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Title: Protein-carbohydrate ingestion alters Vps34 cellular localization independent of changes in kinase activity in human skeletal muscle

Authors: Nathan Hodson
Jess R Dent
Zhe Song
Mary O'Leary
Tom Nicholson
Simon Wyn Jones
James Murray
Stewart Jeromson
Lee D Hamilton
Leigh Breen
Andrew Philp

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Running Title: Vps34 regulation in human skeletal muscle

Abstract: The Class III PI3Kinase, Vps34, has recently been proposed as a nutrient sensor, essential for activation of the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1). We therefore investigated the effects of increasing nutrient availability through protein-carbohydrate (PRO-CHO) feeding on Vps34 kinase activity and cellular localization in human skeletal muscle. Eight young, healthy males (21 {plus minus}

0.5yrs, 77.7{plus minus}9.9kg, 25.9{plus minus}2.7kg/m2, mean {plus minus} SD) ingested a PRO-CHO beverage containing 20/44/1g PRO/CHO/FAT respectively, with skeletal muscle biopsies obtained at baseline and 1h and 3h post-feeding. PRO-CHO feeding did not alter Vps34 kinase activity, but did stimulate Vps34 translocation toward the cell periphery (PRE (mean{plus minus}SD) - 0.273{plus minus}0.040, 1h - 0.348{plus minus}0.061, Pearson's Coefficient (r)) where it co-localized with mTOR (PRE - 0.312{plus minus}0.040, 1h - 0.348{plus minus}0.069, Pearson's Coefficient (r)). These alterations occurred in parallel to an increase in S6K1 kinase activity (941{plus minus}466% of PRE at 1h post-feeding). Subsequent in vitro experiments in C2C12 and human primary myotubes displayed no effect of the Vps34-specific inhibitor SAR405 on mTORC1 signalling responses to elevated nutrient availability. Therefore, in summary, PRO-CHO ingestion does not increase Vps34 activity in human skeletal muscle, whilst pharmacological inhibition of Vps34 does not prevent nutrient stimulation of mTORC1 in vitro. However, PRO-CHO ingestion promotes Vps34 translocation to the cell periphery, enabling Vps34 to associate with mTOR. Therefore, our data suggests that interaction between Vps34 and mTOR, rather than changes in Vps34 activity per se may be involved in PRO-CHO activation of mTORC1 in human skeletal muscle.

New Findings: What is the central question of the study? Is Vps34 a nutrient-sensitive activator of mTORC1 in human skeletal muscle? What is the main finding and its importance? We show that altering nutrient availability, via protein-carbohydrate feeding, does not increase Vps34 kinase activity in human skeletal muscle. Instead, feeding increased Vps34-mTORC1 co-localization in parallel to increased mTORC1 activity. These findings may have important implications in the understanding nutrient-induced mTORC1 activation in skeletal muscle via interaction with Vps34.

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Protein-carbohydrate ingestion alters Vps34 cellular localization independent of changes in kinase activity in human skeletal muscle

Nathan Hodson[1#], Jessica R. Dent[1#], Zhe Song[1], Mary F. O'Leary[1], Thomas Nicholson[2], Simon W. Jones[2], James T. Murray[3], Stewart Jeromson[4], D. Lee Hamilton[5], Leigh Breen[1], Andrew Philp[1,6,7].

1. School of Sport, Exercise and Rehabilitation Sciences, University of Birmingham, UK.
2. Institute of Inflammation and Ageing, University of Birmingham, UK.
3. Trinity Biomedical Sciences Institute, Trinity College, Dublin, Ireland.
4. Physiology, Exercise and Nutrition Research Group, School of Sport, Faculty of Health, Stirling University, Stirling, UK.
5. Institute for Physical Activity and Nutrition (IPAN), School of Exercise & Nutrition Sciences, Deakin University, Geelong 3216, Australia.
6. Garvan Institute of Medical Research, NSW, Australia.
7. St Vincent's Clinical School, UNSW Medicine, UNSW Sydney, NSW, Australia.

* Corresponding author:

Andrew Philp, Ph.D.

Mitochondrial Metabolism and Ageing Laboratory

Healthy Ageing Theme

Garvan Institute of Medical Research

384 Victoria Street, Darlinghurst, NSW, 2010, Australia

Phone: +61 (0) 292958249 Email: a.philp@garvan.org.au

[#]Authors contributed equally

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Abstract

The Class III PI3Kinase, Vps34, has recently been proposed as a nutrient sensor, essential for activation of the mechanistic target of rapamycin (mTOR) complex 1 (mTORC1). We therefore investigated the effects of increasing nutrient availability through protein-carbohydrate (PRO-CHO) feeding on Vps34 kinase activity and cellular localization in human skeletal muscle. Eight young, healthy males (21 ± 0.5yrs, 77.7±9.9kg, 25.9±2.7kg/m^2, mean ± SD) ingested a PRO-CHO beverage containing 20/44/1g PRO/CHO/FAT respectively, with skeletal muscle biopsies obtained at baseline and 1h and 3h post-feeding. PRO-CHO feeding did not alter Vps34 kinase activity, but did stimulate Vps34 translocation toward the cell periphery (PRE (mean±SD) - 0.273±0.040, 1h - 0.348±0.061, Pearson's Coefficient (r)) where it co-localized with mTOR (PRE – 0.312±0.040, 1h – 0.348±0.069, Pearson's Coefficient (r)). These alterations occurred in parallel to an increase in S6K1 kinase activity (941±466% of PRE at 1h post-feeding). Subsequent *in vitro* experiments in C2C12 and human primary myotubes displayed no effect of the Vps34-specific inhibitor SAR405 on mTORC1 signalling responses to elevated nutrient availability. Therefore, in summary, PRO-CHO ingestion does not increase Vps34 activity in human skeletal muscle, whilst pharmacological inhibition of Vps34 does not prevent nutrient stimulation of mTORC1 *in vitro*. However, PRO-CHO ingestion promotes Vps34 translocation to the cell periphery, enabling Vps34 to associate with mTOR. Therefore, our data suggests that interaction between Vps34 and mTOR, rather than changes in Vps34 activity *per se* may be involved in PRO-CHO activation of mTORC1 in human skeletal muscle.

Introduction

Amino acids (AAs) are critical to skeletal muscle plasticity, acting as both substrates in the process of muscle protein synthesis (MPS) as well as initiating the signaling pathways which activate this cellular process (Fujita et al., 2007; Witard et al., 2014; Wolfe, 2006). Carbohydrate (CHO) ingestion can also elevate MPS via insulin action (Borsheim et al., 2004), and a combination of these nutrients is believed to act synergistically on MPS following exercise (Miller et al., 2003). 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) (Dickinson et al., 2011; Drummond et al., 2009), an evolutionarily conserved serine/threonine kinase complex which stimulates translation initiation and elongation (Kim et al., 2002; Ma & Blenis, 2009; Wang et al., 2001) in response to increased nutrient provision.

The canonical mechanism by which AAs stimulate mTORC1 activity is thought to be through the elevation of mTORC1 complex co-localization with the lysosome (Sancak et al., 2010) *in vitro*, or through mTORC1/lysosomal trafficking *in vivo/vitro* (N Hodson et al., 2017; Korolchuk et al., 2011; Song et al., 2017). However, how nutrients stimulate mTORC1 activity in human skeletal muscle is still poorly understood. A potential nutrient-sensitive activator of mTORC1 is the vacuolar protein sorting 34 (Vps34), a class III PI3Kinase. The primary function of Vps34 is the production of phosphatidylinositol 3-phosphate (PI(3)P) through the phosphorylation of phosphatidylinositol (Backer, 2016), a product responsible for the recruitment of various proteins to phospholipid bilayers (i.e. plasma and lysosomal membranes) (Gillooly et al., 2001). A role for Vps34 in nutrient sensing was first proposed by Byfield et al. (Byfield et al., 2005), who reported that overexpression of Vps34 in HEK293 cells elicited a 2-fold increase in S6K1 activity, a common readout of mTORC1 activation. Conversely, siRNA targeting Vps34

abolished insulin-stimulated S6K1^{Thr389} phosphorylation (Byfield et al., 2005). Nobukuni et al. (Nobukuni et al., 2005) 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. In addition, recent *in vitro* evidence suggests that Vps34 colocalises with mTOR, close to cellular membranes, following insulin stimulation (Hirsch et al., 2014), and is required for nutrient-stimulated translocation and activation of mTORC1 (Hirsch et al., 2014). As such, Vps34 represents a novel candidate as a nutrient-sensitive activator of mTORC1.

With regard to skeletal muscle, 3h and 24h exposure to leucine (5mM) and insulin (100nM) elevated Vps34 protein content and mTOR^{Ser2448} and S6K1^{Thr389} phosphorylation in human primary myotubes (Gran & Cameron-Smith, 2011), however it is unknown if an elevated Vps34 protein content corresponds to enhanced kinase activity. However, supra-physiological levels of AA's do increase Vps34 kinase activity in C2C12 myotubes (MacKenzie et al., 2009). In addition, high frequency electrical contraction has been reported to increase Vps34 activity in rodent *Tibialis Anterior* muscle (MacKenzie et al., 2009), whereas sprint exercise and protein ingestion failed to activate Vps34 in human skeletal muscle (Rundqvist et al., 2013). Overall, such data implicates a possible role for Vps34 in nutrient/contraction sensing within skeletal muscle. However, a more detailed investigation in human skeletal muscle is required.

Therefore, our primary aim was to investigate if AA/CHO feeding could affect Vps34 activity and cellular localisation in human skeletal muscle. We hypothesised that Vps34 activity would increase in response to AA/CHO feeding in parallel to increases in mTORC1 signaling. Our secondary aim was to examine whether inhibition of Vps34 kinase activity *in vitro* with the

specific inhibitor SAR405 (Pasquier, 2015; Ronan et al., 2014) would attenuate nutrient-activation of mTORC1.

Methods

Ethical Approval

Ethical approval for the current study was obtained from NHS West Midlands Black Country Research Ethics Committee (15/WM/0003) and conformed to the standards set out in the Declaration of Helsinki (7th version), other than registration in a database. All participants provided written informed consent prior to participation in the study.

Participants

Eight young, healthy males (age – 21 ± 1.3yrs, weight – 77.7±9.9kg, BMI – 25.9±2.7kg/m^2, mean ± SD, n=7) volunteered to partake in the current study. All participants were considered healthy (as assessed by a general health questionnaire) and recreationally active (~3 exercise sessions per week) but not involved in a structured exercise training program. Exclusion criteria encompassed current cigarette smokers, recreational drug users (including anabolic steroids), the presence of neuromuscular disease and any medication/condition that may affect nutrient digestion/absorption i.e. inflammatory bowel disease.

Study Design

On the day of the experimental trial, participants reported to the laboratory following an overnight fast (~10h) and having refrained from strenuous exercise and alcohol consumption in the prior 48h. Upon arrival, participants were placed in a supine position and a 21G cannula was inserted into the antecubital vein of one arm to allow for repeated blood sampling. At this point

an initial baseline blood sample was obtained from all participants. A skeletal muscle biopsy sample was then taken from the *vastus lateralis* of a randomised leg using the Bergstrom percutaneous needle technique, modified for suction (Tarnopolsky et al., 2011). Participants then consumed a commercially available protein-carbohydrate beverage (Gatorade Recover®, Gatorade, Chicago, IL, USA.) providing 20/44/1g of protein, carbohydrate and fat respectively (0.26±0.03g/kg protein, 0.57±0.07g/kg carbohydrate, n=7). Further venous blood samples were taken every 20 minutes for a 3h post-prandial period and subsequent skeletal muscle biopsy samples were obtained at 1h and 3h following beverage ingestion. Muscle samples were blotted free of excess blood and dissected free of any excess adipose and connective tissue, then immediately frozen in liquid nitrogen and stored at -80ºC until analysis. A separate piece of muscle tissue was placed in optimal cutting temperature (OCT) compound (VWR, Lutterworth, UK.) and frozen in liquid nitrogen-cooled isopentane before storage at -80ºC. Blood samples were collected into EDTA-coated vacutainers (BD, Franklin Lakes, NJ, USA.) and then centrifuged at 1000g for 15min to separate plasma. Plasma was then aliquotted into micro-centrifuge tubes and stored at -80ºC until analysis. The experimental design is depicted in Figure 1.

Blood analyses

Plasma insulin concentrations were quantified using a commercially-available ELISA kit (IBL International, Hamburg, Germany.) as per the manufacturer's instructions. Plasma leucine concentrations were determined via gas chromatography-mass spectrometry (GC-MS) using an internal standard method, as previously described (McKendry et al., 2016), following the conversion of plasma free amino acids to their N-tert-butyldimethyl-silyl-N-methyltrifluoracetamide (MTBSTFA) derivative.

S6K1 and AKT Kinase Activity Assays

S6K1 and AKT kinase activity assays were conducted as described previously (McGlory et al., 2014) with the following antibodies; S6K1 – SCBT no.2708 (Santa Cruz Biotechnologies, Dallas, TX, USA.) & AKT (DSTT, Dundee, UK). Briefly, a ~30mg piece of muscle tissue was homogenized on ice in RIPA buffer (50 mmol/l Tris·HCl pH 7.5, 50 mmol/l NaF, 500 mmol/l NaCl, 1 mmol/l sodium vanadate, 1 mmol/l EDTA, 1% (vol/vol) Triton X-100, 5 mmol/l sodium pyrophosphate, 0.27 mmol/l sucrose, and 0.1% (vol/vol) 2-mercaptoethanol and Complete protease inhibitor cocktail (Roche)). Cellular debris was then removed via centrifugation at 13000g for 15min (4ºC). Protein concentrations of samples was then determined via bicinchoninic acid (BCA) protein assay. Immunoprecipitation of the target protein was then conducted on 200µg protein for 2h at 4ºC, with agitation, in homogenization buffer (50 mM Tris·HCl pH 7.5, 0.1 mM EGTA, 1 mM EDTA, 1% (vol/vol) Triton X-100, 50 mM NaF, 5 mM NaPPi, 0.27 M sucrose, 0.1% -mercaptoethanol, 1 mM Na3(OV)4, and 1 Complete (Roche) protease inhibitor tablet per 10 mL) combined with 2.5µL Protein G Sepharose beads and appropriate antibody. Immunoprecipitates were subsequently washed twice in high-salt buffer (homogenization buffer with 0.5M NaCl added) and once in assay buffer (50 mM Tris·HCl pH 7.4, 0.03% Brij35, and 0.1% -mercaptoethanol). Immunoprecipitates were then resuspended in 10µL assay buffer and activity assay commenced every 20 seconds through the addition of a assay mix (assay buffer + 100µM ATP + 10mM $MgCl_2$ + 32γATP + synthetic substrate (S6tide - KRRRLASLR at 30 µM & Crosstide - GRPRTSSFAEG at 30µM for S6K1 and AKT assays respectively). Every 20s reactions were stopped through spotting on to chromatography paper, immersion in 75mM phosphoric acid and drying. Chromatography paper was immersed in GoldStar LT Quinta Scintillation fluid (Meridian Biotechnologies, Chestefield, UK) and spots

were counted in a Packard 2200CA TriCarb Scintillation Counter (United Technologies) as fmol·min^{-1}·mg^{-1}.

Vps34 Kinase Activity Assay

Vps34 kinase activity assays were conducted as previously described (MacKenzie et al., 2009) from 25mg muscle homogenized in Cantley lysis buffer. Vps34 was immunoprecipitated overnight at 4ºC from tissue lysates containing ~1mg total protein using 2µg anti-Vps34 antibody (sheep antibody produced by Dr. James T. Murray, Trinity College Dublin) before immobilisation on Protein G Sepharose beads for 1h. Immunoprecipitates were then washed 3 times in Cantley lysis buffer (10 mM Tris pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton-X 100, 10% glycerol, 100 mM NaF), once in Tris-LiCl (10 mM Tris, pH 7.5, 5 mM LiCl, 0.1 mM Na2VO4) and twice in TNE (10 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 0.1 mM Na2VO4) and then resuspended in 60 µl TNE+ (TNE, 0.5 mM EGTA, pH 8.0, 1 : 1000 2-mercaptoethanol). Samples were then incubated with 20µg Vps34 antigen peptide for 10min before 10µl of 30 mM MnCl2 and 10µl of 2 mg/ml phosphoinositol were added to provide substrate for the reaction. Reactions then commenced through the addition of 5µL assay buffer (400 µM unlabelled ATP, 12.5 µCi of 32γATP, 4.3 µl water) for 10 minutes at 30ºC. Reactions were terminated by the addition of 20µL 8M HCl, phase separated using 1:1 chloroform and methanol and the lower phase spotted on to an aluminium-backed 60 A silica Thin Layer Chromatography (TLC) plate (Merck, Damstadt, Germany). This was then run in a TLC chamber solvent system to determine 32γP transfer to substrate. This kinase activity assay has previously been utilized in both rodent (MacKenzie et al., 2009) and human (Rundqvist et al., 2013) skeletal muscle tissue, and is able to detect physiological alterations in Vps34 kinase activity. In addition, previous studies have validated the specificity of this assay through the

absence of phosphorylation when Phosphatidylinositol 4-phosphate and Phosphatidylinositol 4,5-bisphosphate were used as substrates (MacKenzie et al., 2009).

Immunohistochemistry

Immunohistochemical analysis was conducted as described previously (Song et al., 2017). In short, 5µm sections of muscle tissue were sectioned at -25ºC using a Bright 5040 Cryostat (Bright Instrument Company Ltd., Huntingdon, UK) and transferred to room temperature (RT) glass slides (VWR international, UK) and allowed to airdry for ~1h. Sections from each time point for each participant were sectioned onto the same slide in duplicate to remove slide-to-slide variation during analysis. Muscle sections were subsequently fixed in a 3:1 solution of acetone and ethanol, washed 3 times in Phosphate Buffered Saline (PBS) before incubation in relevant primary antibodies (antibodies and dilutions in Table 1) diluted in 5% Normal Goat Serum (NGS) to prevent non-specific secondary binding for 2h at RT. Subsequently, sections were again washed in PBS and then incubated in corresponding secondary antibodies (details in Table 1) for 1h at RT. Following further washes, slides were then incubated in Wheat Germ Agglutinin (WGA – conjugated to 350nm fluorophore) for 30min at RT in order to mark the sarcolemmal membrane. After a final wash in PBS, slides were then mounted in Mowiol® 4–88 (Sigma-Aldrich, Poole, UK) to protect fluorophores and a glass coverslip was applied. Slides were then left to dry overnight in a dark cabinet prior to image capture. All primary antibodies other then Vps34 have previously been validated for specificity by our group (Song et al., 2017). Pilot stains were also conducted with and without the presence of the Vps34 primary antibody to ensure no non-specific binding of the secondary antibody.

Image Capture and Analysis

158 Stained muscle sections were observed under a Nikon E600 widefield microscope using a
159 40x0.75NA objective under three colour filters achieved by a SPOT RT KE colour three shot
160 CCD camera (Diagnostic Instruments Inc., MI, USA) illuminated by a 170W Xenon light source.
161 In the current study, UV (340-380nm) excitation filter was utilized to visualize WGA, TxRed
162 (540-580nm) for mTOR visualization and FITC (465-495nm) for LAMP2 or Vps34 depending
163 on the stain conducted. For each time point, approximately 8 images were taken per section, each
164 consisting of ~8 muscle fibers. As sections were analysed in duplicate, approximately 130
165 muscle fibres per time point per participant were included in analysis. Image processing and
166 quantification was completed on ImageProPlus 5.1 software (Media Cybernetics, MD, USA.)
167 with all variables kept consistent for all sections on a given slide and investigators blinded to
168 conditions throughout. Prior to co-localisation analysis, all images underwent a no neighbour
169 deconvolution algorithm as a filter. Pearson's correlation coefficient (Image-Pro software) was
170 used to quantify co-localization of proteins stained in different channels. This method of
171 assessing co-localization was utilized as it measures co-localization on a pixel-by-pixel basis and
172 is relatively free of user bias (Dunn et al., 2011).

173 *In vitro experiments*

174 C2C12 myoblasts were purchased from American Type Culture Collection (ATCC, Manassas,
175 VA, USA.) and cultured on 150mm culture plates in high glucose Dulbecco's minimum essential
176 medium (DMEM, ThermoFisher Scientific, Waltham, MA, USA.) supplemented with 10%
177 foetal bovine serum (FBS, Hyclone, VWR, Lutterworth, UK.) and 1% penicillin-streptomycin
178 (PS, ThermoFisher Scientific). When 80% confluent, cells were trypsinized (0.05% Trypsin-
179 EDTA, ThermoFisher Scientific) and seeded onto 6-well plates at a density of $2x10^5$cells/well.
180 Myoblasts were then cultured until ~95% confluency (~36h) at which time media was changed

to elicit differentiation of myoblasts to myotubes (DMEM supplemented with 2% horse serum (HS, Hyclone, VWR) and 1% PS). Differentiation was allowed to occur for 5 days, with media replaced every other day, until myotubes were fully formed.

At this point, myotubes were nutrient deprived in Earl's Balanced Salt Solution (EBSS, ThermoFisher Scientific) for ~14h, with a subset of myotubes maintained in DMEM (2%HS, 1%PS) to serve as a 'baseline' condition. Following nutrient deprivation, a subset of myotubes were collected and the remaining myotubes were split into 2 conditions, serum recovery and serum recovery + Vps34 inhibition. Vps34 inhibition was achieved via the addition of the specific Vps34 inhibitor SAR405 (10µM) for 1h prior to serum recovery, a concentration and incubation time previously shown to fully inhibit Vps34 kinase activity *in vitro* (Ronan et al., 2014). Serum recovery occurred through the removal of EBSS and addition of DMEM (2%HS) for 30min prior to collection. Before collection, myotubes were washed twice in ice-cold PBS, before being scraped into 150 µL ice-cold sucrose lysis buffer (50 mM Tris, 1 mM EDTA, 1 mM EGTA, 50 mM NaF, 5 mM $Na_4P_2O_7$-$10H_2O$, 270 mM sucrose, 1 M Triton-X, 25 mM β-glycerophosphate, 1µM Trichostatin A, 10 mM Nicatinamide, 1 mM 1,4-Dithiothreitol, 1% Phosphatase Inhibitor Cocktail 2 (Sigma), 1% Phosphatase Inhibitor Cocktail 2 (Sigma), 4.8% cOmplete Mini Protease Inhibitor Cocktail; (Roche). Lysates were then immediately frozen in liquid nitrogen and stored at -80ºC until analysis. Three experiments were conducted at 3 separate passage numbers equalling n=9 for statistical analysis.

Human primary myoblasts were isolated from 4 patients (age 61±6yrs, BMI 28.7±0.65kg/m^2, mean±SD) as previously described (O'Leary et al., 2017). Cells were passaged at 60% confluency on 0.2% gelatin-coated 100mm culture plates in Hams F10 media (ThermoFisher Scientific, supplemented with 20% FBS and 1% PS) to prevent spontaneous fusion of myoblasts

to myotubes, and at passage 3 were seeded onto 6-well plates at a density of $5x10^4$ cells/well. Myoblasts were then cultured to 80-90% confluency, at which time media was changed to induce differentiation to myotubes (F10 supplemented with 6% HS and 1% PS). Once myotubes were fully formed (6-10days), experiments were conducted in a similar fashion to those described above for C2C12 myotubes with certain alterations. Baseline conditions for human primary myotubes were ~14h incubation in Hams F10 media (20%FBS, 1%PS) and serum recovery experiments were conducted for 30min in Hams F10 (20%FBS, 1%PS) following ~14h EBSS incubation. All other experimental variables were consistent between C2C12 and human primary experiments and cells were collected in an identical fashion. Experiments were run in triplicate for myotubes isolated from each patient and the mean of these results utilized for statistical analysis.

Cell lysates were subsequently homogenised by sonication (3x15s at 50% maximal wattage) and centrifuged at 8000g for 10mins at 40ºC to remove insoluble material. Protein content of these lysates was then determined by DC protein assay (BioRad, Hercules, CA, USA.) and samples were diluted to a desired protein concentration in 1x Laemmli sample buffer and boiled at 95ºC for 5 minutes to denature proteins.

Immunoblotting

Immunoblotting analysis was conducted as described previously (Stocks et al., 2019). Briefly, equal amounts of protein were loaded into 8-15% polyacrylamide gels and separated by SDS-PAGE. Proteins were then transferred to BioTrace NT nitrocellulose membranes (Pall Life Sciences, Pensacola, FL, USA.) and stained with Ponceau S as a loading control. Membranes were then blocked in 3% skimmed-milk diluted in Tris-buffered Saline with tween (TBST) for

1h at RT. Following washing in TBST, membranes were then incubated overnight in relevant primary antibodies, subsequently washed again and incubated in corresponding HRP-conjugated secondary antibodies (anti-rabbit IgG #7074, Cell Signaling Technologies (CST), Danvers, MA, USA. 1:10000). Enhanced chemiluminescence HRP detection kit (Merck-Millipore, Watford, UK.) was used to quantify antibody binding. Each phosphorylated protein visualized was expressed in relation to its total protein content, after each target had been normalized to a loaded control (Ponceau). All primary antibodies utilized for immunoblotting were purchased from CST and diluted at 1:1000 in TBST unless stated otherwise: 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) and p-4EBP1$^{Thr37/46}$ (#9459).

Statistical analysis

Alterations in enzyme kinase activity, protein-protein colocalization, plasma insulin and plasma leucine concentrations were analysed utilizing a repeated measures analysis of variance (ANOVA) with one within-subject factor (time). Changes in phosphorylation status of proteins in human primary myotubes was also analysed with a repeated measures ANOVA with one within-subject factor (condition). A one-way ANOVA with one between-subject factor (condition) was used to analyse changes in phosphorylation status of proteins in C2C12 myotubes. Greenhouse-Geisser corrections were applied to F values if data did not pass Mauchly's test of sphericity. If a significant main effect was found, *post-hoc* analysis was conducted on comparisons determined *a priori* with the Holm-Bonferroni correction for multiple comparisons. Significance for all variables was set at $p<0.05$ and data are presented as mean±SD unless otherwise stated.

Results

Blood Analyses

For plasma insulin analysis, physiological results from 7 out of 8 participants were obtained and, as such, statistical analysis was completed on n=7. A significant time effect was observed for changes in plasma insulin concentrations ($p<0.001$), however, following *post hoc* analysis no differences between individual time points were noted ($p>0.05$, Figure 2A). A significant time effect was also observed for plasma leucine concentrations ($p<0.001$). Plasma leucine concentrations were elevated above basal levels at 20min post-feeding (0.113±0.018 vs. 0.187±0.047mmol/L, $p=0.02$, Figure 2B) and remained above baseline (all $p<0.012$) until 160min post-feeding (0.118±0.017, $p=0.51$).

Kinase Activity Assays

A significant time effect was observed for S6K1 activity ($p=0.001$), with S6K1 activity significantly higher 1h post-feeding compared to PRE and 3h post-feeding (1h – 941±466% of PRE, 3h – 149±65.6% of PRE, $p=0.003$ & $p=0.004$ respectively, Figure 2C). Activity of S6K1 also trended toward being greater at 3h post-feeding compared to PRE ($p=0.07$, Figure 2C). A significant time effect was also apparent for AKT activity ($p=0.05$, Figure 2D). Following *post hoc* analysis, however, no differences in AKT activity between individual time points was apparent (1h – 207±132% of PRE, 3h – 118±43.8% of PRE, $p>0.05$, Figure 2D). Finally, no differences in Vps34 activity were noted at any time point (1h - 124±31.4% of PRE, 3h – 123±30.1% of PRE, $p>0.05$, Figure 2E).

Co-localization

No time effect for mTOR co-localization with LAMP2 (lysosomal marker) was found ($p=0.347$, Figure 3B) suggesting these proteins are co-localized independently of a nutritional stimulus. A significant time effect was observed for mTOR co-localization with WGA (membrane marker, $p=0.026$). Following feeding, mTOR-WGA co-localization increased by 17% at 1h before returning to basal values by 3h, however, following *post hoc* analysis, no alterations were significant (PRE – 0.181±0.021, 1h – 0.212±0.041, 3h – 0.184±0.027, PRE vs. 1h $p=0.090$, 1h vs. 3h $p=0.067$, Figure 3C). Vps34 co-localization with WGA exhibited a trend toward a time effect ($p=0.053$) and subsequent *post hoc* analysis revealed that co-localization was greater 1h post-feeding compared to PRE feeding levels (0.347±0.060 vs. 0.273±0.040, $p=0.043$, Figure 4B), however no other differences were apparent ($p>0.05$). Finally, there was a significant effect of time observed for mTOR co-localization with Vps34 ($p=0.045$). Here, following *post hoc* analysis no differences between individual time points were apparent ($p>0.05$), although a trend toward a greater mTOR-Vps34 co-localization 1h post-feeding compared to 3h was noted (0.348±0.069 vs. 0.315±0.044, $p=0.067$, Figure 4C).

In vitro experiments

In C2C12 myotubes, a significant effect of treatment was found for $S6K1^{Thr389}$ phosphorylation ($p<0.001$, Figure 5A). Here, nutrient/serum withdrawal significantly attenuated $S6K1^{Thr389}$ phosphorylation compared to baseline levels (34% reduction, $p<0.001$), whereas phosphorylation was elevated by 65% and 50% in serum recovery (SR) and SR+SAR405 treatments respectively (both $p<0.001$, Figure 5A) with no difference between these two conditions ($p=0.26$). A treatment effect was also noted for $4EBP1^{Thr37/46}$ phosphorylation ($p<0.001$), however subsequent *post hoc* analysis revealed nutrient/serum withdrawal only significantly altered phosphorylation

compared to baseline (~28% reduction, p=0.015, Figure 5B). Again, no difference between SR and SR+SAR405 was observed (p=0.57).

A significant treatment effect was also noted for both RPS6$^{Ser235/236}$ and RPS6$^{Ser240/244}$ phosphorylation (both p<0.001). RPS6$^{Ser235/236}$ phosphorylation was significantly reduced by nutrient/serum withdrawal (33%, p<0.001, Figure 5C), whereas SR elicited a significant elevation in RPS6$^{ser235/236}$ phosphorylation above baseline levels (32.7% increase, p=0.038, Figure 5C). A trend toward SR+SAR405 eliciting an elevation in RPS6$^{Ser235/236}$ phosphorylation, compared to baseline, was also observed (29.5% increase, p=0.05) with no difference between the response of this treatment compared to SR (p=0.83). Following *post hoc* analysis of RPS6$^{Ser240/244}$ phosphorylation, only serum/nutrient withdrawal altered phosphorylation status in relation to baseline (18% reduction, p<0.001, Figure 5D). No difference between SR and SR+SAR405 was observed (p=0.80). Representative immunoblots are displayed in Figure 5E.

In human primary myotubes, a significant treatment effect was noted for S6K1^{Thr389} phosphorylation (p<0.001). Here, serum/nutrient withdrawal reduced S6K1^{Thr389} phosphorylation by ~70% compared to baseline (p=0.026, Figure 6A). SR and SR+SAR405 both elevated S6K1^{Thr389} phosphorylation above baseline levels (92% & 54%, p=0.026 & 0.035 respectively. Figure 6A). A trend for a greater response in SR, compared to SR+SAR405, was also observed (p=0.069). A treatment effect for 4EBP1$^{Thr37/46}$ phosphorylation was also observed (p=0.004), however, following *post hoc* analysis no differences in 4EBP1$^{Thr37/46}$ phosphorylation between individual treatment conditions was apparent (p>0.05, Figure 6B). Significant treatment effects were also observed for RPS6$^{Ser235/236}$ and RPS6$^{Ser240/244}$ phosphorylation, however *post hoc* analysis for both these variables did not reveal differences between individual treatments

(p>0.05, Figures 6C & 6D respectively). Representative immunoblots are displayed in Figure 6E.

Discussion

The class III PI3Kinase, Vps34, has been proposed as a nutrient/amino acid sensitive regulator of mTORC1 activity (4, 10, 22). To examine Vps34 action in human skeletal muscle, we examined changes in Vps34 activity and cellular localization following PRO-CHO ingestion *in vivo* and assessed the effect of the Vps34 inhibitor SAR405 on anabolic responses to nutrient availability *in vitro* in C2C12 and human primary myotubes. We observed that PRO-CHO ingestion altered Vps34 localization, promoting translocation to the cell periphery and co-localization with mTORC1. Of note, these changes occurred independent of alterations in Vps34 kinase activity. In parallel, our *in vitro* studies demonstrated that the Vps34 specific inhibitor SAR405 did not affect nutrient stimulated mTORC1-related signaling. Together, these observations suggest a change in Vps34 cellular location, rather than an increase in kinase activity, may contribute to mTORC1 nutrient sensing in human skeletal muscle (Figure 7). However, the presence of a Vps34 specific inhibitor does not prevent nutrient stimulation of mTORC1 *in vitro*.

The finding that PRO-CHO ingestion did not significantly increase Vps34 kinase activity was contrary to our hypothesis and contrasts with previous studies (Byfield et al., 2005; Nobukuni et al., 2005). Previously, it has been shown that high-frequency electrical stimulation, a potent stimulator of mTORC1 activity, elevated Vps34 kinase activity in rodent skeletal muscle, a response suggested by the authors to be mediated by contraction-induced elevations in intracellular leucine (MacKenzie et al., 2009). Given the increase in plasma leucine reported in the current study, we would expect our feeding protocol to result in similar increases in

intramuscular leucine (Apro et al., 2015). In human skeletal muscle, there is only one previous study to have assessed Vps34 kinase activity (Rundqvist et al., 2013). Sprint exercise combined with PRO-CHO 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. Importantly, in combination with our findings, this suggests that Vps34 kinase activity is not solely activated by leucine in human skeletal muscle and may suggest that a contraction stimulus is needed to activate this kinase. However, as this is only the second study to investigate Vps34 kinase activity in human skeletal muscle, it is not to possible to know if the ~24% increase we observed here is physiologically relevant. As such further investigations regarding Vps34 kinase activity in human skeletal muscle are required.

In an attempt to further clarify the role of Vps34 in mTORC1 activation in skeletal muscle, we completed *in vitro* experiments in both C2C12 and human primary myotubes, utilising the Vps34 specific inhibitor SAR405 (Ronan et al., 2014). In support of our findings *in vivo*, we observed no effect of SAR405 administration on mTORC1-related signaling responses to serum recovery in C2C12 or human primary myotubes, suggesting Vps34 kinase activity may not be necessary for downstream mTORC1 signaling. One limitation of this approach was that we did not validate the efficacy of SAR405 on Vps34 kinase activity in our hands. Instead, we used experimental conditions previously reported to consistently abolish Vps34 kinase activity in HeLa and lung/renal carcinoma cell lines (H1299 and ACHN cells) (Pasquier, 2015; Ronan et al., 2014), as no investigations using SAR405 in muscle cells have been conducted. Nevertheless, we assume that Vps34 kinase activity was inhibited using SAR405 in our *in vitro* experiments. It is also important to acknowledge that our in vitro experiments were not designed to mimic our *in vivo* study, but to test if the presence of a Vps34 specific inhibitor would prevent or reduce

markers of mTORC1 activity in response to an anabolic stimulus. As such, our data indicates that downstream mTORC1 signalling is not affected by SAR405, a purported Vps34 kinase inhibitor.

Recent work from our lab (N Hodson et al., 2017; Song et al., 2017), and others (Korolchuk et al., 2011) suggests that mTORC1 activation in skeletal muscle involves the translocation of mTOR-lysosome complexes to peripheral regions of the cell (Nathan Hodson & Philp, 2019). As such, it seems mTOR-lysosomal translocation is an integral component of mTORC1 activation (Korolchuk et al., 2011) which leads to elevations in rates of muscle protein synthesis (Dickinson et al., 2011). Furthermore, we have displayed that this translocation is specific to mTORC1, rather than mTORC2, in response to anabolic stimuli in human skeletal muscle (N Hodson et al., 2017). Here, we report a similar process by which mTOR-LAMP2 co-localize in the fasted state, prior to mTOR-LAMP2 complex translocation post PRO-CHO ingestion. Vps34 has previously been implicated in mTOR translocation *in vitro*, where it is required for the recruitment of mTOR to lamellipodia (cellular projections of motile cells) in response to insulin stimulation, co-localizing with mTOR in these regions (Hirsch et al., 2014). In the current study, we also found Vps34 translocation toward the cell periphery following nutrient provision, with a trend toward a time effect noted for Vps34-WGA co-localization ($p=0.053$). In this context, Vps34-WGA co-localization increased significantly above basal fasted levels 1 hour post-feeding ($p=0.043$) before returning to basal fasted levels 3 hours post PRO-CHO ingestion. Therefore, our observation that Vps34 translocation, and localization with mTORC1, occurs in human skeletal muscle indicates that Vps34 may act as a scaffold for mTORC1 recruitment toward the cell periphery, with an increase in Vps34 kinase activity not required for this process. It is also possible that this translocation of Vps34 directs its basal kinase activity to where it is

required and therefore an increase in kinase activity itself is not needed. However, based on our *in vitro* data, the presence of a Vps34 inhibitor does not affect mTORC1 activation in response to nutrients. That said, further investigations are still required to determine the complete role of Vps34 localisation and/or kinase activity in mTORC1 activation *in vivo*.

From the current data it is not possible to conclude whether Vps34 and mTORC1 translocate in tandem or independently before co-localizing, or the physiological relevance of these events in human skeletal muscle. We are also unable to delineate whether the translocation events we observe are a result of the CHO or AAs within the beverage. Both insulin and AAs have been observed to independently elevate Vps34 kinase activity (Byfield et al., 2005; Nobukuni et al., 2005), and initiate the translocation of the kinase to peripheral regions (Hirsch et al., 2014; Hong et al., 2017), *in vitro*. Therefore, we decided to include both CHO and AAs within the beverage in the current study to provide a greateranabolic stimulus. A potential mechanism as to how Vps34 may regulate mTORC1 translocation and activation has recently been proposed by Hong and colleagues (Hong et al., 2017) who suggested that the product of Vps34 kinase activity, PI(3)P, may regulate lysosomal positioning via its receptor, FYCO1 (Hong et al., 2017). In this model, AAs increase the association between FYCO1 and lysosomes, whereas the ablation of this protein caused the clustering of mTOR-positive lysosomes to perinuclear regions and attenuated mTORC1 activity irrespective of nutrient availability (13). Other potential mechanisms as to how Vps34 may regulate mTORC1 activity include via Tuberous Sclerosis Complex 2 (TSC2) ubiquitination (Mohan et al., 2016) and leucyl t-RNA synthetase (LRS)-regulated mTORC1 activation (Yoon et al., 2016), however each of these processes require further investigation to determine their relevance for mTORC1 activity in skeletal muscle.

In conclusion, we report that PRO-CHO ingestion does not increase Vps34 activity in human skeletal muscle, whilst the administration of a Vps34-specific inhibitor *in vitro* does not prevent nutrient stimulation of mTORC1. However, PRO-CHO ingestion did promote Vps34 translocation to the cell periphery, where Vps34/mTOR co-localize. Therefore, our data suggests that cellular trafficking of Vps34 may result from increased PRO-CHO availability and occur in order to increase Vps34 association with mTOR. Future research studying the effects of resistance exercise, independently or in combination with AA/CHO ingestion may be required to fully understand the role of Vps34 in nutrient sensing and skeletal muscle anabolism.

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Authors Contributions

N.H. & A.P. conceived the study. J.R.D. Z.S. L.B. & A.P. designed and conducted *in vivo* experiments. N.H. conducted and completed analysis for all *in vitro* experiments. N.H. J.R.D. Z.S. S.J. D.L.H. J.T.M. M.F.O. T.N. & S.W.J. performed analysis. N.H. completed data processing and statistical analysis. N.H. J.R.D. & A.P. drafted the manuscript. All authors edited

the manuscript, approved the final version and accept responsibility for the work presented herein.

Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure Legends

Figure 1. Schematic of Experimental Protocol for Human Trial.

Figure 2. The effect of protein-carbohydrate feeding on plasma insulin and leucine concentrations and enzyme kinase activities. Insulin concentrations (A) are presented as µU/ml and Leucine concentrations (B) are presented as mM. Kinase activity of S6K1 (C), AKT (D) and Vps34 (E) are presented as % of PRE. For A & B, Ψ denotes a significant effect of time ($p<0.05$) and *denotes a significant difference at this time point compared to 0 ($p<0.05$). For C, D & E *denotes a significant difference at this time point compared to PRE ($p<0.05$), $^{\#}$denotes a significant difference at this time point compared to 3h ($p<0.05$) and Ψ denotes a significant effect of time ($p<0.05$). All values are presented as mean±SD. Data analyzed on SPSS using Repeated Measures ANOVA with Holm-Bonferroni *post hoc* comparisons conducted on Microsoft Excel. Insulin – n=7, Leucine – n=8. Kinase acitivies – n=8

Figure 3. The effect of protein-carbohydrate ingestion on mTOR-LAMP2 and mTOR-WGA co-localization. Representative images of mTOR (red), LAMP2 (green) and WGA (blue) stains at each time point are provided (A). Quantification of mTOR-LAMP2 (B) and mTOR-WGA (C) co-localization is presented as Pearson's correlation coefficient. Data in B and C are presented as mean±SD. Ψ denotes a significant effect of time ($p<0.05$). Data analyzed on SPSS using Repeated Measures ANOVA with Holm-Bonferroni *post hoc* comparisons conducted on Microsoft Excel. All analyses – n=8.

Figure 4. The effect of protein-carbohydrate ingestion on mTOR-VPS34 and mTOR-WGA co-localization. Representative images of mTOR (red), VPS34 (green) and WGA (blue) stains at each time point are provided (A). Quantification of VPS34-WGA (B) and mTOR-VPS34 (C) co-localization is presented as Pearson's correlation coefficient. Data in B and C are presented as mean±SD. Ψ denotes a significant effect of time ($p<0.05$). *denotes a significant difference at this time point compared to PRE ($p<0.05$). Data analyzed on SPSS using Repeated Measures ANOVA with Holm-Bonferroni *post hoc* comparisons conducted on Microsoft Excel. All analyses – n=8.

Figure 5. The effects of serum/nutrient withdrawal (~14h) and subsequent serum recovery (30 min), +/- SAR405, on anabolic signalling in C2C12 myotubes (n=9/group). S6K1^{Thr389} (A), 4EBP1$^{Thr37/46}$ (B), RPS6$^{Ser235/236}$ (C) and RPS6$^{Ser240/244}$ (D) phosphorylation were quantified in relation to their total proteins and ponceau staining was used as a loading control. Representative images are also provided (E). Data is presented in relation to baseline as Mean±SD. *denotes a significant difference in this treatment compared to B ($p<0.05$). Data analyzed on SPSS using One-Way ANOVA with Holm-Bonferroni *post hoc* comparisons conducted on Microsoft Excel. All analyses – n=9. B = Baseline, SW = Serum Withdrawal & SR = Serum Recovery.

Figure 6. The effects of serum/nutrient withdrawal (~14h) and subsequent serum recovery (30 min), +/- SAR405, on anabolic signalling in human primary myotubes (n=4). S6K1^{Thr389} (A), 4EBP1$^{Thr37/46}$ (B), RPS6$^{Ser235/236}$ (C) and RPS6$^{Ser240/244}$ (D) phosphorylation were quantified in relation to their total proteins and ponceau staining was used as a loading control. Representative images are also provided (E). Data is presented in relation to baseline as Mean±SD. *denotes a significant difference in this treatment compared to B ($p<0.05$). Ψ denotes a significant effect of treatment ($p<0.05$). Data analyzed on SPSS using Repeated Measures ANOVA with Holm-Bonferroni *post hoc* comparisons conducted on Microsoft Excel. All analyses – n=4. B = Baseline, SW = Serum Withdrawal & SR = Serum Recovery.

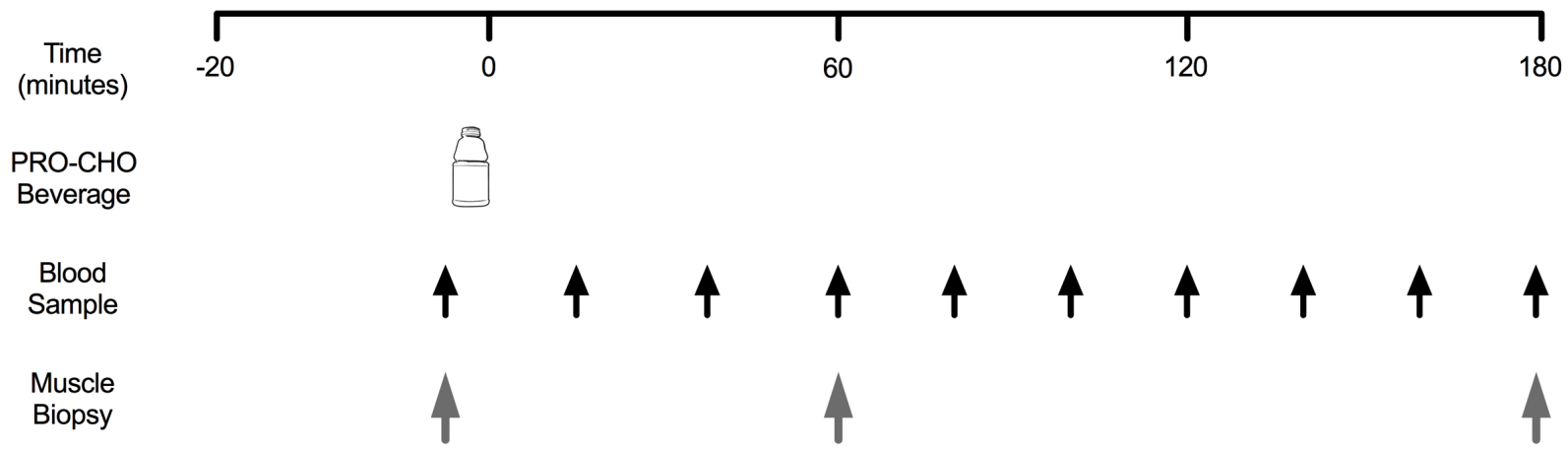
Figure 7. Proposed mechanism of Vps34 nutrient sensing. In times of low nutrient availability e.g. the post absorptive state, mTORC1 and Vps34 reside independently in the cytosol, with mTORC1 associated with the lysosome. As a result, mTORC1 activity is low whereas Vps34 is active, producing phosphatidylinositol-3-phosphate (PI(3)P). When nutrient availability is high e.g. following PRO-CHO feeding, intramuscular amino acid concentrations rise and elevated plasma insulin stimulates insulin-related intramuscular signalling pathways. This elicits translocation of mTORC1-lysosome complexes and Vps34 to peripheral regions where they colocalize. This results in elevated mTORC1 activity which stimulates protein synthesis, whereas Vps34 activity is maintained at pre-feeding levels.

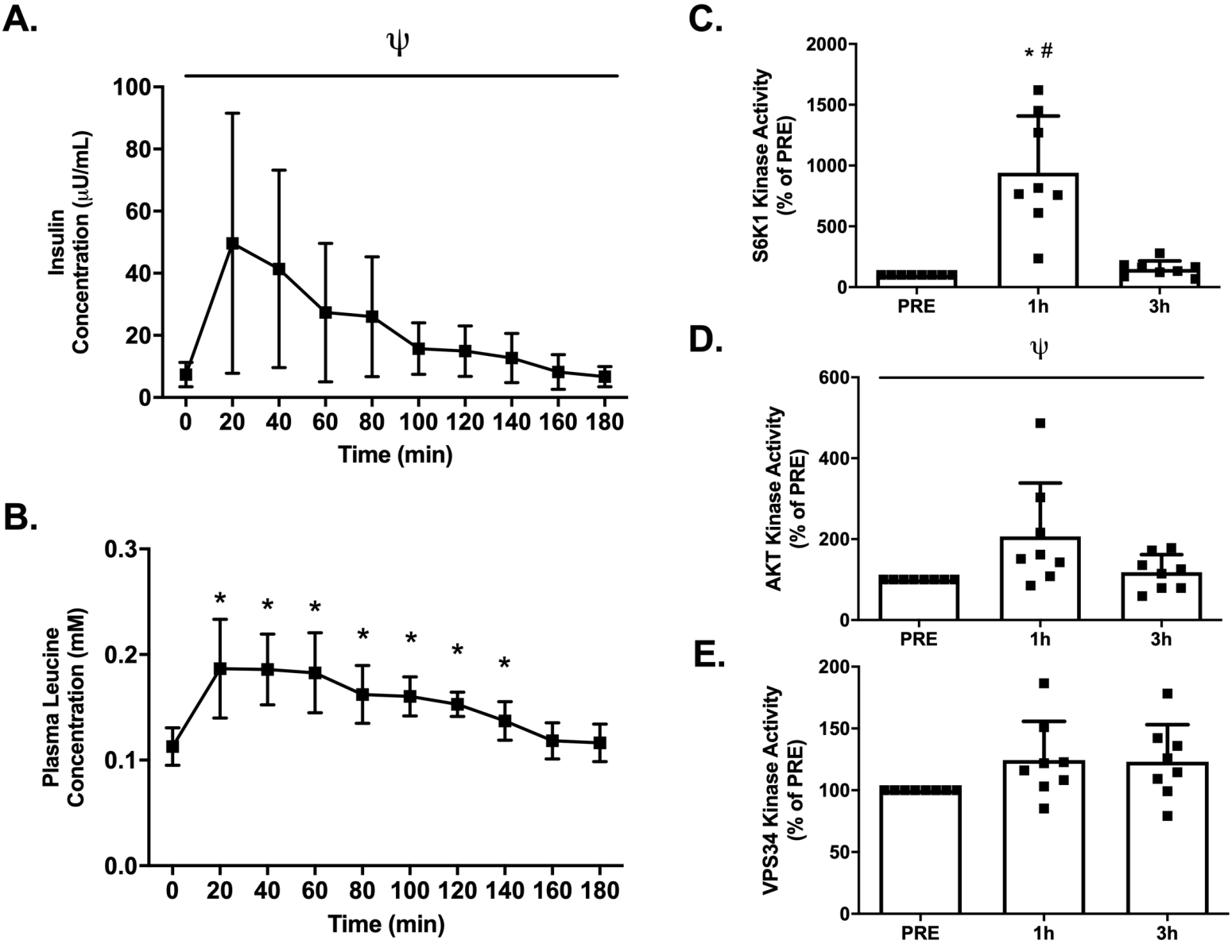
Primary Antibody	Source	Dilution	Secondary Antibody	Dilution
Monoclonal anti- mTOR antibody with mouse antigen, isotype IgG γ1 kappa	Millipore, 05-1592	1:200	Goat anti-mouse IgG γ1 kappa Alexa®594	1:200
Polyclonal anti- Lamp2 antibody with rabbit antigen, isotype IgG	Abgent, AP1824d	1:100	Goat anti-rabbit IgG(H+L) Alexa®488	1:200
Monoclonal anti-Vps34/PIK3C3 antibody with rabbit antigen, isotype IgG	Cell Signaling Technology #3358	1:20	Goat anti-rabbit IgG(H+L) Alexa®488	1:200
Wheat Germ Agglutinin-350	W11263, Invitrogen	1:20	Alexa Fluor® 350 Conjugated	N/A

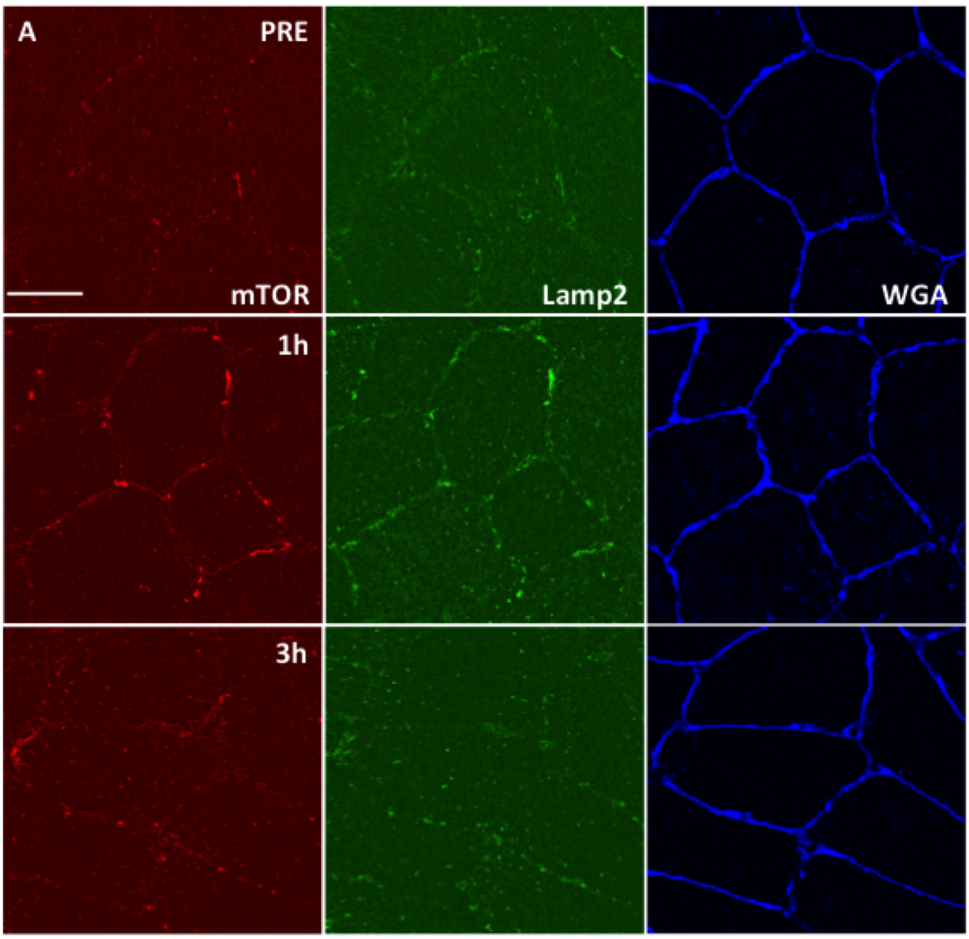
Table 1. Summary of Antibodies used for Immunofluorescent Analysis

Primary Antibody	Source	Dilution	Secondary Antibody	Dilution
Monoclonal anti- mTOR antibody with mouse antigen, isotype IgG γ1 kappa	Millipore, 05-1592	1:200	Goat anti-mouse IgG γ1 kappa Alexa®594	1:200
Polyclonal anti- Lamp2 antibody with rabbit antigen, isotype IgG	Abgent, AP1824d	1:100	Goat anti-rabbit IgG(H+L) Alexa®488	1:200
Monoclonal anti-Vps34/PIK3C3 antibody with rabbit antigen, isotype IgG	Cell Signaling Technology #3358	1:20	Goat anti-rabbit IgG(H+L) Alexa®488	1:200
Wheat Germ Agglutinin-350	W11263, Invitrogen	1:20	Alexa Fluor® 350 Conjugated	N/A

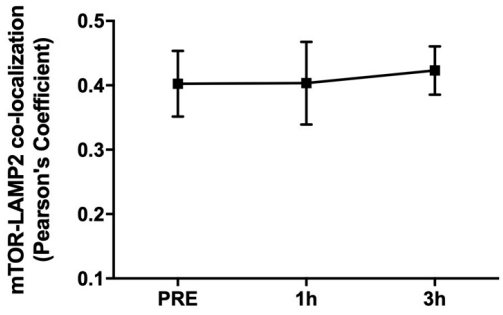
Table 1.


Time
(minutes)
-20
0
60
120
180
PRO-CHO
Beverage
Blood
Sample
Muscle
Biopsy

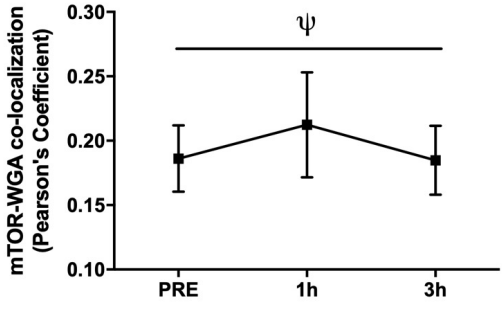

A.
ψ
Insulin Concentration (μU/mL)
100
80
60
40
20
0
0 20 40 60 80 100 120 140 160 180
Time (min)
B.
Plasma Leucine Concentration (mM)
0.3
0.2
0.1
0.0
* * * * * * *
0 20 40 60 80 100 120 140 160 180
Time (min)
C.
S6K1 Kinase Activity (% of PRE)
2000
1500
1000
500
0
* #
PRE
1h
3h
D.
ψ
AKT Kinase Activity (% of PRE)
600
400
200
0
PRE
1h
3h
E.
VPS34 Kinase Activity (% of PRE)
200
150
100
50
0
PRE
1h
3h

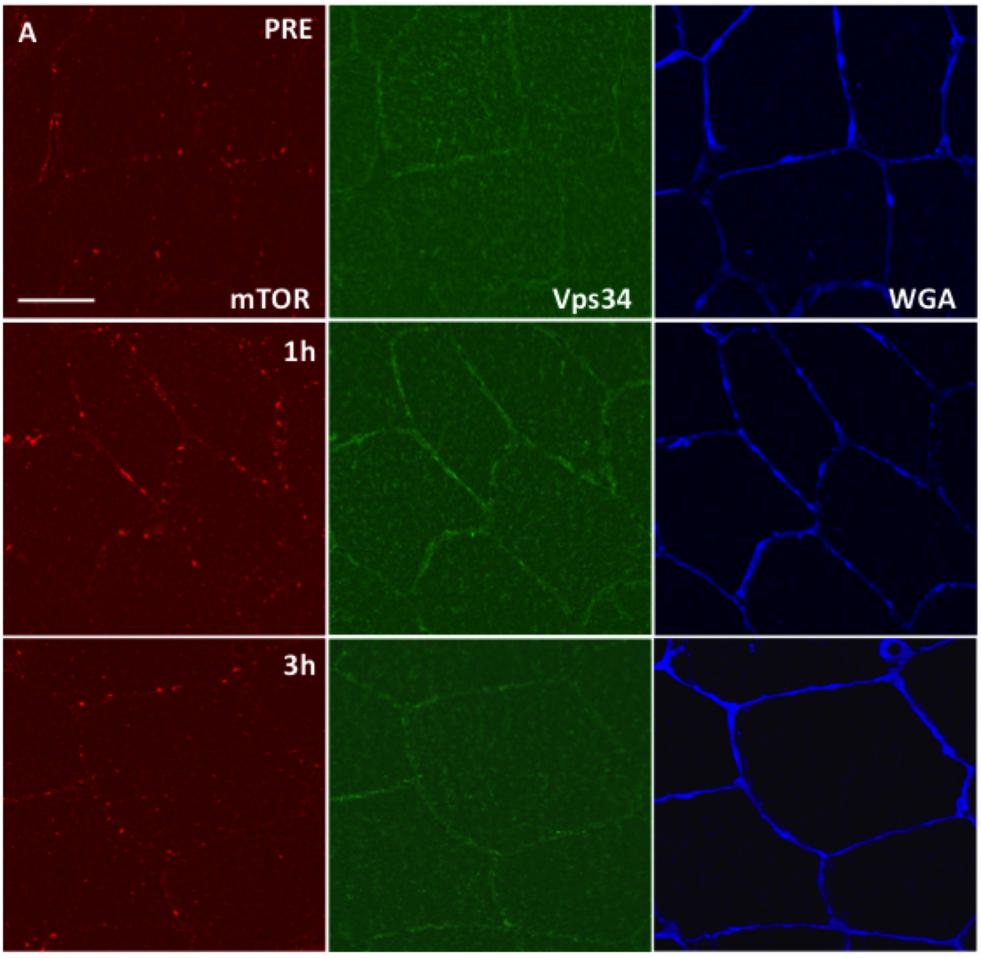

A
PRE
mTOR
Lamp2
WGA
1h
3h

B.

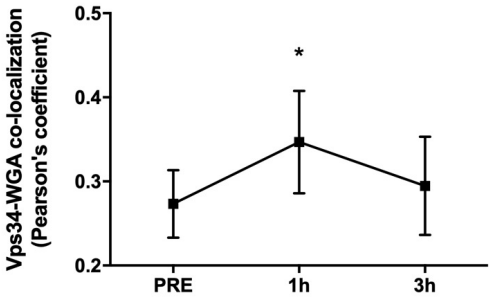

mTOR-LAMP2 co-localization
(Pearson's Coefficient)
0.5
0.4
0.3
0.2
0.1
PRE
1h
3h

C.

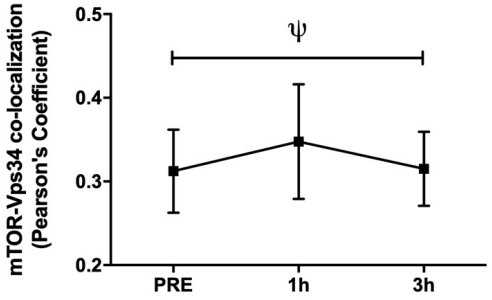

mTOR-WGA co-localization
(Pearson's Coefficient)
ψ
0.30
0.25
0.20
0.15
0.10
PRE
1h
3h

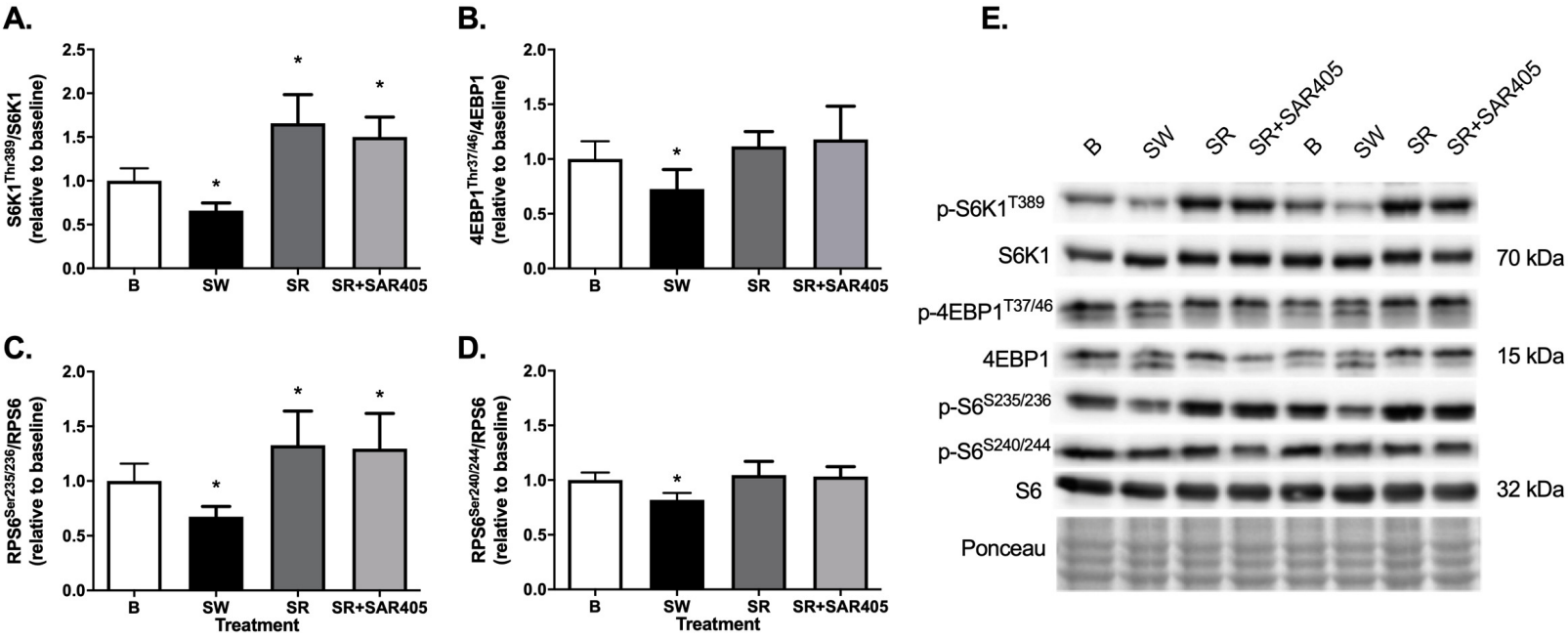

A
PRE
mTOR
Vps34
WGA
1h
3h

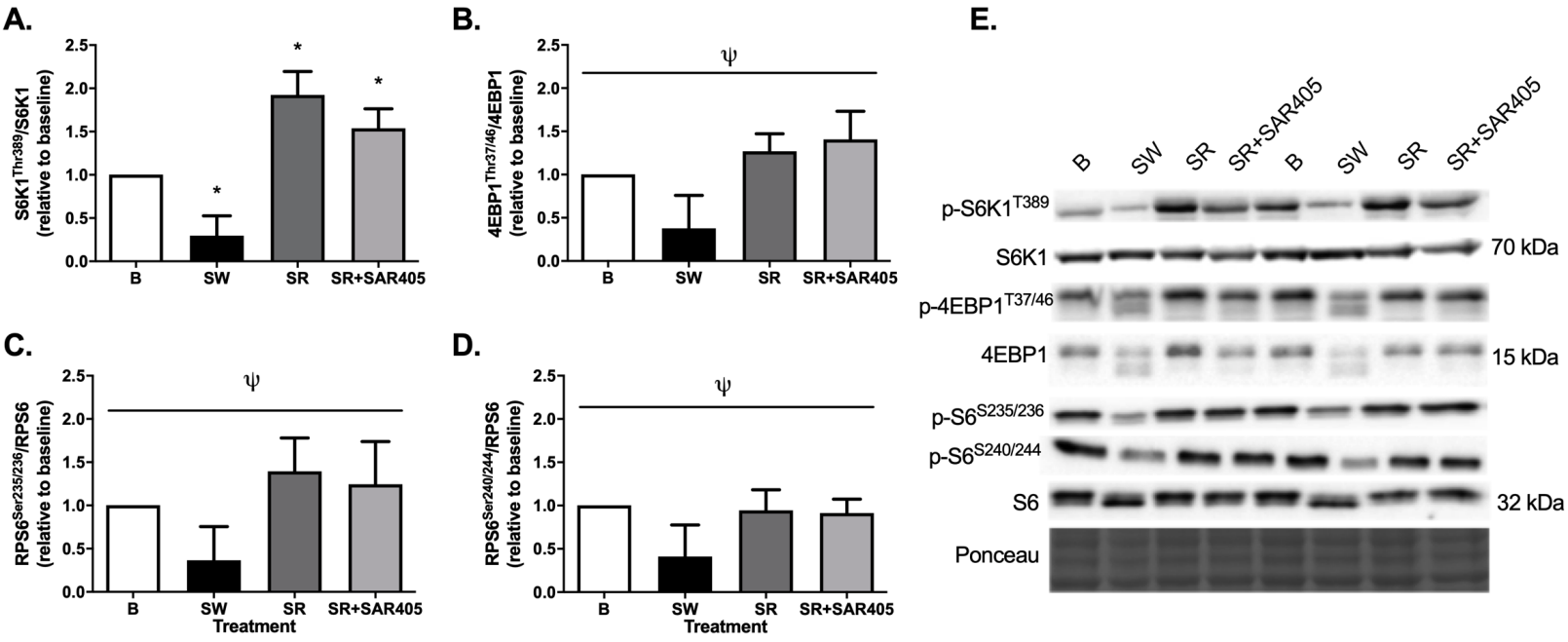
B.

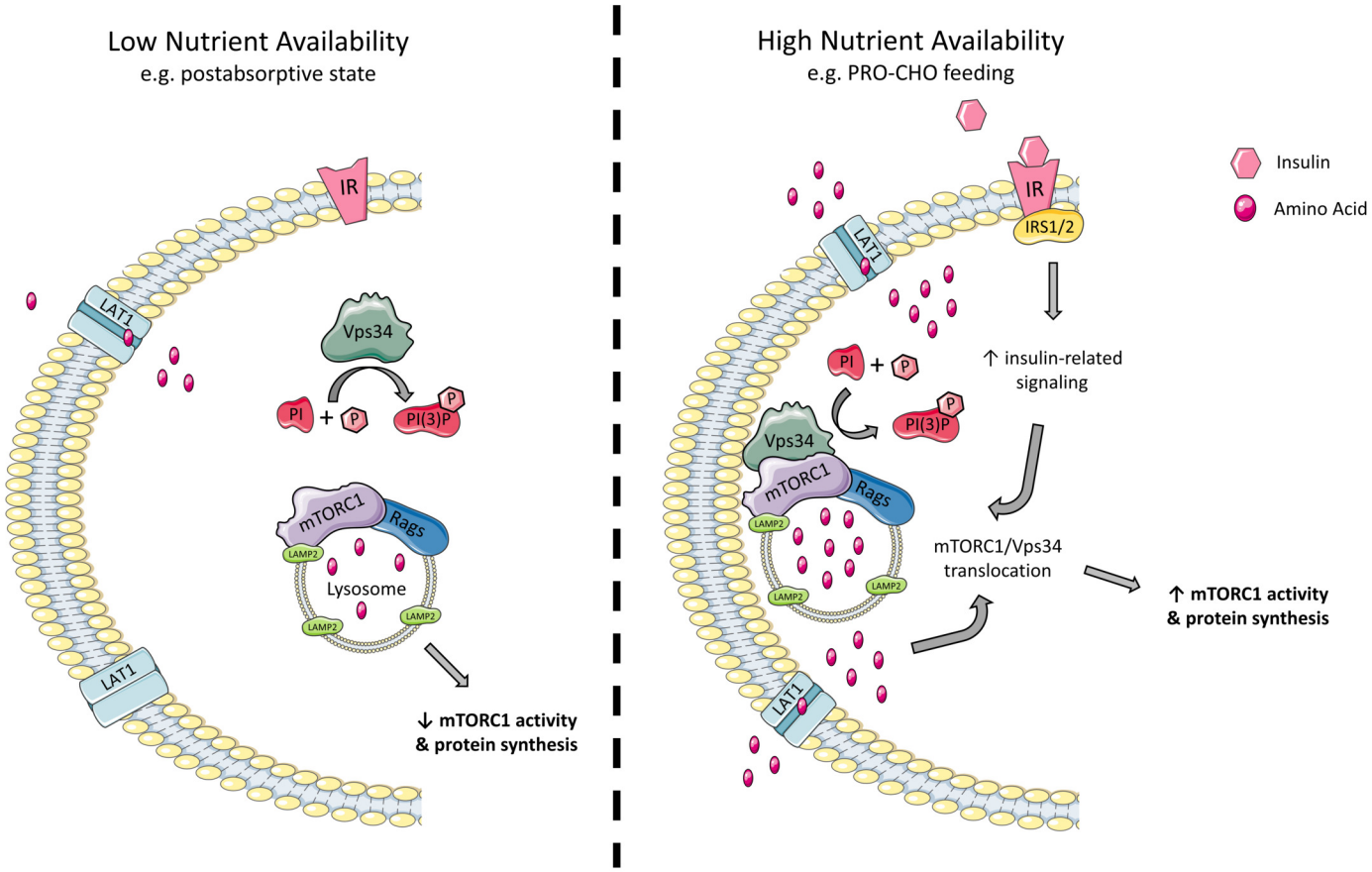

Vps34-WGA co-localization
(Pearson's coefficient)
0.5
0.4
0.3
0.2
*
PRE
1h
3h

C.


mTOR-Vps34 co-localization
(Pearson's Coefficient)
0.5
0.4
0.3
0.2
ψ
PRE
1h
3h


A.
S6K1Thr389/S6K1 (relative to baseline)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
B
SW
SR
SR+SAR405
B.
4EBP1Thr37/46/4EBP1 (relative to baseline)
2.0
1.5
1.0
0.5
0.0
*
B
SW
SR
SR+SAR405
C.
RPS6Ser235/236/RPS6 (relative to baseline)
2.0
1.5
1.0
0.5
0.0
*
*
*
B
SW
SR
SR+SAR405
Treatment
D.
RPS6Ser240/244/RPS6 (relative to baseline)
2.0
1.5
1.0
0.5
0.0
*
B
SW
SR
SR+SAR405
Treatment
E.
B
SW
SR
SR+SAR405
B
SW
SR
SR+SAR405
p-S6K1T389
S6K1
70 kDa
p-4EBP1T37/46
4EBP1
15 kDa
p-S6S235/236
p-S6S240/244
S6
32 kDa
Ponceau


A.
S6K1Thr389/S6K1 (relative to baseline)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
*
B
SW
SR
SR+SAR405
B.
4EBP1Thr37/46/4EBP1 (relative to baseline)
ψ
C.
RPS6Ser235/236/RPS6 (relative to baseline)
Treatment
D.
RPS6Ser240/244/RPS6 (relative to baseline)
E.
B
SW
SR
SR+SAR405
p-S6K1T389
S6K1
70 kDa
p-4EBP1T37/46
4EBP1
15 kDa
p-S6S235/236
p-S6S240/244
S6
32 kDa
Ponceau


Low Nutrient Availability
e.g. postabsorptive state
IR
LAT1
Vps34
PI
P
PI(3)P
mTORC1
Rags
LAMP2
Lysosome
↓ mTORC1 activity
& protein synthesis
High Nutrient Availability
e.g. PRO-CHO feeding
IRS1/2
↑ insulin-related
signaling
mTORC1/Vps34
translocation
↑ mTORC1 activity
& protein synthesis
Insulin
Amino Acid