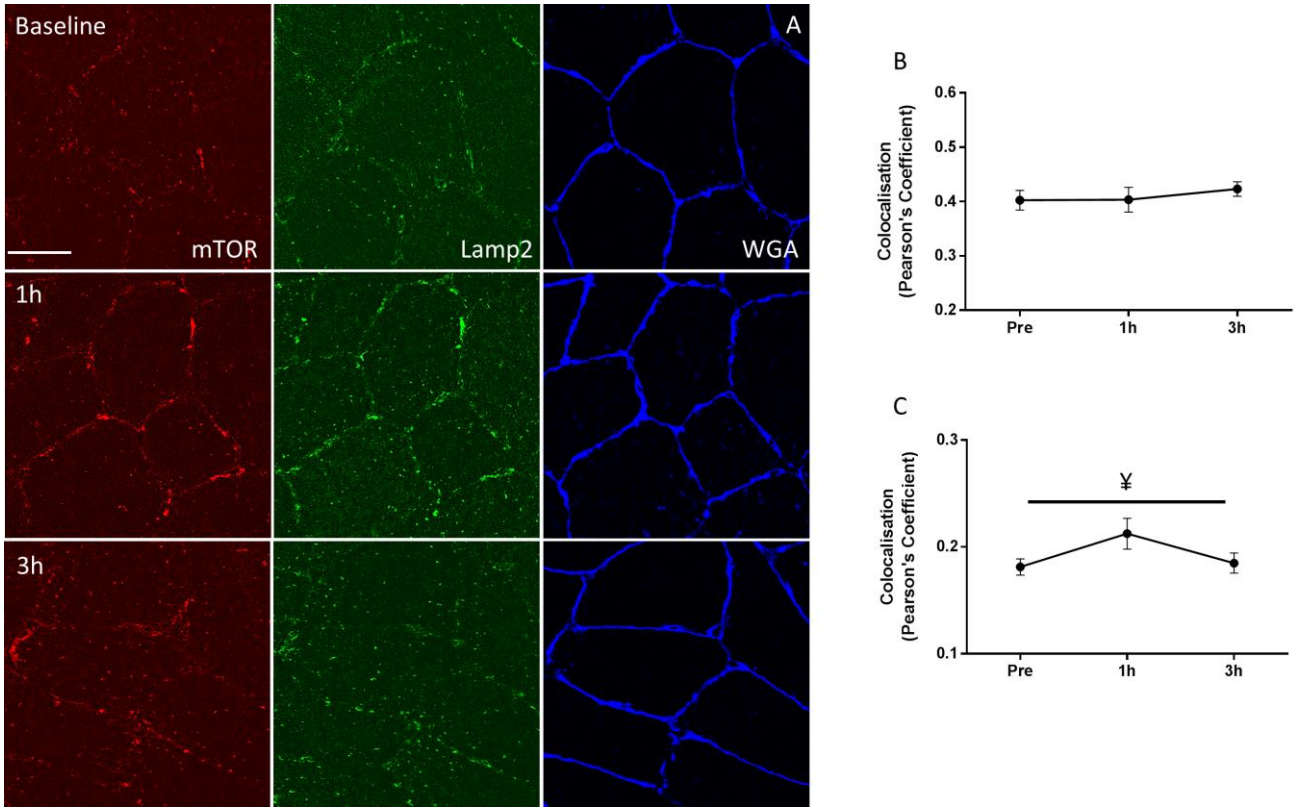

Figure 5.6. The effects of protein-carbohydrate ingestion on mTOR-LAMP2 and mTOR-WGA colocalisation (n=8). Representative images of mTOR, LAMP2 and WGA stains at each time point are provided (A). mTOR stain is shown in the first column of images (red), LAMP2 in the second (green) and WGA in the third (blue). Quantification of mTOR-LAMP2 (B) and mTOR-WGA (C) colocalisation was completed. Data in B and C are Mean±SEM. ¥Significant Time effect ($p<0.05$).

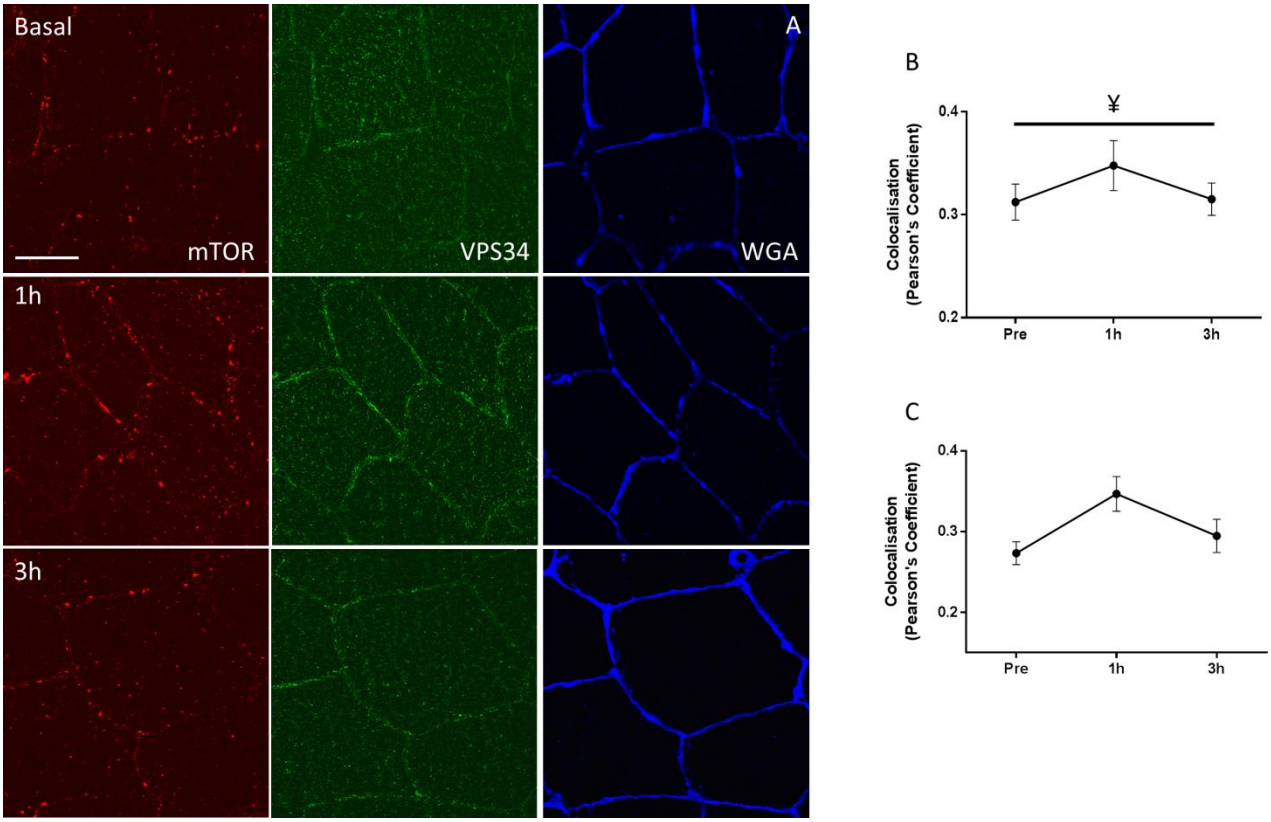

Figure 5.7. The effects of protein-carbohydrate ingestion on mTOR-VPS34 and mTOR-WGA colocalisation (n=8). Representative images of mTOR, VPS34 and WGA stains at each time point are provided (A). mTOR stain is shown in the first column of images (red), VPS34 in the second (green) and WGA in the third (blue). Quantification of mTOR-VPS34 (B) and VPS34-WGA (C) colocalisation was completed. Data in B and C are Mean±SEM. ¥Significant time effect ($p<0.05$).

5.5 – Discussion

Herein, we report that the Vps34 specific inhibitor SAR405 does not affect mTORC1 activation following serum recovery in human primary or C2C12 myotubes. These findings suggest Vps34 kinase activity may not be essential for mTORC1 activity *in vitro*. Further to these findings, we show that Vps34 kinase activity is not affected by protein-carbohydrate feeding in human skeletal muscle. The feeding protocol however, did alter Vps34's cellular location, prompting a translocation toward the cell periphery and

inducing an increase in its colocalisation with mTOR. The alterations in the cellular locations of these proteins coincided with an augmentation of mTORC1 activity. Together, these observations suggest a change in Vps34 cellular location, rather than its kinase activity, may contribute to AA sensing in human skeletal muscle.

The notion that Vps34 contributes to AA sensing is based on studies utilising non-muscle immortalised cell lines(15, 16, 22) which may not necessarily mimic a 'normal' physiological response of skeletal muscle. Furthermore, the importance of Vps34 kinase activity in skeletal muscle cells has not yet been directly tested. In an attempt to elucidate the importance of Vps34 kinase activity in muscle we utilised C2C12, an immortalised mouse myoblast cell line, and isolated human primary myotubes (23). The effect of the Vps34-specific inhibitor SAR405 on the activation of mTORC1 in response to serum recovery, following overnight withdrawal, was then tested to examine the importance of Vps34 kinase activity in this response. In C2C12 myotubes, SAR405 had no effect on the activation of mTORC1 (Fig. 5.2). This evidence suggests that Vps34 kinase activity does not contribute in to mTORC1 activation in this immortalised muscle cell line. This data contradicts other reports *in vitro* where SAR405 administration, at low concentrations (3μM), in HEK293 cells completely ablated S6K1 phosphorylation in response to AAs (22). Therefore, our finding that SAR405 had no effect on mTORC1 activation in C2C12 myotubes is contrary to our working hypothesis. There are a number of potential reasons for this lack of effect. First our assumption is that Vps34 is activated in muscle cells in response to serum stimulation. Currently we do not have evidence to support this and so the model used needs further validation to its suitability to study vps34 activity *in* vitro. Second, the SAR405 dose incorporated in our studies may not be sufficient to inhibit Vps34 activity in myotubes. C2C12 myoblasts have a much higher metabolism than

HEK293, proliferating and differentiating at a greater rate (24). We assumed that the dose previously reported to inhibit vps34 *in vitro* would be sufficient to inhibit Vps34 in our experimental models, however without direct or surrogate readouts of Vps34 activity we are unable to confirm this point at this stage. Furthermore, it is possible that our serum recovery protocol exerted such a large effect on mTORC1 activation that the 'threshold' for maximal signalling effects was already reached irrespective of any possible effects of the Vps34 inhibitor. This suggests that even if SAR405 did cause some inhibition of mTORC1 activity it did not reduce this below the threshold level and therefore maximal S6K1 phosphorylation still occurred. Interestingly, one previous report suggests that Vps34 activity may actually be reduced in C2C12s in the presence of AAs(25) which may explain why no effect of SAR405 was apparent, however these findings have not been replicated in other cell lines(15, 16). In fact, the addition of leucine to C2C12 myotubes elevates Vps34 kinase activity to a similar extent as did eccentric contractions in rodent skeletal muscle (19). These contradictory findings, combined with our own, advocated the need to investigate the importance of Vps34 kinase activity in human primary myotubes.

Subsequently, we also did not find an effect of SAR405 on mTORC1 activation in human primary myotubes. Here, however, the administration of this inhibitor reduced S6K1 Thr389 phosphorylation, in relation to baseline levels, by ~42%, yet this attenuation did not reach significance ($p=0.069$). This effect did not extend downstream of S6K1 or to any other mTORC1 target. This is the first study to directly investigate the effects of Vps34 activity on mTORC1 signalling in primary myoblasts. Previous *in vitro* data reported a complete ablation of mTORC1 activity in the presence of Vps34 inhibitors (22) or siRNA targeting this kinase(15, 16, 26-28), suggesting Vps34 may completely

govern mTORC1 activation in response to AAs or insulin. The data gathered in the current study contradicts these findings however, as we did not directly measure Vps34 kinase activity we cannot fully conclude its importance in this scenario. Interestingly, several other candidates for nutrient sensors to mTORC1 have been purported including Sestrins(26-28), MAP4K3(29) and leucyl-tRNA synthetase (LRS)(30, 31). Of note, when each of these other 'nutrient sensors' were inhibited in HEK293 or HeLa cells, a complete ablation of the S6K1^{Thr389} response was also reported(28-30), suggesting all these proteins coordinate into one signal pathway to activate mTORC1. A second, more plausible, explanation is that simple nature of these cellular monolayers allows the knockdown of a single protein to have a drastic effect on cellular signalling, whereas in a more 'complex' *in vitro* system, such as human primary myotubes, these proteins may display smaller contributory roles. Though the importance of Vps34 kinase activity has not been previously investigated in human primary myotubes, the effect of leucine and insulin administration on Vps34 protein content has been reported. Gran and Cameron-Smith(18) reported that leucine and/or insulin exposure for 0.5, 3 or 24h all elevated Vps34 protein content, an effect that occurred alongside mTORC1 activation. The significance of this increase in Vps34 protein is difficult to deduce as it is impossible to know whether it occurred upstream or downstream of the altered mTORC1 signalling. Nevertheless, this data does suggest Vps34 content is regulated somewhat by nutrient provision, however Vps34 kinase activity was not measured and as such conclusions regarding this aspect of its regulation cannot be drawn. Together, our findings show that the use of the Vps34 specific inhibitor SAR405 has no significant effect on mTORC1-related signalling following serum recovery in C2C12 or human primary myotubes. As such future research regarding the potential importance of Vps34 kinase activity in specific tissues is required.

We next investigated whether Vps34 kinase activity was altered by protein-carbohydrate feeding in vivo in human skeletal muscle. Contrary to our hypothesis, Vps34 kinase activity did not change across the postprandial time course. Previously, it has been shown that eccentric contractions elevated Vps34 kinase activity in rodent skeletal muscle, and this was believed to be due to the elevation in intracellular leucine that occurred here(19). Based on the elevations in plasma leucine, our feeding protocol would have likely increased intramuscular leucine, therefore stimulating Vps34 kinase activity however, this was not apparent. In human skeletal muscle, there is only one previous study which assessed Vps34 kinase activity(32). Here, sprint exercise combined with protein-carbohydrate ingestion did not alter kinase activity whereas exercise in the fasted state elicited a trend toward elevated activity ~1.5h following the final exercise bout. This implies that protein-carbohydrate feeding itself may attenuate Vps34 kinase activity, a notion we did not find in the current study. A potential explanation for these contradictory findings is the large number of complexes in which Vps34 is comprised(13). As the only class III PI3 Kinase in mammalian cells, Vps34 resides in many complexes of varying functions. For example, when associated with Beclin-1 and Atg14, Vps34 will regulate phagophore nucleation and aid the induction of autophagy(33), whereas an association Rab5/7 will allow Vps34 to regulate endocytic sorting(34). Further to this, and as described earlier, Vps34 seems to regulate anabolic responses nutrient provision (15, 16). Importantly, these processes, in particular mTORC1 activation and autophagosome nucleation, are oppositely regulated by nutrient provision. This could result in protein-carbohydrate feeding eliciting opposing effects on certain Vps34 complexes and, as our method of measuring Vps34 kinase activity does not distinguish between these complexes, may explain the absence of a change in this measure. It may also explain the

differing results from previous analysis where eccentric contractions in rodents could have simultaneously activated both anabolic and catabolic Vps34 complexes(19), whilst the addition of protein-carbohydrate feeding following sprint exercise(32) may inhibit catabolic and activate anabolic Vps34 complexes resulting in similar findings to those we report. As our understanding of the *in vivo* regulation of Vps34 kinase activity is in its infancy, further research is imperative to fully elucidate this kinase's role in skeletal muscle.

The current most widely accepted model of AA-induced mTORC1 activation is based on recruitment of mTORC1 to the lysosome where it can bind with its direct activators Rheb and PA(8, 35-38). However recent evidence from our laboratory (10, 11), and others (12) suggests that mTORC1 activation in skeletal muscle seems to involve the translocation of mTORC1-lysosome complexes to peripheral regions of the cell, an event which coincides with elevated mTORC1 activity(10, 11) and elevated interaction between mTORC1 and Rheb(10). Recently, Vps34 has been implicated in this process *in vitro*, where Vps34 is required for the recruitment of mTOR to cellular projections in response to insulin stimulation and in fact colocalises with mTOR in these regions (17). We investigated this paradigm in human skeletal muscle through the use of immunofluorescence microscopy partnered with colocalisation analysis. Firstly, we found no change in mTOR-LAMP2 colocalisation following protein-carbohydrate feeding, in agreement with our previous data displaying mTOR to be constantly associated with the lysosome(10, 11). Interestingly, these findings have been contradicted recently by other groups(39, 40), reporting an increase in mTOR-LAMP2 localisation following resistance exercise or essential AA feeding. The discrepancy in findings is difficult to explain however, could be due to differences in antibodies utilised and

quantification methods (Pearson's vs. Mander's correlation coefficients). In the current study we next observed mTOR-positive structures move toward the cell periphery, inferred by the time effect noted for mTOR-WGA (marker of the cell membrane) localisation. This time effect was most likely driven by the 17% increase noted at 1 hour following feeding; an alteration which occurred in concurrence with an increase in mTORC1 activation, reinforcing our hypothesis that mTORC1 translocation is a key event in this kinase's activation(10, 11). Notably, this change in mTOR-WGA localisation following protein-carbohydrate feeding is similar to an effect we have previously observed (11), showing a reproducibility in our measurements. The colocalisation of Vps34 with WGA exhibited a similar pattern as that of mTOR-WGA, with a trend toward a time effect noted ($p=0.053$). Here, Vps34-WGA colocalisation increased significantly above baseline 1 hour post-feeding before returning to baseline 3 hours after drink consumption. This investigation is the first to our knowledge which has examined Vps34 cellular location in skeletal muscle, however a translocation toward the cell periphery has been previously observed *in vitro* in response to insulin administration(17). Moreover, the product of Vps34 kinase activity (PI(3)P) is also visualised in these areas and the translocation of mTORC1 to peripheral regions is believed to be PI(3)P dependent(17, 41, 42). Our finding that Vps34 translocation also occurs in human skeletal muscle suggests that this may direct Vps34 kinase activity toward these regions in order to recruit mTORC1. Specifically, this may further clarify why global Vps34 kinase activity remained unchanged in the current study, as an alteration in the location at which PI(3)P production occurs rather than an increase in total PI(3)P presents a more efficient mechanism of mTORC1 peripheral recruitment.

Notably, we also report an effect of protein-carbohydrate feeding on mTOR-Vps34 colocalisation where an overall time effect was observed. Again an increase in this variable 1 hour following feeding (11%) most likely drove this effect and a trend toward a greater colocalisation at this time point, compared to 3 hours post-feeding ($p=0.067$. This data implies that protein-carbohydrate feeding elicits both mTORC1 and Vps34 translocation toward the cell periphery where these proteins associate. This association has been reported in NIH3T3 cells following insulin exposure (17) and our similar observations in human skeletal muscle further reinforces the notion that Vps34 could regulate nutrient-induced mTORC1 trafficking and activation(15, 16). A recent study in HeLa cells suggests that PI(3)P may regulate lysosomal positioning via its receptors Protrudin and FYCO1(42). Specifically, AAs induce an increased association of FYCO1 with lysosomes and the ablation of this protein, or Protrudin, caused the clustering of mTOR-positive lysosomes to perinuclear regions and attenuated mTORC1 activity, irrespective of nutrient availability(42). This intricate control mechanism of lysosomal positioning by the product of Vps34 activity may extend to human skeletal muscle and explain the results we present here, and previously (10, 11), regarding both mTORC1 and Vps34 translocation. With this current *in vivo* study design we are unable to conclude whether Vps34 translocation occurs before, or in fact causes, mTORC1 translocation. Intriguingly, several other mechanisms by which Vps34 may regulate mTORC1 have been presented. Mohan et al.(41) utilised a Vps34 mutant, which exhibited elevated kinase activity, in NIH3T3 cells finding an increased amount of PI(3)P in peripheral regions. This active form of Vps34 was found to co-immunoprecipitate with TSC1, most likely via both protein's association with phosphatidylinositol 3-phosphate 5-kinase (PIKFYVE). The formation of this complex removed TSC1 from its association with

TSC2 and led to ubiquitination and breakdown of this protein(41). The consequential removal of TSC2-induced inhibition of Rheb would then allow for a greater activation of mTORC1 through direct binding of Rheb to the catalytic domain, as described previously (43-46). Yoon et al.(22) reported a differing mechanism whereby Vps34 may exhibit its AA sensing role through leucyl t-RNA synthetase (LRS). Here it was suggested that, during times of high AA availability, leucine will bind to LRS and activate this enzyme. LRS will then bind to and activate Vps34 which will subsequently cause the translocation of phospholipase D1 (PLD1) to lysosomal membranes(22). Here this enzyme will produce PA, another direct mTORC1 activator, which can bind to the FKB domain of mTOR and enhance its kinase capabilities(37, 38, 47, 48). This mechanism seems to rely on an increase in Vps34 activity, a finding we failed to report here and as such this mechanism may be unlikely in the present study. Furthermore, it was recently purported that PLD1 may not regulate PA production in skeletal muscle as once believed(49). Nevertheless, as each of these potential mechanisms have only been studied *in vitro,* further investigations are warranted to fully understand their contribution to human skeletal muscle protein synthetic response *in vivo*.

5.6 – Conclusions

In conclusion, we present evidence that treatment of C2C12 and primary human myoblasts with the Vps34 kinase inhibitor SAR405 does not alter serum-induced activation of mTORC1. Further, in human skeletal muscle *in vivo*, we show that a change in Vps34 cellular location, rather than activity, occurs in response to protein/carbohydrate ingestion in the basal state. Specifically, in response to feeding, Vps34 and mTOR translocate toward the cell periphery, an event coinciding with elevated mTORC1 activity. Previous *in vitro* evidence suggests mTORC1/lysosomal translocation may be a

result of increased Vps34 kinase activity in peripheral regions and as such future research should focus on elucidating this mechanism in skeletal muscle. Additionally the effects of resistance exercise, a paradigm previously shown to activate Vps34 *in vivo* in rodents, on the measures described herein should be investigated to fully understand Vps34's role in AA sensing.

5.7 – References

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Chapter 6

THE EFFECTS OF RESISTANCE EXERCISE AND/OR PROTEIN-CARBOHYDRATE FEEDING ON VPS34 & LAT1 REGULATION IN HUMAN SKELETAL MUSCLE

6 THE EFFECTS OF RESISTANCE EXERCISE AND/OR PROTEIN-CARBOHYDRATE FEEDING ON VPS34 & LAT1 REGULATION IN HUMAN SKELETAL MUSCLE

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6.1 – Abstract

The mechanistic target of rapamycin is believed to be the central regulator of protein synthesis in human skeletal muscle. Due to this much research has focussed on identifying the upstream mechanism by which mTORC1 is regulated in response to anabolic stimuli. Recent evidence has suggested that vacuolar protein sorting 34 (Vps34) is implicated in sensing variations in nutrient levels and signalling these to mTORC1. Furthermore, amino acid transport, particularly through the primary leucine transporter LAT1, has been purported to contribute to skeletal muscle anabolism. Therefore the aim of the current study was to investigate the effect of unilateral resistance exercise and/or protein carbohydrate feeding on the regulation of these two proteins in relation to mTORC1 signalling. 8 young, healthy males (age – 21.3±3.6yrs, BMI – 22.5±2.1kg/m^2, Body fat % - 11.1±3.3%) completed a trial whereby unilateral knee extensions were completed followed by consumption of a protein carbohydrate beverage (20g PRO, 40g CHO, 0.6g FAT). Muscle biopsies were taken at rest and then bilaterally at 1h and 3h post exercise/feeding in order to create distinct feeding alone (FED) and exercise + feeding (EXFED) conditions. The regulation of mTORC1 and Vps34 was then analysed via immunoblots for related targets and LAT1 regulation was analysed via immunohistochemistry and immunoblot. Following exercise/feeding mTORC1 related signalling ($S6K1^{Thr389}$, $S6^{Ser235/236}$, $S6^{Ser240/244}$) was elevated in both conditions but to a greater extent, and for a more prolonged period, in EXFED, suggestive of greater mTORC1 activation. Vps34 protein content and a surrogate marker of its activity ($SGK3^{Thr320}$) were unchanged in either group ($p>0.05$), suggesting mTORC1 can be activated without changes in these parameters. Interestingly a negative association between $Vps34^{Ser249}$ and $S6K1^{Thr389}$ phosphorylation was found (r=-0.43,

p=0.009) indicative of a link between mTORC1 activity and the regulation of this post-translational modification which is purported to regulate autophagy induction. Interestingly, this finding add further complexity to Vps34s purported association to mTOR signalling as it may lie both upstream and downstream of this kinase. Finally, LAT1 fibre type differences were maintained across the post-exercise/feeding period and large amounts of positive intracellular LAT1 staining was also apparent at each time point in type II fibres (55-67% greater, all $p<0.0001$). This identifies a need for further research regarding the role of this intracellular LAT1 expression. Overall, these data suggest mTORC1 activity can be stimulated without alterations in Vps34 kinase activity and protein content or LAT1 fibre type differences. Further the identification of a potential relationship between mTORC1 activity and $Vps34^{Ser249}$ phosphorylation provides a potential mechanism by which Vps34 complexes of differing roles may be regulated oppositely by anabolic stimuli.

6.2 – Introduction

The protein synthetic response in skeletal muscle can be activated via nutritional, mechanical and hormonal stimuli (1, 2). Under normal physiological conditions, the two principal stimuli thought to increase muscle protein synthesis (MPS) are amino acid (AA) feeding (3-5) and resistance-type exercise (6). These stimuli both act initiate signalling pathways which elevate MPS (7), whilst AAs can simultaneously inhibit MPB (2). In fact, both AA feeding and resistance exercise exhibit synergistic effects on muscle protein turnover whereby net protein balance (NPB), the difference between MPS and MPB, can only become positive following resistance exercise if AAs are also ingested (5). Interestingly, these two anabolic stimuli exert their positive effects on MPS via the same protein kinase, the mechanistic target of rapamycin (mTOR), specifically mTOR complex 1 (mTORC1) (7-9). In fact, if this serine/threonine kinase is inhibited both AA feeding and resistance exercise are no longer able to stimulate MPS (10, 11). Accordingly, recent research has focussed on understanding the mechanisms by which AA feeding and resistance exercise activate mTORC1 in human skeletal muscle.

Byfield et al (12) first identified vacuolar protein sorting 34 (Vps34), as a potential regulator of mTORC1 activation in human skeletal muscle, and hypothesised it to have a role in AA sensing. Vps34 converts phosphatidylinositol into phosphatidylinositol 3-phosphate (PI(3)P) (13), a compound implicated in the translocation of several proteins to cellular and/organelle membrane (14), and is essential for maximal mTORC1 activation in response to insulin and AAs *in vitro* (12, 15, 16). Furthermore, inhibiting the kinase activity of Vps34 inhibits mTOR translocation to the cell periphery following insulin stimulation (17) whilst simultaneous reducing mTORC1 activity. We have

investigated the role of Vps34 in AA sensing in Chapter 5 of this thesis finding that its kinase activity does not seem to contribute to mTORC1 activation in C2C12 or human primary myotubes. Furthermore, in human skeletal muscle, protein-carbohydrate feeding initiated the translocation of Vps34 and mTOR toward the cell periphery where these two proteins colocalised. Interestingly Vps34 kinase activity was unaltered by protein-carbohydrate feeding in human skeletal muscle, suggesting that it may be the change in Vps34's cellular location that may contribute to its role in AA sensing through the recruitment of mTORC1 to peripheral regions. Of note, Vps34 kinase activity has been shown to increase in response to eccentric contractions in rodent skeletal muscle (18), and this was purported to be a result of the change in intracellular leucine concentrations noted following these contractions. However it may also be that a contractile stimulus rather than AA alone is required to achieve full activation of Vps34 in skeletal muscle. The protein expression, post-translational regulation and kinase activity of Vps34 are yet to be investigated in response to resistance type exercise in human skeletal muscle. Recently, a new phosphorylation site on Vps34 (ser249) has been identified which is specific to the serine/threonine kinase ULK1 (19). ULK1 is negatively regulated by mTORC1 through direct phosphorylation at ser757 (20-22) and as such this novel Vps34 phosphorylation site is an intriguing avenue for investigation in human skeletal muscle. Moreover, the phosphorylation status of serine/threonine-protein kinase 3 (SGK3) at Thr320 is believed to be regulated by Vps34 kinase activity (23), however is yet to be studied in human skeletal muscle. Therefore elucidating how these two novel post-translational modifications are regulated following resistance exercise and/or protein-carbohydrate feeding could provide valuable insight into Vps34 action in human skeletal muscle.

The increase in intramuscular AAs following resistance exercise and/or AA feeding can occur via two processes, first an elevation in intramuscular protein breakdown (6, 24, 25) or second through an increase in AA transport into the muscle (26). As mTORC1 inhibits autophagy through phosphorylation of ULK1, Atg13 and Atg14 (22, 27-29), it seems plausible that an elevated AA transport may be a prominent contributor. In skeletal muscle, the major AAs implicated in skeletal muscle anabolism are transported into the cell by the permease L-Type amino acid transporter 1 (LAT1) (30), also known as the solute carrier family 7 member 5 (SLC7A5), via a bi-transport system which concomitantly exports glutamine from the cell (31). Studies of the global protein expression of this AA transporter, in skeletal muscle, following anabolic stimuli have yielded varying results (32-35), possibly due to the need of the transporter to be associated with the plasma membrane to be fully functional. To combat this, our laboratory has recently characterised this protein in resting skeletal muscle through the use of immunofluorescent microscopy (36). Here, we reported that LAT1 was more highly expressed in type II muscle fibres, and in these fibres it resided both in intracellular and peripheral regions (36). As LAT1 may be more active following anabolic stimuli we believe it is important to understand how the distribution of this protein may be altered by resistance exercise and/or protein-carbohydrate feeding.

In this study, we utilised a unilateral exercise model combined with protein-carbohydrate feeding to elicit both feeding alone and feeding + exercise conditions within the same individual. We then aimed to elucidate how increasing AA availability in either basal or post-exercise conditions altered Vps34, LAT1 and mTORC1 activity. We hypothesised that Vps34 phosphorylation/activity would be associated with an increase in mTORC1 activity. Further we hypothesised that LAT1 translocation to the

cell periphery would occur in type II fibres in response to feeding + exercise, but not feeding alone.

6.3 – Methods

6.3.1 Subjects

Eight young, healthy males (age – 21.3±3.6yrs, BMI – 22.5±2.1kg/m^2, Body fat % - 11.1±3.3%) volunteered to partake in the study after being deemed healthy based on responses to a general health questionnaire. Participants were informed of all experimental procedures to be undertaken and any risks involved before written informed consent was obtained.

6.3.2 Experimental Design

Study participants visited the School of Sport, Exercise and Rehabilitation Sciences at the University of Birmingham on three separate occasions. The first visit consisted of anthropometric measurements followed by a 10 repetition maximum (10RM) unilateral strength test on a Knee extension machine (Gym Gear Elite Series, Lancashire, UK.). Participants reported to the laboratory in a fasted state where body mass was determined to the nearest 0.1kg on a digital scale and height was measured, without shoes, using a stadiometer. Body fat % was determined using bioelectrical impedance (BodyStat QuadScan 4000, Isle of Man, British Isles.) following a period of 5 minutes resting in the supine state. 10RM strength testing consisted of 10 repetitions of knee extensions at ascending loads until 10 repetitions could no longer be completed. Each set was separated by a period of 3 minutes rest. The final load at which the participant completed 10 successful repetitions was recorded as the 10RM for the participant. The

leg used for this test was randomised for each participant and this leg was used each exercise bout in the remaining visits.

After a period of 5-7 days, subjects reported to the School of Sport, Exercise and Rehabilitation Sciences at 08:00h following a 10h overnight fast, having not consumed alcohol or caffeine for 24 hours and having not completed strenuous lower-body exercise for 48 hours. This visit consisted of an identical exercise bout to that which would be completed in the final experimental visit and was aimed at familiarising the participant with the exercise protocol. This was conducted in order to minimise potential effects of unaccustomed exercise, rather than the exercise protocol itself, on variables measured in the final trial. This session also allowed for verification of the 10RM load determined in the initial laboratory visit. The exercise bout itself consisted of 10 sets of 10 repetitions of unilateral knee extensions at 10RM interspersed by 2 minutes of rest, designed to elicit volitional failure on the 10^{th} repetition of each set. If volitional failure occurred before the 10^{th} repetition, load was adjusted such that 10 repetitions were maintained in subsequent sets. Based on the loads lifted during this session, the starting load for the final experimental session was adjusted if required to allow for better load maintenance throughout the exercise session. Following completion of this exercise bout participants were free to leave the laboratory.

The final visit took place 5-7 days following the familiarisation session and subjects arrived at the laboratory at 7.30am in an identical state to that of the previous session. Upon arrival 21G cannulas were inserted into the antecubital vein of each forearm. One cannula was used for repeated blood sampling throughout the trial and the other was used to administer a primed continuous infusion of L-[ring$^{13}C_6$]phenylalanine (prime dose 2 $\mu mol \cdot kg^{-1}$; continuous infusion 0.05$\mu mol \cdot kg^{-1} \cdot min^{-1}$; Cambridge Isotope

Laboratories, Andover, MA, USA). The infusion began after a basal, resting blood sample had been obtained. Further blood samples were obtained every 30 minutes for 150 minutes prior to the exercise bout. Subsequent samples were then obtained immediately post-exercise and 15, 30, 45, 60, 90, 120, 150 and 180 minutes post exercise/beverage consumption. Unfortunately, analysis utilising these measures was not completed before submission of this thesis and therefore will not be further discussed. Subjects rested in a supine position before a skeletal muscle biopsy was obtained, under local anaesthesia (1% lidocaine), from the *vastus lateralis* of the subject's non-exercising leg using the Bergstrom technique (37). Muscle samples were process for immunoblotting and immunohistochemical analysis as described previously (Chapter 2.3). Following this, subject's completed an identical unilateral exercise bout to that undertaken in the familiarisation session, with loads again adjusted to ensure 10 repetitions were completed in each set. The mean starting load for this bout was 52±6.8kg and, on average, loads had decreased by ~24% by the final set. Immediately following the exercise bout, subjects consumed a protein-carbohydrate beverage (Optimum Nutrition 2:1:1 Recovery, Downers Grove, IL, USA.) containing 20/40/0.6g of protein carbohydrate and fat respectively. A small amount of L-[ring$^{13}C_6$]phenylalanine was added to the beverage (enriched to 4%) to minimise changes in plasma $^{13}C_6$ phenylalanine enrichment. Subjects then remained in a supine, rested state for 3 hours, with bilateral skeletal muscle biopsies taken at 1 and 3 hours following exercise/feeding. This protocol allowed for divergent within-subject conditions of protein-carbohydrate feeding alone (FED) and resistance exercise combined with protein-carbohydrate feeding (EXFED) to be investigated within the same trial. Each skeletal muscle biopsy was processed for both immunoblotting and

immunohistochemical analysis and was stored at -80°C until needed. The experimental trial protocol is depicted in Figure 6.1.

6.3.3 Antibodies

For immunoblotting analysis all primary antibodies were purchased from Cell Signaling Technologies (CST) and diluted at 1:1000 in TBST unless stated otherwise. Antibodies utilised were: p-mTOR^{Ser2448} (#2971), mTOR (#2983), p-TSC2^{Thr1461} (#3611), p-TSC2^{Ser939} (#3615), TSC2 (#3612), p-AKT^{Thr308} (#2965), p-AKT^{Ser473} (#4060), AKT (#4691), p-Vps34^{Ser249} (#13875), Vps34 (#3358), p-S6K1^{Thr389} (#9205, 1:500), S6K1 (#2708), p-rpS6$^{Ser235/236}$ (#4858), p-rpS6$^{Ser240/244}$ (#5364), rpS6 (#2217), p-4EBP1$^{Thr37/46}$ (#9459, 1:500), 4EBP1 (#9452, 1:500), ULK1 (#8054, 1:500), p-ULK1^{Ser757} (#6888), p-SGK3^{Thr320} (#5642), SGK3 (#8156), p-eEF2^{Thr56} (#2331), eEF2 (#2332), GAPDH (#5174) and LAT1 (Abcam, #Ab85226, 1:500). All primary antibodies were produced in rabbits and as such anti-rabbit IgG HRP-linked secondary antibody (CST #7074) was utilised. Antibody binding was detected via the use of enhanced chemiluminescence horseradish peroxidase substrate detection kit (Millipore, Watford, UK).

For immunohistochemical analysis the antibodies utilised were as follows: LAT1 (1:100, Abcam, #Ab85226), MHC1 (1:250, #A4.840, DSHB, Iowa, USA) and Wheat Germ Agglutinin (1:20, WGA-350, #11263, ThermoFisher, UK). Corresponding secondary antibodies were goat anti-mouse goat anti-mouse IgM Alexa®594 (#A21044, ThermoFisher, UK, targeting MHC1) and goat anti-rabbit IgG(H+L) Alexa®488 secondary antibodies (#A11008, ThermoFisher, UK, targeting LAT1). WGA-350 was pre-conjugated to a Alexa®350 fluorophore and as such now secondary antibody was needed.

6.3.4 Immunoblotting

Identical amounts of protein were separated on 8-15% polyacrylamide gels by SDS-PAGE as described in Chapter 2 (Section 2.5.2). Proteins were then transferred to BioTrace NT nitro-cellulose membranes (Pall Life Sciences, UK.) at 100V for 1h, blocked in 3% milk/TBST (5% Bovine Serum Albumin (BSA) for LAT1 and p-TSC2^{S939}) and incubated overnight in the desirable primary antibodies. The following day, membranes were washed in TBST and incubated in corresponding secondary antibodies (diluted 1:10000 in TBST) at room temperature for 1h. Chemiluminescence horseradish peroxidase reagent kit (Merck-Millipore) was used to quantify protein content following IgG binding. Images were captured with a G:Box Chemi-XR5 (Syngene (A Division of Synoptics Ltd.), Cambridge, UK) imaging system and blot bands were quantified using GeneTools software (Syngene, Cambridge, UK). Each protein target was expressed in relation to a gel control (combination of all samples) and a loading control (eEF2 or GAPDH dependent on weight of target protein) and each phosphorylation site is presented in relation to its corresponding total protein abundance.

6.3.5 Immunohistochemistry

Skeletal muscle samples were processed for immunohistochemical analysis as described in Chapter 2 (Section 2.3). Following this, skeletal muscle sections were fixed, blocked and stained as described previously (Section 2.4). MHC1(594nm)-LAT1(488nm)-WGA(350nm) stains were visualised as described in section 2.4.5 of this thesis. Image analysis for colocalisation and fibre-type specific fluorescence intensity of these targets

was conducted using ImagePro or Fiji (ImageJ) software respectively, as described in section 2.4.6.2.

6.3.6 Statistics

Changes in intramuscular signalling and fibre type-specific LAT1 fluorescence intensity were examined using a repeated measures ANOVA with two within-subject factors (time and condition). Differences in LAT1 fluorescence intensity between fibre types were analysed using independent t-tests at each time point. When a significant main or interaction effect was found, *post-hoc* tests were completed with the Holm-Bonferroni correction for multiple comparisons. Significance for all variables analysed was set at $p \leq .05$. Data are presented as means±SEM unless otherwise stated.

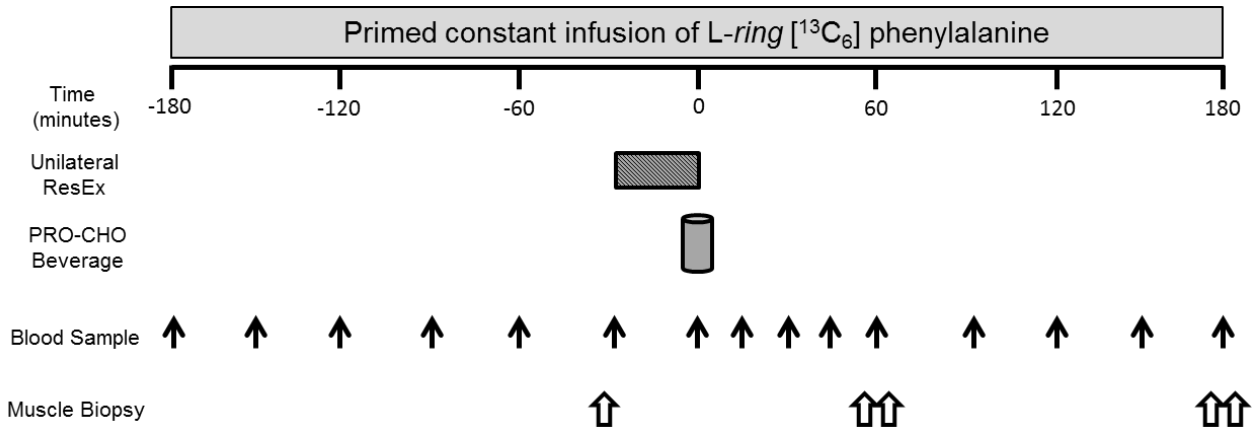

Figure 6.1. Experimental trial design

6.4 – Results

6.4.1 Intramuscular signalling upstream of mTORC1

A significant interaction effect was observed for AKT phosphorylation at Thr308 ($p=0.011$). Phosphorylation at this site was significantly greater, in both conditions, 1h

following exercise/feeding compared to PRE and 3h time points (EXFED – 7.6±1.2 fold above PRE, FED – 4.4±0.7 fold above PRE, all p<.05, Fig 6.2a). A time effect was noted for AKT^{Ser473} phosphorylation (p<.001) suggesting that, when both conditions are combined, phosphorylation at this site was greater at the 1h time point compared to both PRE and 3h (p<0.01, Fig 6.2b). TSC2^{Thr1462} phosphorylation remained unchanged in either condition at any time point (p>0.05, Fig 6.2c). A significant interaction effect was noted for TSC2^{Ser939} phosphorylation (p=0.023), however following *post hoc* tests no differences between time points/conditions were significant (p>0.05, Fig 6.2d). In addition, significant condition (p=0.011) and time (p=0.028) effects were observed for this variable. This suggests that when time points are combined, greater TSC2^{Ser939} phosphorylation is apparent following EXFED compared to FED. Furthermore, when conditions were combined, the extent of TSC2^{Ser939} phosphorylation at the 1h time point was significantly greater than 3h (p=0.027, Fig 6.2d).

6.4.2 Intramuscular signalling downstream of mTORC1

A significant time effect was observed for mTOR^{Ser2448} phosphorylation (p<.0.001). Pairwise comparisons displayed that protein-carbohydrate feeding, irrespective of accompanying resistance exercise, elicited an increase in mTOR^{Ser2448} phosphorylation ay 1h post feeding (p=0.003, Fig 6.3a) which remained above PRE values at 3h post-feeding (p=0.012, Fig 6.3a). Furthermore the extent of mTOR^{S2448} phosphorylation at the 1h time point was greater than 3h, when condition were combined (p=0.027, Fig 6.3a). A significant interaction effect was observed for S6K1^{Thr389} phosphorylation (p=0.004). Here, both feeding alone, and combined with resistance exercise, elicited significant elevations in S6K1^{Thr389} phosphorylation above PRE and 3h values at 1h (EXFED – 43.3±4.4 fold, p<0.001, FED – 27.9±5.4 fold, p<0.01, Fig 6.3b). These

elevations remained above PRE at 3h in both conditions (EXFED – 13.5±3.0 fold, FED – 3.8±0.8 fold, both p=0.012, Fig 6.3b). Furthermore, at both 1h and 3h, the extent of S6K1^{Thr389} phosphorylation was greater in the EXFED condition compared to FED ($p<0.05$, Fig 6.3b).

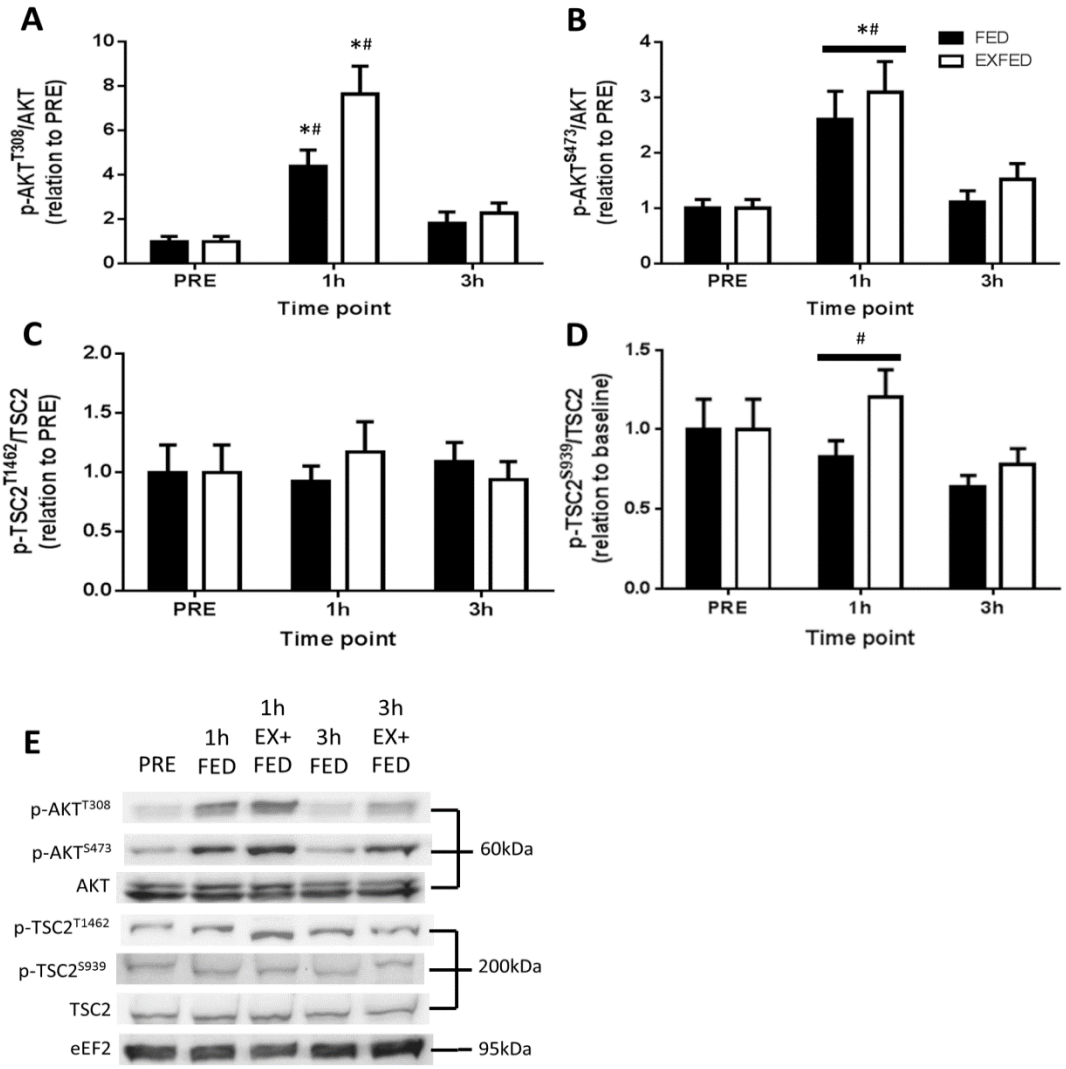

Figure 6.2. The effect of resistance exercise and/or protein-carbohydrate feeding on AKT/TSC2 signalling (n=8). AKT^{Thr308} (A), AKT^{Ser473}(B), TSC2^{Thr1462} (C) & TSC2^{Ser939} (D) phosphorylation at rest (PRE) and following protein-carbohydrate feeding with (EXFED – open bars) or without (FED – black bars) prior resistance exercise. Each phosphorylation site is displayed in relation to its corresponding total protein. Representative images are also displayed (E). *significantly different to corresponding PRE ($p<0.05$). #significantly different to corresponding 3h ($p<0.05$). Black lines denote time effect. Data presented as Mean±SEM.

A significant interaction effect was also observed for $S6^{Ser240/244}$ phosphorylation (p=0.007). Here, both FED and EXFED increased $S6^{Ser240/244}$ phosphorylation above PRE values at 1h (EXFED – 19.2±4.1 fold, p=0.016, FED – 17.4±4.3 fold, p=0.028, Fig 6.3c). $S6^{Ser240/244}$ phosphorylation remained above PRE values at 3h in both conditions, however was only lower than 1h in the FED condition (p=0.042, Fig 6.3c), suggesting a maintenance of phosphorylation at this site with resistance exercise. Furthermore, at 3h, $S6^{Ser240/244}$ phosphorylation was greater the EXFED condition compared to FED (p=0.027, Fig 6.3c). $S6^{Ser235/236}$ phosphorylation also exhibited a significant interaction effect (p=0.014), where both conditions elicited significant elevations above PRE at 1h (EXFED – 53.3±8.1 fold, p=0.003, FED – 35±8.9 fold, p=0.041, Fig 6.3d). Phosphorylation at this time point was also greater in the EXFED condition compared to FED (p=0.043, Fig 6.3d). $S6^{Ser235/236}$ phosphorylation returned to PRE values in the FED condition at 3h, however there was a trend toward greater phosphorylation, compared to PRE, at this time point in the EXFED condition (39.6±13.1 fold, p=0.067), again suggesting a possible synergistic effect of feeding combined with resistance exercise.

An interaction effect was noted for $eEF2^{Thr56}$ phosphorylation (p=0.006), showing a reduction in phosphorylation at this site, in both conditions at 1h compared to PRE (EXFED – 0.58±0.03 fold of PRE, p=0.006, FED – 0.73±0.05 fold of PRE, p=0.035, Fig 6.3e). In addition $eEF2^{Thr56}$ phosphorylation was lower at this time point in the EXFED condition compared to FED (p=0.038, Fig 6.3e). Phosphorylation at this site returned to PRE values in the FED condition at 3h (p=0.289), however a trend toward lower phosphorylation, compared to PRE, in the EXFED condition was noted at this time point (0.66±0.05 fold of PRE, p=0.064). Further trends were also apparent when

comparing between 1h and 3h in the FED condition (p=0.06) and between conditions at 3h (p=0.057). Finally, there were no alterations in 4EBP1$^{Thr37/46}$ phosphorylation at any time point or between conditions (p>0.05, Fig 6.3f).

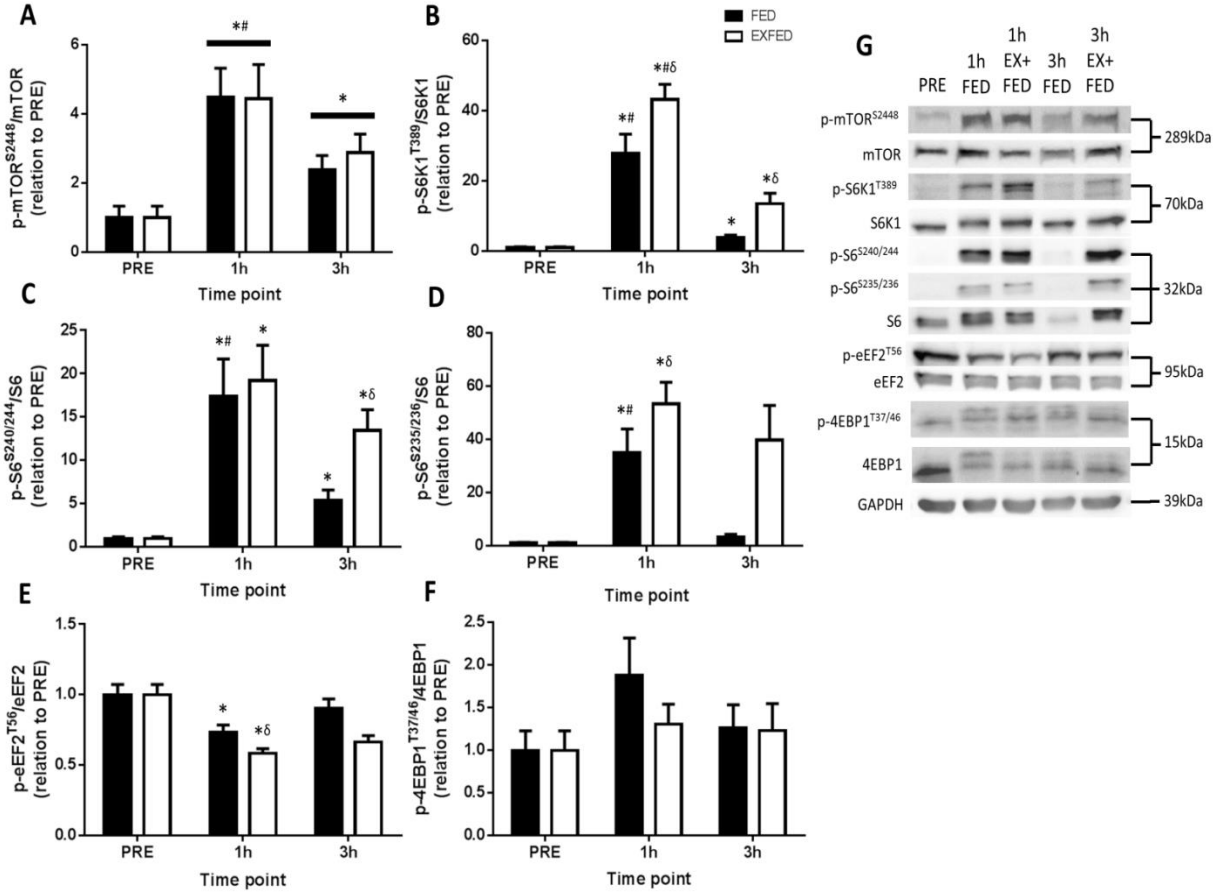

Figure 6.3. The effect of resistance exercise and/or protein-carbohydrate feeding on mTORC1-related signalling (n=8). mTOR^{Ser2448} (A), S6K1^{Thr389} (B),S6$^{Ser240/244}$ (C), S6$^{Ser235/236}$ (D), eEF2^{Thr56} (E) & 4EBP1$^{Thr37/46}$ (F) phosphorylation at rest (PRE) and following protein-carbohydrate feeding with (EXFED – open bars) or without (FED – black bars) prior resistance exercise. Each phosphorylation site is displayed in relation to its corresponding total protein. Representative images are also displayed (G). *significantly different to corresponding PRE (p<0.05). #significantly different to corresponding 3h (p<0.05). δsignificantly different to corresponding FED (p<0.05). Black lines denote time effect. Data presented as Mean±SEM.

6.4.3 Vps34-related intramuscular signalling

Vps34^{Ser249} phosphorylation was reduced at 1h in both conditions (EXFED – 0.29±0.05 fold of PRE, FED – 0.55±0.15 fold of PRE) before increasing back toward baseline at 3h but did not achieve significance (time effect - p=0.084, Fig 6.4a). Both total Vps34 protein content and SGK3^{Thr320} phosphorylation remained unaltered across the time course in both conditions (p>0.05, Fig 6.4b & c). A significant time effect was observed for ULK1^{Ser757} phosphorylation (p=0.001). Here, protein-carbohydrate feeding, irrespective of prior resistance exercise, elevated ULK1^{Ser757} phosphorylation above PRE and 3h values at 1h (EXFED – 3.4±0.8 fold, FED – 3.1±0.6 fold, p<0.05, Fig 6.4d). Correlation analysis displayed that the extent of Vps34^{Ser249} phosphorylation at all time points was significantly correlated to that of S6K1^{Thr389} (r = -0.43, p=0.009, Fig 6.4e) but not that of ULK1^{Ser757} (r = -0.01, p=0.95, Fig 6.4f).

6.4.4 LAT1 fibre type distribution and protein content

LAT1 fluorescence intensity was greater in type II fibres (MHC1-negative), compared to type I fibres (MHC1-positive), at each time point (on average 1.6±0.02 fold greater, all p<0.001, Fig 6.5b). In type I fibres, LAT1 fluorescence intensity did not change at any time point in either condition (p>0.05). However, in type II fibres, a significant time effect was observed (p=0.004, Fig 6.5b) whereby LAT1 fluorescence intensity was reduced at both 1h (p=0.03) and 3h (p=0.028), irrespective of condition. Total LAT1 protein content, measured by immunoblot, remained unchanged across the time course in both conditions (p>0.05, Fig 6.5c).

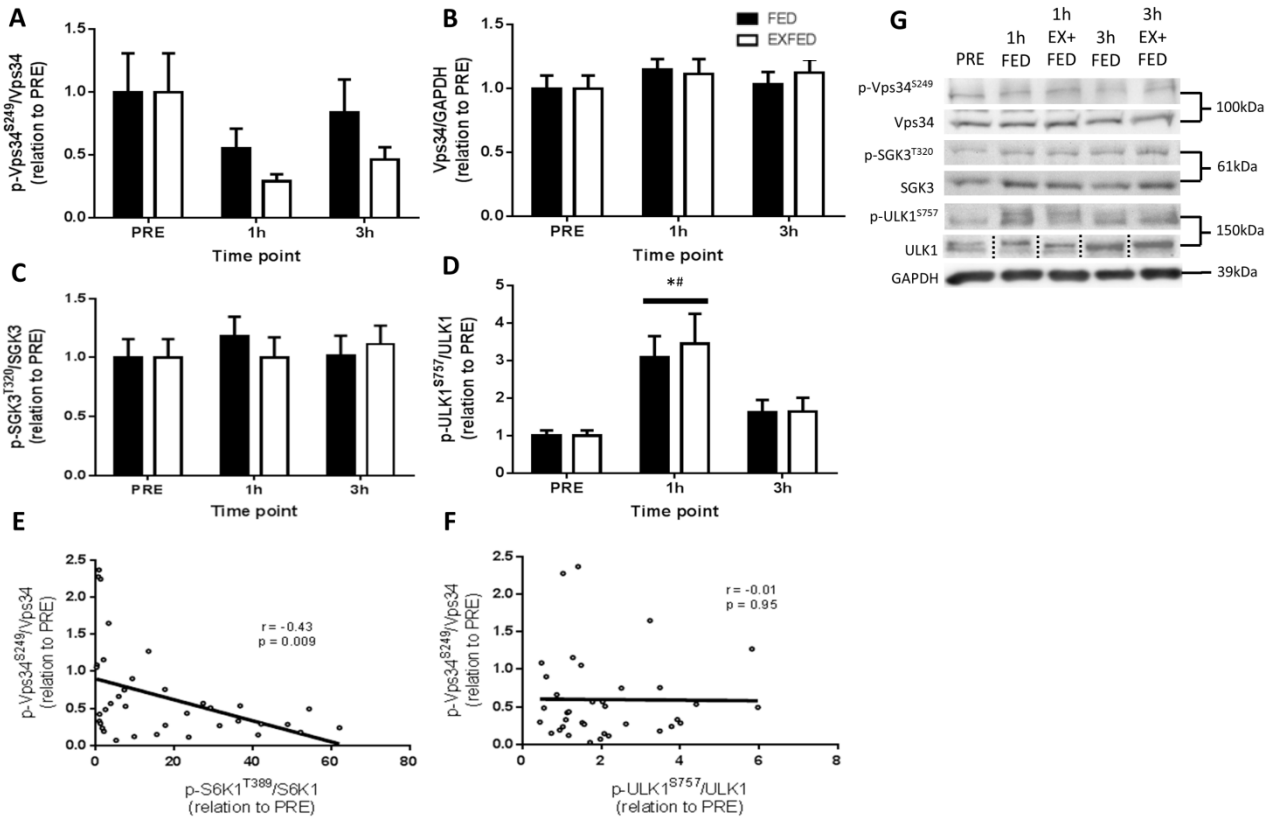

Figure 6.4 The effect of resistance exercise and/or protein-carbohydrate feeding on Vps34-related signalling (n=8). Vps34^{Ser249} (A), Vps34 (B), SGK3^{Thr320} (C) & ULK1^{Ser757}(D) phosphorylation at rest (PRE) and following protein-carbohydrate feeding with (EXFED – open bars) or without (FED – black bars) prior resistance exercise. Each phosphorylation site is displayed in relation to its corresponding total protein. Vps34Ser249 phosphorylation is also correlated to S6K1Thr389 (E) and ULK1Ser757 (F). Representative images are also displayed (G). *significantly different to corresponding PRE ($p<0.05$). #significantly different to corresponding 3h ($p<0.05$). Black lines denote time effect. Data presented as Mean±SEM.

6.5 – Discussion

In the present study, we report that the phosphorylation of SGK3^{Thr320}, a marker of Vps34 kinase activity, and total Vps34 protein content are unaffected by protein-carbohydrate feeding either when consumed alone or after a unilateral bout of resistance

exercise. Furthermore, the phosphorylation of Vps34^{Ser249}, decreased across the trial and this reduction was negatively associated with mTORC1 activity. In addition we also investigated whether LAT1 fibre-type distribution and protein content were affected by resistance exercise and/or protein-carbohydrate feeding. Here we show that the greater LAT1 content in type II fibres, previously reported in basal skeletal muscle, is maintained in both conditions across the trial. Interestingly however, we also find that type II-specific LAT1 expression decreases following protein carbohydrate feeding, irrespective of prior resistance exercise completion, though total LAT1 protein content, measured via immunoblot, remained unchanged.

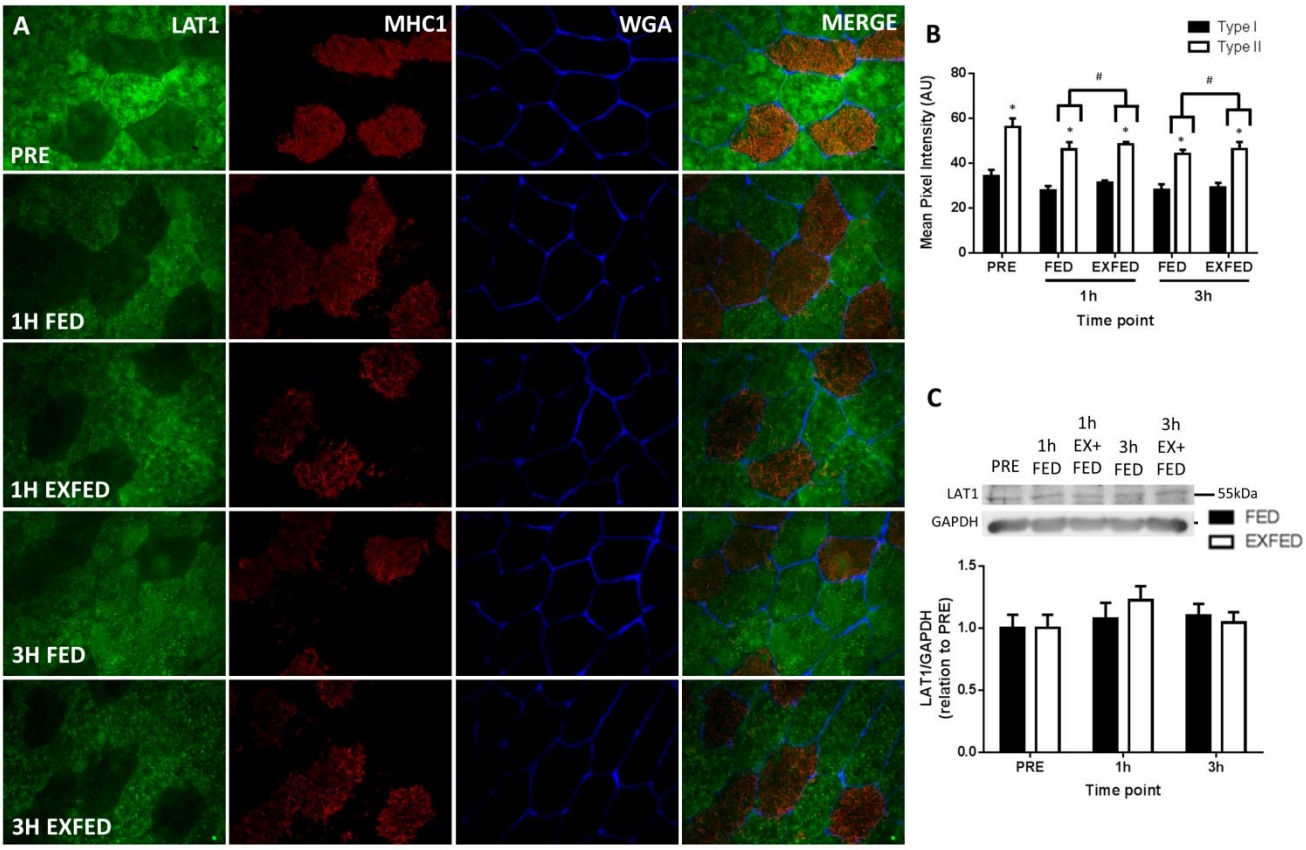

Figure 6.5 The effect of resistance exercise and/or protein-carbohydrate feeding on LAT1 total and fibre-type expression (n=7). LAT1 fibre type specific intensity (B) was quantified in type I (black bars) and type II (open bars) across the timecourse. Representative images of each stain are displayed (A). Total LAT1 protein expression was measured via immunoblot (C) and representative images are provided. *Significantly different from corresponding type I ($p<0.001$). #Significantly different to PRE ($p<0.05$). Data presented as Mean±SEM.

mTORC1 signalling is known to be essential for MPS stimulation following anabolic stimuli i.e. mechanical loading and AA feeding (10, 11). These particular anabolic stimuli have been consistently reported to have synergistic effects both on mTORC1-related signalling and MPS (4, 5, 38-41). Here we provide further confirmation of this synergism, observing elevated phosphorylation of several downstream mTORC1 targets, in the EXFED condition compared to FED. Specifically the phosphorylation of

S6K1^{Thr389}, S6$^{Ser235/236}$ and S6$^{Ser240/244}$ was propagated above FED levels, at varying time points, following EXFED. Furthermore the extent of eEF2^{Thr56} phosphorylation was significantly lower at 1h following EXFED, again indicative of increased mTORC1 activity (42). These findings suggest that the processes of translation initiation and elongation would most likely be greater in this condition (42-44), therefore stimulating greater levels of MPS as described previously (45). In contrast, one direct downstream target of mTORC1, 4EBP1$^{Thr37/46}$, did not exhibit this synergistic pattern, or in fact any alteration in phosphorylation status. Although somewhat counterintuitive due to the high elevation in mTORC1 activation we note on other targets, this finding has been previously described on several occasions (46, 47). 4EBP1 contains 4 sites of mTORC1-dependent phosphorylation (Thr37/46, Ser65 & Ser70) which are phosphorylated in a hierarchical manner before this protein is removed from its interaction with eIF4E (48). 4EBP1$^{Thr37/46}$ are the initial sites of phosphorylation on this target, essentially priming this protein for further phosphorylation (48). It is therefore possible that the time points utilised in this study may have missed this initial 'priming' phosphorylation which may have been subsequently removed when the other sites were phosphorylated. This suggestion can be reinforced through the visual examination of the immunoblot images for 4EBP1 (Fig 6.3g), where this protein consistently exhibited an upward gel shift at every time point other than PRE. This suggests that this protein is hyper-phosphorylated at these time points, most likely at the subsequent mTORC1-dependent phosphorylation sites (Ser65 & Ser70). 4EBP1^{Ser65} phosphorylation has been previously noted to increase both 90 and 180 minutes following resistance exercise and protein feeding, time points where alterations 4EBP1$^{Thr37/46}$ were less apparent (46). These data suggest it may be these secondary phosphorylation sites which may be a

better readout of mTORC1 activity at these time points and therefore further research should aim to elucidate their full regulation in response to anabolic stimuli.

Upstream of mTORC1, we report that AKT phosphorylation at its two major regulatory sites (Thr308 & Ser473) is elevated above baseline and 3h values at 1h, irrespective of prior exercise. These findings suggest this response was predominantly driven by protein-carbohydrate feeding with no propagation of the response by resistance exercise. This notion has been previously described as AKT is predominantly regulated by insulin/growth factors (49). Downstream of AKT lies TSC2 (50) which is believed to be regulated in an AKT-dependent fashion at two sites, Thr1462 and Ser939 (50). Here, no alteration in either phosphorylation site, from PRE values, was apparent although the extent of $TSC2^{Ser939}$ phosphorylation was greater at 1h, compared to 3h, when conditions were combined. These findings were unexpected, particularly due to the large stimulation of AKT phosphorylation observed. Again, this absence of phosphorylation may be due to timings of samples, especially as AKT/TSC2 signalling lies upstream of mTORC1 and a large activation of mTORC1 is already apparent at our first post-intervention time point. Moreover, it is important to note here that it has been proposed that mTORC1 is activated in an AKT-independent manner following resistance exercise in skeletal muscle (51, 52), and therefore alterations in TSC2 phosphorylation at these sites may not actually be relevant in this context. Jacobs et al. (53) describe a mechanism whereby TSC2 is phosphorylated at multiple sites on its RxRxxS∗/T∗ consensus motif following eccentric contractions in rodent skeletal muscle. This phosphorylation then elicits the removal of TSC2 from Rheb and subsequent activation of mTORC1 (54). Further recent work by the same group (55) has identified several new phosphorylation sites on TSC2 that seem to be regulated by

mechanical contraction (Ser664, Ser1155, Ser1254, Ser1364 & Ser1499/52). These sites therefore offer a more appropriate avenue for research regarding upstream mechanisms of mTORC1 activation following muscular contraction and warrant future research in human skeletal muscle.

It is currently unknown how nutrients directly activate mTORC1 in human skeletal muscle. Recently, Vps34, has been reported to contribute to nutrient-induced mTORC1 activation *in vitro* (12, 15), however little research has been conducted on this topic in skeletal muscle. In the previous chapter of this thesis (chapter 5) we reported that the use of a Vps34-specific inhibitor (SAR405) did not affect nutrient-induced mTORC1 activation in both C2C12 and human primary myotubes. In addition, we observed no change in Vps34 kinase activity, in human skeletal muscle, in response to protein-carbohydrate feeding. In fact, instead of a change in kinase activity it seemed alterations in Vps34 cellular location, and subsequent colocalisation with mTOR, may be the mechanism by which Vps34 completes its purported nutrient-sensing role. In the current study, we utilised the phosphorylation of SGK3 at Thr320 as a proxy measure of Vps34 kinase activity. Phosphorylation at this site is dependent on SGK3's ability to associate with PI(3)P (56), the product of Vps34 kinase activity (13), and is significantly reduced in the presence of a Vps34 specific inhibitor (VPS34IN1) (23). The phosphorylation status of the site remained unaltered across the time course of the current study, in both conditions, suggesting these anabolic stimuli have no effect of Vps34 kinase activity. These date are in agreement with those reported in the previous chapter of this thesis, however contradict that reported in rodent skeletal muscle following eccentric contractions. Mackenzie et al. (18) reported that forced eccentric contractions of the tibialis anterior of rodents elicited a 70% increase in Vps34 kinase activity 3h following

said contractions. Furthermore, this alteration in Vps34 kinase activity was associated with the intracellular change in leucine apparent following these contractions, shown when this amount of leucine was added to C2C12 myotubes and a similar Vps34 activation found (18). Importantly, however, the two concentrically contracting muscles analysed, the plantaris and soleus, exhibited much lower or no elevation in Vps34 kinase activity respectively (18). This may explain the discrepancies in results between this study and that of Mackenzie et al. (18) as we sampled skeletal muscle tissue from the *vastus lateralis* of subjects, a muscle that would contract in a concentric manner to complete knee extension exercise. It is possible that eccentric contractions alone can produce a great enough stimulus to elevate Vps34 kinase activity and therefore our exercise protocol may have had no effect. Future research on the effect of contraction type on Vps34 kinase activity in human skeletal muscle is therefore required.

To our knowledge, other than the study described in chapter 5, only one study has investigated the effect of exercise or protein feeding on Vps34 kinase activity in human skeletal muscle. Here, repeated sprint exercise followed by EAA feeding elicited no change in Vps34 kinase activity ~1.5h following the final sprint interval (57). Interestingly however, sprint exercise alone, with no feeding, elicited a trend toward an increase in Vps34 kinase activity. The authors here hypothesise that this may be due to the varying of roles of Vps34 complexes in mammalian cells (12, 13, 17, 58-60). Vps34 complexes are known to contribute both to autophagy induction (58, 61) and mTORC1 activation (12, 15, 59) *in vitro* and therefore EAA ingestion would have opposing effects on the kinase activity of Vps34 in these complexes (13). As a beverage containing fairly high (20g) amounts of protein was consumed by subjects in the current study this may provide further evidence as to why an absence of Vps34 activation is

apparent here. One final potential mechanism as to why Vps34 activity was unchanged here is similar to that hypothesised in the previous chapter of this thesis. It is possible that a change is Vps34 kinase activity is not required to exert an effect upon mTORC1, but that an alteration in its cellular location is sufficient to recruit mTORC1 to peripheral regions and permit its activation, as previously described *in vitro* (17, 62). Specifically, this mechanism occurs through the recruitment of the PI(3)P binding protein FYCO1 to lysosomes, which initiates the translocation of mTORC1-positive lysosomes to the cell periphery (62). These particular mechanisms were not investigated in the current study and therefore require further investigation to fully understand whether they extend *in vivo* in human skeletal muscle.

A second mechanism by which Vps34 may contribute to mTORC1 activation is through a potential increase in its overall protein content. However, similarly to the readout of its activity, there was no change in the total protein expression of Vps34 in the current study. Previous studies in human skeletal muscle, following a combination of sprint exercise and protein feeding have echoed these findings (57) . *In vitro* data in human primary myotubes however, contradicts this notion (63). Leucine or insulin exposure induces a 30-50% increase in Vps34 protein content following 0.5, 3 or 24h of incubation. However, concentrations of insulin and leucine utilised were supraphysiological (100nM and 5mM respectively) (63) and therefore may have driven a greater stimulation of Vps34 protein translation than can be noted in human skeletal muscle following exercise and feeding. Therefore, due to these contradictory findings further research is required into the regulation of Vps34 expression and activity in human skeletal muscle. Nevertheless it can be concluded that overall in this current

study, resistance exercise and/or protein-carbohydrate feeding does not seem to elevate either Vps34 protein content or activity.

Recently, a novel phosphorylation site on Vps34 has been identified, serine residue 249 (19). Further analysis utilising ULK1 knockout (KO) HEK293 cells revealed this to be a ULK1-regulated site (19). As ULK1 is negatively regulated by mTORC1 activity, a potential phosphorylation site on Vps34 which could be regulated by mTORC1 activity is an intriguing notion, especially due to Vps34 purported upstream role of mTORC1. Here, despite a numerical reduction in Vps34^{Ser249} phosphorylation post exercise, there were no significant changes in Vps34^{Ser249} observed. There was however a significant, negative correlation apparent between S6K1^{Thr389} and Vps34^{Ser249} phosphorylation ($r=-0.43$, $p=0.009$, Fig 6.4e), suggesting a downstream link between mTORC1 activity and Vps34 post-translational regulation. The complete role of this post-translational modification has not yet been fully elucidated, however it is believed to contribute to autophagy regulation via Vps34's complex with Beclin-1 and Ambra-1 (19). Therefore, the reduction in phosphorylation at this site, and its association with S6K1^{Thr389} phosphorylation, may provide a further mechanism as to how mTORC1 can inhibit autophagy induction, in addition to its direct inhibitory effect on ULK1, Atg13, Atg14 and TFEB (20, 28, 29, 64, 65). In addition, a reduction in phosphorylation here could conceivably negatively affect the kinase activity of this autophagy-related Vps34 complex and provide further credence to the notion that anabolic and catabolic Vps34 complexes may be oppositely regulated by resistance exercise and protein-carbohydrate feeding.

Further we report that Vps34^{Ser249} phosphorylation is not correlated with ULK1^{Ser757} phosphorylation ($r=-0.01$, $p=0.95$, Fig 6.4f), its direct upstream kinase and a target of

mTORC1 (19, 21). Although this seems counterintuitive, this may be explained by the numerous mechanisms by which ULK1 itself may be regulated. ULK1, in addition to being inhibited by mTORC1, can also be phosphorylated by AMP-activated protein kinase (AMPK) at serine residues 317, 555 and 777 (20, 21, 66) which elicits an increase in ULK1's kinase activity (20, 21). Upon nutrient provision, phosphorylation at these sites is reduced (67) and therefore ULK1 kinase activity would also decrease. Importantly, mTORC1-dependent phosphorylation of ULK1^{Ser757} disturbs the association of AMPK and ULK1 removing its ability to phosphorylate ULK1 at these sites (20). In addition, mTORC1 also regulates ULK1 activity via the phosphorylation of another component in the ULK1-autophagy complex, Atg13 (28). mTORC1 is able to directly phosphorylate Atg13 at serine residue 258 thereby preventing the ULK1 complex from translocating to areas of need (28). It is likely that is an amalgamation of these mTORC1-dependent effects that contribute to a reduction in Vps34^{Ser249} phosphorylation and as such a direct correlation with ULK1^{Ser757} would not be apparent. Nevertheless a significant correlation with S6K1^{Thr389}, considered a reliable readout of mTORC1 activity, suggests an interaction between mTORC1 and Vps34 complexes and as such warrants further investigation.

AA transport is a further mechanism which has been purported to affect mTORC1 activation and therefore MPS (68, 69). Of the myriad of AA transporters which are expressed in human tissue, LAT1 has received the greatest attention due to its role in the transport of leucine, the major anabolic EAA (30, 70). Early research regarding how anabolic stimuli affected this transporter studied total LAT1 protein content in whole muscle lysates. Findings here were variable, with LAT1 protein content increasing at varying time points between 3h-24h post-exercise/feeding in young individuals (32, 33).

Furthermore, the addition of EAA feeding post-exercise seemed to reduce LAT1 protein content in young individuals yet elevate protein content in the skeletal muscle of elderly individuals (33, 34). Here, we report no change in total LAT1 protein content in response to resistance exercise and/or protein-carbohydrate feeding (Fig 6.5c). These differing results, and the notion that LAT1 must be in a specific cellular location (associated with a membrane) in order for its permease activity to be active (71-73), prompted us to examine LAT1 cellular distribution using immunofluorescent techniques (36). We utilised this method in the current study, finding that the elevation in LAT1 protein content in type II fibres was apparent at every time point measured. Interestingly however, when studying the intensity of LAT1 staining in type II fibres, across the trial, a time effect was apparent (Fig 6.5b), suggesting LAT1 staining intensity was reduced at both 1h and 3h post-feeding in this fibre type irrespective of prior resistance exercise. This reduction in staining intensity may be due to a catabolism of LAT1 in type II fibres, however this may not be the case as no reduction in overall LAT1 protein content (immunoblot) was observed. It is possible that LAT1 may have translocated to the plasma membrane in this fibre type and that the nature of our analysis method may not have been sensitive enough to detect this i.e. the identification of fibre borders using WGA may have discounted positive staining within positive WGA staining. Furthermore, due to the large intracellular amounts of LAT1 in type II fibres, and the inability to distinguish between type I and type II plasma membranes, we were unable to measure the localisation of LAT1 proteins with WGA. In the future, it would be beneficial to utilise either immuno-electron microscopy or plasma membrane fractionation to determine the translocation of this AA transporter. A recent study did indeed utilise plasma membrane fractionation to this end and reported an increase in

LAT1 protein content following low-load resistance exercise and protein feeding in a pulse, but not bolus fashion (35). These findings are intriguing and seem to suggest that small frequent protein doses may increase AA transporter translocation compared to bolus feeding. However as this research avenue is still in its infancy, much more research combining a variety of these measures is needed before full conclusions can be drawn.

The current finding that the majority of basal intracellular LAT1 remains intracellular following resistance exercise and/or protein-carbohydrate feeding was somewhat unexpected. As LAT1 must be associated with a membrane to be active, this suggests there may be large amounts of 'inactive' LAT1 within type II fibres which is not associated with a membrane. However, it is possible that this LAT1 may actually be associated with other, intracellular membranes. Recent *in vitro* evidence proposes that LAT1 can associate with the membrane of lysosomes, permitting the influx of leucine into the lysosomes themselves (74). Furthermore, this association of LAT1 with the lysosomal membrane seems to be required for mTORC1 activation, via the inside-out mechanism proposed by the Sabatini laboratory (75), in response to EAAs and/leucine alone (74). This mechanism provides a functional justification for the presence of large amounts of intracellular LAT1, and why this remained following anabolic stimuli. A second possibility is that this intracellular LAT1 may not be able to associate with a membrane due to a lack of its associated glycoprotein CD98. In order for LAT1 to associate with a membrane, it must form a heterodimer with CD98 (71, 73), and therefore if there is a dearth of this protein intracellular LAT1 would not be able to associate with any membranes and remain intracellular. Indeed, it has been previously reported that CD98 protein expression in human skeletal muscle remains unchanged

following EAA consumption (32), and is not elevated until 24h following resistance exercise (33), and as such any change in LAT1 expression may be have no effect AA transport. CD98 expression was not studied in the current study, so conclusions on this cannot be made. As such, future research regarding the intricate regulation of these proteins is warranted.

6.6 – Conclusions

In summary, the data presented herein display that neither the ingestion of protein-carbohydrate alone or following fatiguing resistance exercise affects Vps34 protein content or kinase activity, in spite of a strong induction of mTORC1 signalling in both conditions. In addition, we report a negative association between Vps34Ser249 and S6K1^{Thr389} suggestive of a downstream inhibitory effect of mTORC1 on autophagy-related Vps34 complexes. This provides an intriguing paradox whereby certain Vps34 complexes may contribute to mTORC1 activation following anabolic stimuli while others are inhibited by this activation, a phenomena which warrants more in-depth investigation. Finally, we observed higher protein abundance of LAT1 in type II compared to type I muscle fibres, however neither AA ingestion or resistance exercise altered the cellular localisation of LAT1.In conclusion, our data indicates that activation of Vps34 or cellular trafficking of LAT1 is not required for mTORC1 activation following AA ingestion or resistance exercise in human skeletal muscle. As such, this study provides new insight into the regulation of potential AA/nutrient sensors in human skeletal muscle following anabolic stimuli and suggests that the mechanism(s) responsible for mTORC1 activation by AA's and resistance exercise are still to be elucidated.

6.7 - References

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Chapter 7

GENERAL DISCUSSION

7.1 – Introduction

Skeletal muscle is an essential organ to the human body contributing mechanically to posture maintenance and locomotion as well as metabolically to lipid oxidation and glucose disposal. Therefore maintaining skeletal muscle mass throughout life will positively affect both health and independence (1). Skeletal muscle mass is governed by the balance of two processes, muscle protein synthesis (MPS) and muscle protein breakdown (MPB) both of which vary greatly throughout a day in response to nutrition and physical activity (2, 3). Recent research has suggested that it is MPS which has the greatest effect on Net Protein Balance (NPB), as it seems to be the more plastic process (4) and changes in this variable can account for the majority of muscle loss noted during atrophy (5). Therefore, how this intricate process is regulated at the molecular level is an important area of research.

Central to the regulation of MPS is the mechanistic target of rapamycin (mTOR), a serine/threonine kinase which forms two distinct complexes in skeletal muscle, mTORC1 and mTORC2 (6). Of the two complexes, mTORC1 is the most relevant in the regulation of MPS as its substrates stimulate translation initiation and elongation, in addition to inhibiting catabolic processes (7, 8). Both mechanical loading and protein ingestion are known to increase mTORC1 activity in human skeletal muscle (9-14), whilst inhibition of mTORC1 using the specific inhibitor rapamycin (15, 16) blocks resistance exercise or amino acid-mediated increases in MPS (15, 16). As such it seems that mTORC1 is the main regulatory protein governing MPS in human skeletal muscle. Whilst the requirement of mTORC1 activity for the MPS response in human skeletal muscle is well established, how this process is co-ordinated at the cellular level is less well understood. Therefore the aims of this thesis were:

1. To investigate how resistance exercise and/or protein-carbohydrate feeding affects mTOR localisation and protein-protein interactions in human skeletal muscle.
2. To characterise the cellular location and distribution of the leucine transporter LAT1 in human skeletal muscle in basal and anabolic scenarios.
3. To investigate the regulation of a novel AA sensor, Vps34, in human skeletal muscle *in vitro* and *in vivo*.

This chapter will provide a brief summary of the results presented in this thesis and how they relate to the current knowledge surrounding the regulation of mTORC1 in human skeletal muscle. Furthermore the limitations of the current research will be discussed and recommendations for future research provided.

7.2 – mTORC1-lysosome translocation as a mechanism of mTORC1 activation

The current, most widely accepted model of mTORC1 activation in response to AA availability revolves around the recruitment of mTOR, via Rag proteins, to the lysosomal membrane (17). Here mTOR is in close proximity both to its direct activators (Rheb and PA) as well as an abundant supply of AAs. Recently, Korolchuk et al. (18) provided evidence that in conditions of less extreme nutrient deprivation, which may be similar to that observed in postabsorptive human skeletal muscle, mTOR does not disassociate from lysosomal membranes yet becomes less active (27). Furthermore, upon AA stimulation, mTOR-lysosomal complexes were observed to translocate to the cell periphery, a process coinciding with elevated mTORC1 activity (18). The inhibition of this translocation was shown to completely ablate the effect of AA provision on mTORC1 activation, thereby

suggesting that the translocation of these complexes to the cell periphery is a central mechanism for mTORC1 activation (18).

Previous work from our laboratory has observed a similar phenomena in human skeletal muscle in response to resistance exercise and protein/carbohydrate feeding (14). However, as the immunohistochemical approach used in our previous work could not distinguish between mTOR complexes, we were unable to conclude whether the responses we observed were specific to mTORC1. Consequently, the study described in **Chapter 3**, aimed to answer the questions by utilising a unilateral exercise protocol, combined with protein-carbohydrate feeding, allowing the separation of FED vs. EXFED responses simultaneously in subjects and co-staining mTOR with Raptor and Rictor antibodies to distinguish mTORC1 and mTORC2 complexes.

Our data supported previous work in that resistance exercise combined with protein-carbohydrate feeding induced a translocation of mTOR-lysosomal complexes towards the cell membrane (identified with WGA), which was greater across the time course in this condition compared with feeding alone. Furthermore, mTOR-WGA colocalisation was elevated above baseline at 3h post exercise and protein-carbohydrate feeding, whilst returning to baseline levels with feeding alone. These elevations coincided with elevations in S6K1 kinase activity, a readout of mTORC1 activity. These findings display a synergistic effect of the combination of resistance exercise and protein feeding on mTOR translocation and may provide a mechanism as to why greater mTORC1 activation (S6K1 phosphorylation or kinase activity) is consistently reported in response to these anabolic stimuli (9, 10, 14, 19). Furthermore, the maintenance of elevated mTOR complexes at the cell membrane (identified via mTOR-WGA colocalisation) in the

EXFED condition could contribute to the purported sensitisation of skeletal muscle to subsequent protein feeding (20, 21).

In addition to these findings, we next validated antibodies to probe for the mTOR complex specific proteins Raptor (mTORC1) and Rictor (mTORC2) using mKO tissue for each target protein. These antibodies were then utilised on human tissue sections to investigate mTOR complex-specific translocation following anabolic stimuli. Rictor-WGA localisation remained unchanged in both conditions across the timecourse suggesting mTORC2 localisation does not alter in response to anabolic stimuli. In contrast, Raptor-WGA colocalisation increased to a similar extent to that of mTOR-WGA at 1h post-exercise/feeding before decreasing below baseline values only in the FED condition at the 3h time point, where a difference between conditions was noted. These data therefore suggest that mTORC1, the complex which contributes most to MPS, translocates in response to anabolic stimuli.

mTOR-lysosome translocation was also investigated in **Chapter 5** of this thesis, in response to protein-carbohydrate feeding alone. Data here corroborated that reported in **Chapter 3**, where mTOR-WGA colocalisation was elevated 1h post-feeding before returning to basal levels at 3h post-feeding. This temporal alteration in mTOR-WGA localisation again occurred in parallel to elevations is S6K1 kinase activity, reiterating the potential link between mTOR cellular location and mTORC1 activation (14, 18). Overall, these data regarding mTOR-lysosome translocation display how the observations of Korolchuk et al. (18) transfer to human skeletal muscle and extend the knowledge of how this important kinase is regulated in this tissue.

7.3 – Immunofluorescent Characterisation of LAT1

The process of AA transport is known to be essential for optimal cell function as it allows for the influx of substrates needed to synthesise new proteins (22, 23). In skeletal muscle, this process is particularly critical as AAs are needed to maintain NPB and prevent the loss of muscle mass (24). The most potently anabolic AA is believed to be leucine, which has been shown to activate mTORC1 both *in vitro* (25) and *in vivo* (9, 10). As LAT1 is the main AA transporter responsible for transporting leucine, and other BCAAs, from the bloodstream into muscle, its specific role in AA transport has garnered recent focus (26-28). The majority of previous research has focussed on investigating how anabolic stimuli affect mRNA and protein expression of LAT1 in whole muscle lysates (26-28). These investigations have produced varying results (27, 28), and this is likely in part due to the need for LAT1 to be in a specific cellular location (associated with a membrane) to be active (29). Therefore, by measuring LAT1 protein content in whole muscle lysates it is impossible to deduce whether any change actually affects AA transport. In **Chapter 4** of this thesis we attempted to circumvent this issue by developing an immunofluorescent staining protocol whereby LAT1 could visualised in human skeletal muscle.

To achieve this, we first successfully validated a LAT1 antibody through the use of LAT1 mKO tissue. Staining for LAT1 in human tissue then displayed a strong reactivity close to the periphery in most fibres and a strong intracellular reactivity only in type II fibres. The peripheral location of LAT1 was in agreement with evidence from other tissues including human placenta (30) and several tumour types (31, 32), and is congruent with LAT1s role as an AA transporter. Conversely, the pronounced fibre type difference in intracellular LAT1 expression was surprising and contrary to our hypothesis. We speculated that elevated LAT1 content in type II fibres may contribute to the greater

cross-sectional area observed in these fibres (33). Furthermore, following exercise, AA influx into type II fibres is greater than that of type I fibres (34), a discrepancy that this fibre type difference in LAT1 content may contribute to. It is possible however, that this 'intracellular' LAT1 is actually associated with organelle membranes i.e. lysosomes or endoplasmic reticulum which may require AA transport (35). We also hypothesised that a portion of this intracellular LAT1 may translocate to the cell periphery in response to anabolic stimuli in order to allow this greater post-exercise AA influx in type II fibres (34). We investigated this hypothesis further in **Chapter 6**, finding a feeding–driven reduction in type II fibre LAT1 content, however a fibre-type difference remained. This data suggests that intracellular LAT1 may be reduced following feeding in type II fibres, however total LAT1 protein content measured via immunoblot remained unchanged. We suggest further research is therefore required in order to fully understand the effects of anabolic stimuli on LAT1 protein content. Nevertheless, these data provide a valid method whereby LAT1 can be visualised in human skeletal muscle and can be used in combination with other methods in order to determine the effects of anabolic stimuli on LAT1 regulation (expression and subcellular location).

7.4 – Vps34 regulation in response to anabolic stimuli

Much mTORC1 centered research has focused on identifying upstream 'nutrient sensors' which recognise alterations in the nutrient availability within cells to initiate signalling cascades resulting in elevated mTORC1 activity (36). One purported nutrient sensor is Vps34, which was first implicated in the regulation of autophagy (37), but has since been observed to regulate many other cellular processes including cytokinesis and phagocytosis (38). The notion that Vps34 may regulate mTORC1 response to nutrients was first proposed by Byfield et al. (39) who reported that the siRNA-mediated silencing

of Vps34 completely ablated AA and insulin stimulation of mTORC1 activity in HEK293 cells. Vps34 is a class III PI3Kinase which phosphorylates phosphatidylinositol to form PI(3)P, a compound which can regulate protein trafficking (38). This importance of Vps34 activity to mTORC1 signalling has been corroborated by other groups (40, 41), again finding its a role in mTORC1's response to nutrients. In addition, it has been recently reported that Vps34 could regulate mTORC1-lysosome translocation through the production of PI(3)P (42, 43). This occurs through the recruitment of the PI(3)P binding protein FYCO1 to lysosomal membranes (43). Vps34 kinase activity has also been found to be responsive to forced contractions in rodent skeletal muscle, a response believed to be driven by changes in intracellular leucine (44). Due to the potential of Vps34 to be a nutrient sensor to mTORC1, we studied the effect of protein-carbohydrate feeding on Vps34 kinase activity, translocation and protein-protein interactions in **Chapter 5** of this thesis. In addition, we utilised the Vps34-specific inhibitor SAR405 (45) in both C2C12 and human primary myotubes to ascertain the importance of Vps34 to serum-induced mTORC1 signalling.

The use of SAR405 did not significantly reduce the serum-induced activation of mTORC1 signalling pathways in either cell type investigated. These data suggested that Vps34 kinase activity may not contribute to mTORC1 activation in this cell type. Furthermore, we found Vps34 kinase activity to be unresponsive to protein-carbohydrate feeding, which could be a result of the many different Vps34-complexes apparent in mammalian cells, many of which have opposing functions (38). Nonetheless, we did observe a translocation of Vps34 toward the cell periphery where it associated with mTOR at a time when mTORC1 activity was high. This supports previous *in vitro* data implicating Vps34 in the translocation of mTORC1 to peripheral regions of the cells (42).

Therefore, from this data it seems that it may be the location of Vps34 rather than a change in its activity that contributes to nutrient sensing in skeletal muscle.

We further investigated Vps34 regulation in **Chapter 6** of this thesis, where the effect of resistance exercise and/or protein-carbohydrate feeding on Vps34 phosphorylation and content was studied. Here, using a surrogate measure of Vps34 activity, $SGK3^{Thr320}$, we again found no effect of these anabolic stimuli on Vps34 kinase activity. These findings corroborated the only other study to investigate Vps34 kinase activity in human skeletal muscle when no change was found following sprint intervals and EAA consumption (46). Vps34 protein content was also unchanged in both conditions across the time course, again in agreement with previous human data (46). As mTORC1-related signalling was elevated in both conditions across this trial, and in response to feeding alone in **Chapter 5**, this indicates that a change in overall Vps34 kinase activity or content is not required for the induction of mTORC1-related signalling following anabolic stimuli, questioning its role as a leucine sensor in skeletal muscle. However, we did observe a negative correlation between $S6K1^{Thr389}$ and $Vps34^{Ser249}$ phosphorylation indicating a possible negative regulation of this site by mTORC1. This post-translational modification of Vps34 is believed to lie downstream of ULK1, and is suggested to contribute to autophagy induction (47). This finding is therefore intriguing as it adds a further layer of complexity to the role Vps34 could exhibit in mTORC1 regulation. Specifically, it seems anabolic Vps34 complexes may regulate mTORC1 activation by contributing to its translocation toward the cell membrane, which in turn causes mTORC1 to have a inhibitory effect on autophagy-related Vps34 complexes. In all, this thesis provides further evidence that Vps34 may contribute to nutrient sensing in human skeletal muscle, most likely through altering the translocation of mTOR-lysosome complex to the cell periphery.

7.5 – Limitations

As with any studies in the field of muscle physiology, the studies described in this thesis were conducted in a small number of participants (~8 per study) which could impact the statistical power of our analysis. Nevertheless, power calculations were conducted on the primary measure for each of the studies and the number of participants required were based on this calculation in an attempt to maintain statistical power. Nonetheless, these power calculations suggest enough statistical power for ANOVA testing, not post-hoc analysis and therefore the interpretation of such tests may be unreliable. Ethical obligations and time/equipment constraints would also have prevented the recruitment of more participants. Furthermore, a small n number (n=4) of human primary myotubes donors were utilised in **Chapter 5** of this thesis, which may have impacted results gleaned in these experiments, especially as a trend toward an effect of the Vps34 inhibitor was noted here. Unfortunately, at the time when the experiments were conducted, our laboratory did not possess ethical approval to isolate primary myoblasts from subjects and therefore could only utilise what was made available to us by collaborators. Nevertheless, the statistical power of the experiments would improve greatly from the addition of further primary myoblast donors. The characteristics of these donors (age – 60.8±6.4yrs, BMI – 28.7±0.65kg/m^2) were also different from the participants who completed the *in vivo* aspect of this study (age – 21.1±0.5yrs), and therefore it is possible that results may have differed if similar populations were investigated. Again, as our laboratory did not hold ethical approval at the time of testing for human primary myoblast isolations this was not possible. Therefore, future research should aim to elucidate the effect of SAR405 on mTORC1 signalling in human primary myotubes isolated from young, lean donors.

Another limitation of this thesis is the absence of direct MPS measurements, meaning we can only infer the induction of MPS based on the activation of mTORC1. With the current data it is impossible to elucidate how our measures relate to MPS, however we do expect to complete this analysis in the near future. We did utilise stable isotope methods in the study described in **Chapter 6** of this thesis however, time constraints and equipment malfunctions resulted in the inability for this to be completed. **Chapter 6** of this thesis included a surrogate measure of Vps34 activity, $SGK3^{Thr320}$, which is utilised *in vitro* as a read out of Vps34 kinase activity (48). A direct comparison with Vps34 kinase activity has not yet been completed in human skeletal muscle and as such there is a risk that this measure may not be valid. Kinase activity assays targeting Vps34 are expected to be completed for this study in the near future, however, again time constraints due to the need for this analysis to be completed at a different institution did not allow completion of this analysis in the thesis timeframe.

Finally, it is important to acknowledge that the use of widefield epifluorescence microscopy is not the optimal method for investigating protein-protein interactions. Although this technique is widely used to measure colocalisation/protein-protein interactions, both confocal and immune-electron microscopy techniques produce greater spatial resolution, therefore allowing more accurate analysis of colocalisation. Unfortunately, equipment, costing and time restraints meant this analysis could not be completed. In addition, the marker of the cell membrane utilised in this thesis, WGA, can also bind to sarcoglycans which may be present in intracellular regions of muscle fibres, which could have resulted in unreliable colocalisation values. Each image analysed was observed to be free of any intracellular WGA staining before analysis in order to combat this. Furthermore, as we are only inferring movement toward the cell membrane rather

than an actual association, we believe the use of this marker is valid. Future analysis could utilise a 'cleaner' marker of the cell membrane i.e. dystrophin to understand if results may differ.

7.6 – Future Research

We hypothesise that the process of mTOR-lysosomal translocation toward the cell periphery, in human skeletal muscle, following anabolic stimuli is an important event in the activation of mTORC1 following such stimuli. As such, it is important to investigate this mechanism in populations who exhibit an inability to fully activate mTORC1 in response to anabolic stimuli i.e. elderly (49) and obese (50) individuals. Future research should therefore focus on these populations and determine whether mTOR-lysosome translocation is impaired. This could provide a novel therapeutic target for future interventions aiming to minimize muscle loss and maximize muscle mass gains in these populations. In addition to investigating this mechanism in diverse populations it is also vital to further elucidate why mTOR-lysosome translocation occurs. Evidence does suggest that direct mTOR activators, translation initiation factors and blood vessels are located in the regions to which mTORC1-lysosome complexes translocate (14). Furthermore, MPS itself is believed to occur in these regions, however further investigations are warranted in order to identify where other regulators of mTORC1 signalling are located and whether mTOR associates with them following anabolic stimuli.

Due to the large amounts of intracellular LAT1 staining apparent in type II fibres, it was impossible to determine whether resistance exercise and/or protein-carbohydrate feeding elicited a translocation of this transporter to the cell membrane. It is therefore important

that a combination of methods are utilised to understand the effects of anabolic stimuli on LAT1. This could be achieved through the use of membrane/large sarcolemmal vesicle fractions, a method recently utilised to find an increase in membrane-associated LAT1 following low-load resistance exercise and pulse protein feeding (51). Recent studies have also utilised immunoblotting techniques on individual fibres to study fibre-type differences in target protein content (52). This method could be utilised to further confirm fibre type differences in LAT1 content and how anabolic stimuli may affect this. Further investigations in young, healthy individuals following a variety of anabolic stimuli should be completed using this method to elucidate if LAT1 does translocate in these scenarios. Fractionations of other organelle membranes should also be completed in an attempt to clarify the reasons behind the large amount of intracellular LAT1 in type II fibres. For example, recent *in vitro* evidence suggests LAT1 can associate with lysosomal membranes and these observations should be attempted in human skeletal muscle. Finally, utilising this immunofluorescent staining method, LAT1 expression in diverse population i.e. elderly and diseased should be investigated to better understand the potential mechanisms behind type II fibre atrophy in these populations.

Finally, investigations regarding the contribution of Vps34 to 'nutrient sensing' in human skeletal muscle is still in its infancy; to our knowledge, only 3 studies having investigated this purported sensor. Therefore, future research must continue to investigate the mechanisms by which Vps34 may contribute to nutrient sensing. One potential avenue for such research would be whether anabolic stimuli affects the recruitment of the PI(3)P binding protein FYCO1 to lysosomal membranes, as this is purported to stimulate mTOR-lysosome translocation to the periphery (43). Furthermore as in **Chapter 6** of this thesis a surrogate measure of Vps34 kinase activity was utilised, a comparison between this and

direct Vps34 kinase activity measurements should be conducted to ensure this measure is valid *in vivo*.

7.7 – Reflections

Completing my Ph.D. studies at the University of Birmingham has been an invaluable learning experience which has greatly benefitted myself in my progression into a successful researcher. The knowledge and skilled I have gained, and the contacts I have made, will no doubt prove vital in my ongoing career in research. I have learnt that research can be frustrating at times, yet these occasions are outweighed by those times where research proves to be extremely gratifying. Nevertheless, there are some aspects of the Ph.D. process which I would have done differently if beginning this process again. Firstly, I would have liked to have a greater involvement in human testing and sample collection for the studies presented in this thesis. As human testing and sample collection for the studies presented in **chapters 3-5** were conducted at other institutions or prior to my arrival this was not possible. The opportunity to be more involved with this aspect of research would certainly have improved my research profile and is something I plan to have more involvement with as I proceed in my research career.

Secondly, several parts of my analysis were conducted by 'experts' in that technique at other institutions. Although this resulted in the analysis being conducted efficiently and reliably it meant that I wasn't able to be exposed to these techniques. If beginning this process again I would attempt to visit these laboratories in order to learn these techniques so I could take them forward in my research career. Finally, a major limitation of this thesis is that some analysis planned for the final experimental chapter (**Chapter 6**) was not completed in time for the submission of this thesis. I believe that if testing for this

study had been completed at an earlier date, this analysis may have been undertaken in time. To achieve this, a greater collaborative effort could have been undertaken, however this was not possible particularly as several members of my primary supervisor's laboratory left the university during this time. Nonetheless, if starting this process again, this is an aspect which could have been improved upon.

7.8 – Conclusions

In this thesis we provided evidence that mTOR translocation following resistance exercise and/or protein-carbohydrate feeding is specific to mTORC1. Furthermore, this translocation is greater when exercise and feeding are combined compared to feeding alone and is associated with an increase in S6K1 activity. In addition, we show that following protein-carbohydrate feeding Vps34 also translocates toward the cell periphery and associates with mTOR, and again this is associated with elevated S6K1 activity. Contrary to *in vitro* and rodent data we find no effect of anabolic stimuli on Vps34 kinase activity, or protein content. Moreover, the Vps34-specific inhibitor SAR405 had no effect on serum-stimulated mTORC1 activation, suggesting Vps34's role in nutrient sensing may involve an alteration in its cellular location rather than kinase activity. Intriguingly, we identify a phosphorylation site on Vps34 which may be negatively regulated by mTORC1 and warrants further investigation, adding further complexity to the intricate mechanism of nutrient sensing. We also provide a protocol by which LAT1 can be visualised in skeletal muscle, and observe a greater expression of this transporter in type II fibres, compared to type I, which is maintained throughout the post-exercise/prandial period. Overall these data extend the knowledge base regarding the regulation of mTORC1 in human skeletal muscle, in response to anabolic stimuli, and identify new potential mechanisms by which mTORC1 may be regulated under these conditions.

7.9 – References

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