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The role of salt inducible kinases in the regulation of the mammalian target of rapamycin pathway in adipocytes

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THE ROLE OF SALT INDUCIBLE KINASES IN THE REGULATION OF THE MAMMALIAN TARGET OF RAPAMYCIN PATHWAY IN ADIPOCYTES

Master thesis

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Abbreviations

AMP-Adenosine monophosphate

AMPK –AMP-activated protein kinase

ATCC-American type culture collection

BSA-Bovine serum albumin

CREB-cAMP response element binding protein

CRTC2-CREB regulated transcriptional coactivators

DEPTOR- DEP domain-containing mTOR interacting protein

DMEM- Dulbecco's Modified Eagle Medium

DMSO- Dimethyl sulfoxide

DTT-Dithiothreitol

EDTA-Ethylenediaminetetraacetic acid

EGTA-Ethylene glycol-bis (β-aminoethyl ether)-N, N, N', N'-tetraacetic acid)

ER-Endoplasmic reticulum

FBS-Fetal bovine serum

GAPDH-glyceraldehyde 3-phosphate dehydrogenase

HDAC4-Histone deacetylase 4

IBMX- 3-isobutyl-1-methylxanthine

KRH-Krebs-ringer-HEPES

LC3microtubule-associated protein light chain 3

LDS- lithium dodecyl sulfate

LKB1-Liver kinase B1

MARK-Microtubule-affinity-regulating-kinase

mLst8- mammalian lethal with SEC protein 8

mTOR-mammalian target of rapamycin

NC-negative control

NP-40 -Nonidet P-40

PPAR-peroxisome proliferator-activated receptor γ

PEST-Penicillin and streptomycin

PRAS40- proline rich Akt substrate of 40 KDa

PIA- N-phenylisopropyladenosine

PI3K-phopsho-inositide 3-kinase

PBS-Phosphate-buffered saline

P70s6K-ribosomal protein s6 kinase

QIK-Qin-induced kinase

RICTOR-rapamycin insensitive component of mammalian target of rapamycin

SREBP1-Sterol regulatory element-binding protein 1

SIK-Salt inducible kinase

SREBP1-Sterol regulatory element-binding protein 1

TFEB-Transcription factor EB

TSC2- tuberous sclerosis complex 2

TBSTTris-buffered saline

ULK1-Unc-51 autophagy activating kinase 1

VDCC-Voltage-gated calcium channel

POPULARIZED SCIENTIFIC SUMMARY

Sulochana Kotresh Jali

The role of SIK2 in the regulation of the mTOR pathway in adipocytes

Diabetes mellitus is a metabolic disorder, which results in elevated blood sugar levels due to insufficient secretion of insulin and reduced sensitivity of insulin in target tissues. There are different types of diabetes present with type 1 and type 2 diabetes being at two ends of a spectrum. Years of research have concluded that diabetes is often associated with conditions such as obesity, cardiovascular disorders, modifications of the DNA, different variants of genes and altered signalling of proteins, which often results in modified cellular processes. One of the important cellular processes which are modified is autophagy. Autophagy is a mechanism by which the cell degrades and recycles the damaged components of the cell. Autophagy is highly regulated, for example by the protein TFEB, which is a protein required for autophagy.

Adipose tissue is a major reservoir for storage of fat. Accumulation of fat in other tissues, which sometimes occurs during obesity, can give rise to complex diseases like diabetes. Insulin is an important signal for correct storage of fat in adipocytes, and insulin resistance in adipocytes could, therefore, contribute to incorrect fat storage in obesity. Dysfunctional autophagy is speculated to be one of several possible underlying triggers for insulin resistance in adipose tissue. SIK2, an isoform of salt inducible kinases, is an AMPK-related kinase present in high quantities in adipose tissue. SIK2 is known to play a role in the regulation of metabolic processes but recent studies have also shown that SIK2 regulates TFEB activity. TFEB activity is regulated by the protein mTOR which is also a master regulator of protein synthesis and cell growth in response to nutrient supply. mTOR phosphorylates TFEB and prevents its translocation to the nucleus which results in autophagy genes not being activated. Inhibition of mTOR allows activation of autophagy-related genes. With this as a background, we wish to see whether this regulation of TFEB by SIK2 is via mTOR or other pathways. Our results indicated that AMPK-related kinases do have a role to play in controlling mTOR activity but whether this is via SIK family is still doubtful since inhibition of SIKs did not result in reduced protein mTOR activity.

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ABSTRACT

Salt inducible kinase 2 (SIK2) is found abundantly in adipose tissue and may have a role in the development of diabetes. SIK2 is downregulated in insulin-resistant and obese individuals, thereby providing a new potential target for therapeutic solutions. Dysfunctional adipose tissue is observed in obesity and this is correlated with impaired insulin signalling, which might contribute to imbalanced adipokine secretion and ectopic fat deposition. Degradation and recycling of cellular components are important for the maintenance of the cell, and SIK2 has recently been discovered to participate in the regulation of adipocyte autophagy via the transcription factor TFEB. Phosphorylation of TFEB by mTOR (mammalian target of rapamycin complex 1) results in its inactivity and subsequent downregulation of autophagy-related genes. We investigate here whether SIK2's role in the regulation of TFEB is via mTOR signalling pathway, and do so by analyzing the effect of SIK inhibition on mTOR activity. We found that MRT199665, which is an AMPK and AMPK-related kinase inhibitor, results in inhibition of mTOR activity – seen as reduced phosphorylation of mTOR substrates ULK1 (Unc-51 autophagy activating kinase 1) and P70s6K (P70s6K-ribosomal protein s6 kinase). However, when SIKs were inhibited using SIK inhibitor HG-9-91-01, it resulted in reduced expression levels of these proteins, rather than altered phosphorylation. In human adipocytes, there was no significant mean difference between the negative control and treatment with HG-9-91-01. Overall, we can say that AMPK-related kinases, however not SIKs, appear to have a role to play in controlling mTOR activity.

KEYWORDS: Salt inducible kinase 2, autophagy, diabetes, mTOR

INTRODUCTION

1. Role of autophagy in the pathophysiology of diabetes and obesity

Increased occurrence of obesity and diabetes has drawn a lot of attention globally and requires immediate action by finding new potential drug targets.Type 2 diabetes is a metabolic disorder which is strongly associated with acquired insulin resistance and insufficient insulin production **[1]**. It is known that almost 80% of the diabetic population have obesity. Increase in body mass index increases the risk of diabetes exponentially thereby linking diabetes and obesity**[2]**. Some of the common features seen in obese vs the diabetic populations, which links these two diseases, are the presence of proinflammatory cytokines, insulin resistance, deranged fatty acid metabolism and dysfunctional mitochondria on a systemic level **[2].** Dysfunctional adipose tissue, which is observed in obesity, is associated with impaired insulin signalling in adipocytes, an imbalance in adipokine secretion, and leakage of fatty acids, resulting in ectopic fat deposition in muscle, liver and pancreatic β cells **[3]**.

Autophagy is a process by which the cell adapts to high-stress levels by ingestion of damaged organelles or misfolded proteins. Three different types of autophagy exist and these are microautophagy, chaperon mediated autophagy and macroautophagy **[4]**. Microautophagy usually requires membrane invagination resulting in taking up of the damaged component and delivered to the lysosome. Chaperone mediated autophagy requires proteins which will tag the substrate and deliver them to the lysosome **[5]**. Macroautophagy, hereafter referred to as autophagy requires the formation of autophagosomes which are double-membrane vesicles which later on fuse with lysosomes for the degradation process. In all three cases, the component to be degraded is delivered to the lysosome **[4,5]**. In diabetic persons the autophagy marker microtubule-associated protein light chain 3 (LC3) was detected strongly in the adipose tissue obtained from the visceral and subcutaneous fat, pointing towards enhanced autophagy **[6]**. One study where autophagy was used as a therapeutic target shows that stimulation of autophagy in mice prevents diabetes progression and **β**-cell apoptosis *in vivo* **[7].** Autophagy is suggested to be involved in the pathophysiology of diabetes. For example, studies have shown that autophagy has a pathological role to play in type 2 diabetic heart **[8].** Hormones such as insulin and glucagon inhibit and stimulate autophagy respectively thereby suggesting that autophagy has a role in the development of diabetes and

obesity **[9].** Moreover, studies have shown that in obese populations, enhancement of autophagy experimentally has different effects on adipocyte function **[9]**. Autophagy in adipocyte can stop the pro-inflammatory reaction, which is observed in obesity, but high stimulation of autophagy can result in excess adipocyte energy storage and cause cell death **[9].** Autophagsome content was increased in adipocytes from obese and diabetic populations and autophagic flux was upregulated in obese patients undergoing bariatric surger **[10,11].** But contrasting results have been obtained in rodents where autophagy was decreased *in vitro* and in the adipose tissue post-feeding with high-fat diet **[12].** Autophagy related protein complexes in adipose tissue were upregulated by at least 2-fold in diabetic populations **[13]**

Why is it important to focus on autophagy in adipocytes? One article states that proteins involved in the autophagy machinery are also required for the differentiation of white adipocytes **[14]**. Autophagy is known to regulate adipogenesis but the exact mechanism is not known. One hypothesis is that, upregulated autophagy increases the stability of peroxisome proliferator-activated receptor γ 2 (PPAR γ 2), which is the main regulator of adipocyte differentiation and adipogenesis **[15,16]**. Adipose tissue of adult mammals consists of various types of cells which include adipocytes, fibroblasts, immune cells etc in varying portions. But in obesity, the portion of adipocytes is decreased hence it is important to study isolated adipocytes **[9]**. The notion behind whether autophagy is protective or involved in the development of diabetes is still debated, this requires more investigation **[17]**. Autophagy is thus a very important cellular process in adipocytes as it is interlinked with diabetes and obesity.

2. mTOR and its role in the regulation of translation and autophagy

Under normal conditions or in healthy individuals, insulin inhibits autophagy by activating a protein called mammalian target of rapamycin (mTOR) **[10]**. mTOR forms two protein complexes known as mammalian target of rapamycin complex 1 (mTORC1) (rapamycin sensitive) hereby refered as mTOR and mammalian target of rapamycin complex 2 (mTORC2) (rapamycin-insensitive) with other proteins such as proline-rich Akt substrate of 40 KDa (PRAS40), DEP domain-containing mTOR interacting protein (DEPTOR) and raptor in the case of mTORC1 and mammalian lethal with SEC protein 8 (mLst8), DEPTOR and rapamycin-insensitive companion of mammalian target of rapamycin (RICTOR) in the case of mTORC2 **[18,19,20,21,22]**.

mTOR is a regulator of autophagy and autophagy-related genes. It does so by multisite phosphorylation of transcription factor EB (TFEB) at Ser142 and 138 which is downstream of mTOR **[23]**. In diabetic populations the converse is observed where mTOR is inhibited **[10]** hence in diabetic populations there is a reliance on autophagic processes to maintain the adenosine triphosphate (ATP) concentration in adipocytes **[10]**. Therefore this requires us to study the autophagy controlling proteins such as mTOR and TFEB.

mTOR is a 289-kDa protein kinase which is a part of the phosphoinositide family (PI3K) family **[24].** Previous work **[25]** states that mTOR is one of the main regulators of cellular growth, proliferation, translation of proteins and metabolic homeostasis and is enhanced during tumour formation and insulin resistance. Altered activity of mTOR pathway can lead to diabetes, obesity, and cancer **[24].** The translation machinery is controlled by mTOR as mentioned above, and mTOR manifests this control via two proteins. These proteins are eukaryotic translation initiation factor 4E binding protein 1 (4E-BP1) and P70s6K, which are phosphorylated by mTOR at Thr37/46/70 and Ser83/101/112 (4E-BP1) **[26]**, and Thr389 (P70s6K), respectively. These mTOR substrates are responsible for the synthesis of proteins **[27].** mTOR also known to tune transcription factors such as Sterol regulatory element-binding protein 1 (SREBP1) **[28]** and PPARγ **[29],** which are required for lipid and cholesterol homeostasis.

Some of the important signals which modifiy mTOR activity are amino acids, oxygen levels and energy status of the cell **[24].** An upstream regulator of mTOR is cellular energy levels, which involves adenosine monophosphate-activated protein kinase (AMPK). Fluctuation in glucose avialiabilty can cause changes in ATP levels in the cells which activate AMPK. AMPK, in turn, acts on mTOR via inhibition of tuberous sclerosis complex 2 (TSC2) dependent and independent ways, where TSC2 has an inhibitory effect on mTOR **[30]**. The TSC2 independent pathway includes AMPK phosphorylating Raptor at Ser863, thereby inhibiting mTOR **[31].**

mTOR inhibits autophagy by inhibitory phosphorylation on Unc-51 autophagy activating kinase 1 (ULK1). ULK1 is a protein which is required for autophagosome formation and is one of the downstream proteins on which mTOR acts. mTOR phosphorylates ULK1 at Ser757 thereby preventing autophagosome formation **[22,32]**. AMPK, on the other hand, promotes autophagy by

phosphorylating ULK1 at Ser 317 and Ser 777, while mTOR breaks this step by phosphorylating ULK1 at Ser 777 as seen in **fig 1 B [32]. Fig 1 A** shows mTOR control over autophagy besides ULK1, mTOR controls autophagy via TFEB . TFEB phosphorylation at Ser211 by mTOR renders it inactive by retaining TFEB in the cytoplasm. TFEB dephosphorylation occurs as a consequence of mTOR inhibition, TFEB then translocates to the nucleus where it helps in the transcription of autophagy-related genes **[23,33,34]**.

Considering the central role of mTOR in the control of autophagy, it is of importance to investigate new pathways regulating mTOR activity. Recently, a new AMPK-related kinase called salt inducible kinases (SIKs) has come into the picture. The SIK family is known to have metabolic functions and are implicated in diabetes and obesity **[35].** Interestingly, it has also recently been shown to be involved in TFEB and autophagy regulation **[36]**.

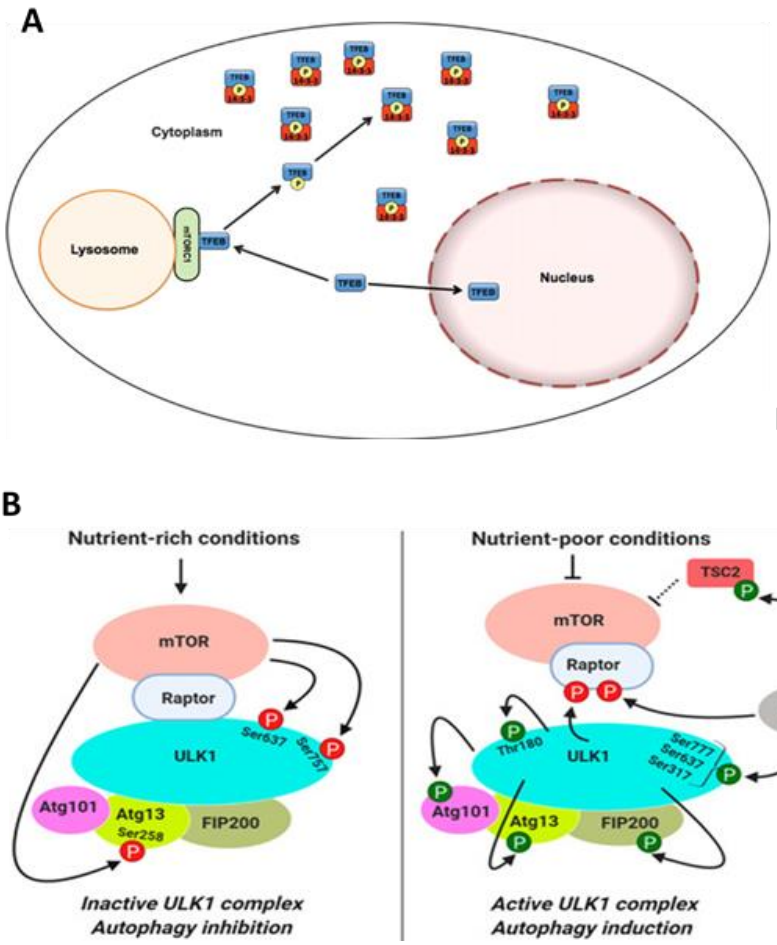


Figure 1 mTOR and its downstream effects of autophagy
(A) Depicts the TFEB regulation by mTOR and its translocation to the nucleus **[37**] and (B) represents the phosphorylation of ULK1 by mTOR and AMPK under nutrient rich and poor conditions respectively **[38]**

3. AMPK and AMPK-related kinases

To guarantee cell survival it is vital for the adenosine triphosphate (ATP) production level in the cell to be optimal. AMPK is activated when there is a slight change in the AMP/ADP levels due to for example physiological stresses, unavailability of nutrients etc **[39]**. Binding of AMP or ADP to AMPK triggers the phosphorylation of AMPK at Thr172 by liver kinase B1 (LKB1), leading to its activation **[35]**. Post phosphorylation, AMPK phosphorylates its downstream substrates to activate catabolic and inhibit anabolic pathways to restore ATP levels **[39]**. One catabolic pathway

which is of our interest is autophagy**.** Recently, studies have shown that LKB1 is a regulator not only of AMPK but another 12 AMPK-related kinases, such as SIKs, which have a similarity in their substrate preference *in vitro* **[40].**

Contrary to AMPK, AMPK-related kinases do not necessarily react to changed energy levels but respond to other signals **[41].** The SIK family consists of three isoforms namely SIK1,2,3 respectively. SIK3 has a broad expression whereas SIK2 is present in abundance in adipocytes **[35]**. A central role for SIKs is to control gene expression downstream of CREB (cAMP response element-binding protein), through their action on CREB-regulated transcription coactivators (CRTC) **[35]**. Other transcriptional co-regulators shown to be controlled by SIKs are the class IIa histone deacetylase (HDACs) **[35,42,43].** Through these and other substrates, SIK isoforms are in involved in the regulation of glucose homeostasis **[35,42]**.

β cells of the pancreas, which are essential for producing insulin, become one of the malfunctioning biological features of diabetes **[42].** Glucose-stimulated insulin secretion in mouse studies is promoted by SIK2 by activating voltage-dependent calcium channels (VDCC) and allowing the influx of calcium required for insulin secretion **[42,43]**. In the liver it was demonstrated that HG-9-91-01, an ATP-competitive chemical inhibitor of the SIK family, resulted in dephosphorylation of CRTC2 and HDAC4. This dephosphorylation of CRTC2 and HDAC4 resulted in their translocation to the nucleus and increased transcription of genes required for gluconeogenesis **[35].**

Our interest lies in the role of SIK2 in adipocytes. The most abundant amongst the SIK isoforms is SIK2, both in white and brown adipocytes and SIK2 is also upregulated during adipogenesis **[44,45].** From various studies, it was concluded that SIK2 enhances insulin signalling in adipocytes **[35**]. In obese individuals, SIK2 expression in adipocytes was found to be reduced **[46].** The insulin-induced phosphorylation of protein kinase B (PKB), a critical protein in the insulin signalling pathway, was reduced in a global SIK2 knockout mouse model, further stating that SIK2 is required for insulin signalling **[48].** Such knockout models also exhibited deformities such as macrophage infiltration, reduced adiponectin and Glut4 mRNA expression in adipocytes **[48].** In human adipocytes, inhibition of SIKs by both small interfering RNA (siRNA) and chemical

inhibition by HG-9-91-01 resulted in reduced phosphorylation of PKB, along with less glucose uptake at the basal level as well as when stimulated by insulin **[46].**

Recent studies have revealed that SIK2 is required for initiation and autophagosome-lysosome fusion stages of autophagy, which was indicated by inhibition of autophagic flux in 3T3-L1 adipocytes after SIK2 inhibition by HG-9-91-01 and siRNA **[36]**. Further investigation which supported these results is that SIK2 inhibition by siRNA as well as pharmacologically resulted in decreased TFEB protein levels **[36]**. It was hypothesized that this control of TFEB by SIK2 might be via HDAC4 or other novel substrates of SIK2 **[36].** Along with the reduction in expression, it was also observed that there was a molecular weight shift of the band denoting TFEB, this shows that TFEB was dephosphorylated after inhibition of SIK2 in 3T3-L1 adipocytes **[36].** TFEB being a well-established downstream target of mTORC1, we hypothesize that the dephosphorylation of TFEB observed after SIK inhibition could be mediated via inhibition of mTORC1. As a step in testing this hypothesis, the aim of this thesis was thus to investigate whether pharmacological inhibition of SIKs affects mTORC1 activity.

MATERIALS AND METHODS

1.3T3-L1 cell culture and differentiation

3T3-L1 fibroblasts (ATCC, CL-173) were cultured in Dulbecco's modification of Eagle's medium (DMEM) (Sigma, D6429) supplemented with 1 % of penicillin-streptomycin solution (Sigma, P0781) and 10% fetal bovine serum (FBS) (Sigma, F7524). Post seeding on 10 cm plates, the cells were subcultured every 2 days and media was changed every 48 hours. 48 hours post reaching 80% confluency, the cells were differentiated to adipocyte-like cells by the addition of growth medium supplemented with Insulin (10 μg/ml) (Sigma, 12643), dexamethasone (1 μM) (Sigma, D4902) and isobutyl methylxanthine (IBMX) (520 μM) (Sigma, 17018). After 3 days, the differentiation medium was changed to the normal growth medium. On day 8 to 15 post differentiation, cells were reseeded in 12 well plates using trypsin (Sigma, T3924) and collagenase

type 1 (2 mg/ml in PBS; Gibco, 17100-017). The cells were then treated with respective inhibitors in DMEM with Glutamax (Gibco, 10569-010)

2. 3T3-L1 cell stimulation and lysate preparation

The cells were then treated with chemical inhibitors HG-9-91-01 (MedChem Express, HY-15776) and MRT199665 (kindly shared by the MRC protein phosphorylation unit, Dundee, Scotland)**[47]** in a 37°C incubator with 5% CO2, in DMEM with Glutamax (Gibco, 10569-010) for one hour. The adipocytes were treated with the inhibitors in Glutamax media to prevent the degradation of glutamate to ammonium and pyroglutamate, which can be toxic for the cells and for the cells to have better access to glutamine **[48].** Dimethyl sulfoxide (DMSO) was used as negative control and Torin 1 (100 nM) (MedChem Express, HY-13003) which is an inhibitor of mTOR was used as positive control.

For HG-9-91-01 and MRT-199665 experiments the cells were stimulated with concentrations as indicated in the figures. The cells were washed with PBS twice and the plates were kept on ice and 70µl -100µl of lysis buffer, containing [EDTA (1 mM), EGTA (1 mM), Na-pyro-phosphate (5 mM), sucrose (0.27 M), NaF (50 mM), Tris-base (50 mM), Na-ortho-vanadate (1 mM), NP40 (Nonidet P-40) (1 % w/v)] was added. The cells were scraped and lysates were transferred to an Eppendorf tube. The lysates were then vortexed and centrifuged at 13000g for 15 minutes in 4℃. After centrifugation, the supernatant between the fat layer and the pellet was transferred into a new Eppendorf tube and stored at -80 ℃ for further protein quantification and sample preparation for western blotting.

3. Primary human cell isolation, stimulation and lysate preparation

Human adipocytes were isolated from abdominal subcutaneous tissue shown in **fig 2** from female subjects who were operated for breast reconstructive surgery with written consent from the respective individuals. The study was approved by an ethical review board at Lund University (approval no.2017/920). Clean specimens of human adipose tissue were weighed and placed in Krebs buffer solution (KRH buffer [hepes (250 Mm), Glucose (200 mM), adenosine (100 µM), bovine serum

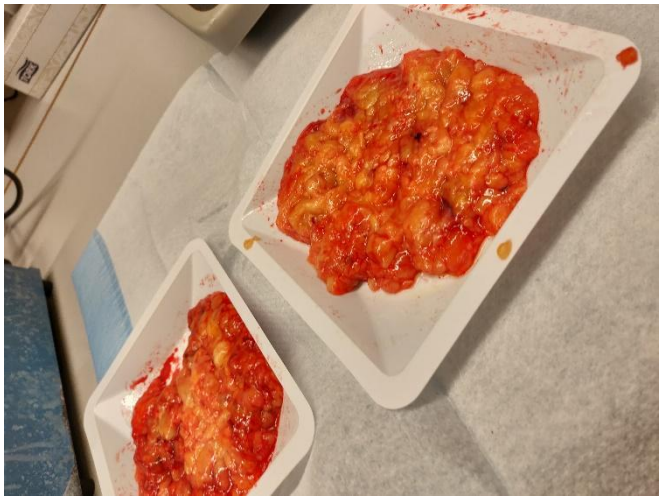


Figure 2 Adipose tissue from human subjects for primary cell isolation
Adipose tissue from which the human adipocytes are isolated after cleaning and removal of blood vessels and connective tissues.

albumin (BSA) (7% w/v)) (Saveen Werner, B2000-100)) . Adipose tissue was cut several times to obtain smaller pieces. The smaller pieces of adipose tissue was then digested using collagenase (1 mg/ml) at 37℃ in a water bath for approximately 30 minutes at 140 rpm. The digested tissue was then filtered using a nylon mesh and 10-15 ml of KRH buffer was used to wash the cells and collect them in 15 ml tubes. Cells were washed in KRH buffer another 2-3 times. The lipocrit was estimated and cells were then incubated overnight (37℃ incubator with 5% CO2) in DMEM to which PEST (1:100), 3.5 % (w/v) BSA and 200 nM N-phenylisopropyladenosine (PIA) (1:5000) were added. The medium was then filtered using Sterile filter (0.2 µM). 24 hours post isolation the cells are resuspended in fresh media, (400 µl of cells and 2 ml of media) and were stimulated with different concentrations of HG-9-91-01 as indicated in the figures in scint vials at 37 °C in the water bath with shaking at 80 rpm. Treatments were stopped by the addition of 5 ml of KRH buffer (w/o BSA) and the cells are washed with KRH (w/o BSA) 2 times. Lysates were prepared by adding lysis buffer (defined above), vortexed and centrifugated at 13000g for 15 minutes in 4℃. After centrifugation, the supernatant between the fat layer and the pellet was transferred into a new Eppendorf tube and stored at -80℃ for further protein quantification and sample preparation for western blotting.

3. Western Blotting

The protein concentration of the lysates was measured using the Bradford method. The samples for western blot analysis were prepared by adding 1x NuPAGE LDS sample buffer (Thermo fisher scientific, NP0007) and 75 mM DTT (Sigma, D9163). Lysates underwent electrophoresis on pre-cast Novex Bis-tris 4-12% gradient gels (Thermo fisher scientific, NP0322BOX) and separated proteins were transferred to nitrocellulose membrane (Amersham Protran 0.45 NC, GE healthcare). After transfer, the membranes were stained using Ponceau (0.1% w/v ponceau in 5% acetic acid) and then blocked using dry milk (10% w/v) in TBS-Tween (TBS-T) (0.1%w/v) for a minimum of 30 minutes. After blocking, the membranes were incubated with primary antibodies, diluted in (5% w/v) BSA in TBS-T, overnight at 4℃. After washing, membranes were incubated in Horseradish peroxidase-conjugated secondary antibodies for 1 hour and then washed again. Protein bands were developed using chemiluminescence reagents (Super West Pico chemiluminescent substrate, Thermo scientific 34078 and Super Signal West Femto Maximum sensitivity substrate, Thermo scientific, 34094) in a Bio-Rad molecular imager chemiDoc XRS+device, and BioRad image lab software 5.2.1 was used for protein quantification. The antibodies used for the western blot analysis include Beta Actin (Sigma, A5441)**,** ULK1 (cell signalling 8054T) (D8H5), pULK1 (Ser757) (cell signalling) 14202T, P70s6K (cell signalling 9202S), p-P70s6K (T389) (cell signalling 9205S), pHDAC4 (Ser246) (Abcam 12172), HDAC4 (Cell signalling 3443S).

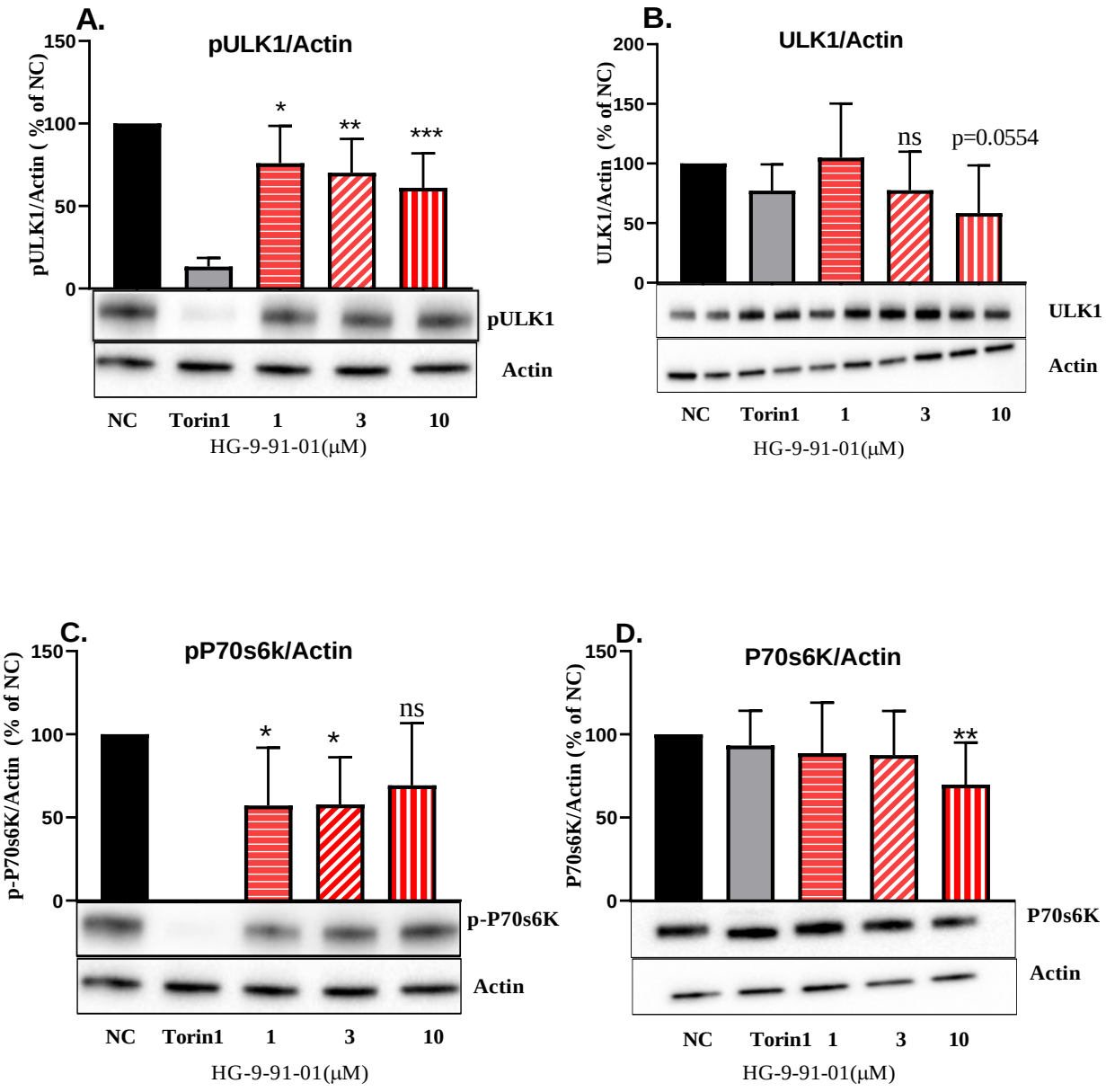
4. Statistical analysis

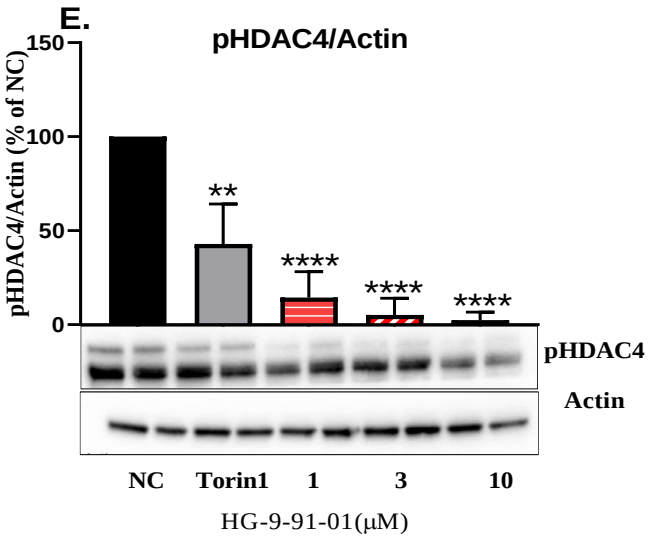
All the experiments were performed at least three times and expressed as mean ± standard deviation. Statistical analysis was performed by ordinary one-way ANOVA with holm-sidak's multiple comparison test or by unpaired t-test (Graph Pad). P values <0.05 were considered statistically significant and are marked with asterisks (*) in the figures (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p<0.0001$).

RESULTS

1. HG-9-91-01 treatment in 3T3-L1 adipocytes causes reduced protein levels of ULK1 and P70s6k in 3T3-L1 adipocytes

To examine the potential role of SIK2 in the regulation of mTORC1 activity, we treated 3T3-L1 adipocytes with the pharmacological SIK inhibitor HG-9-91-01 and investigated total and phosphorylation levels of ULK1, P70s6K and HDAC4.

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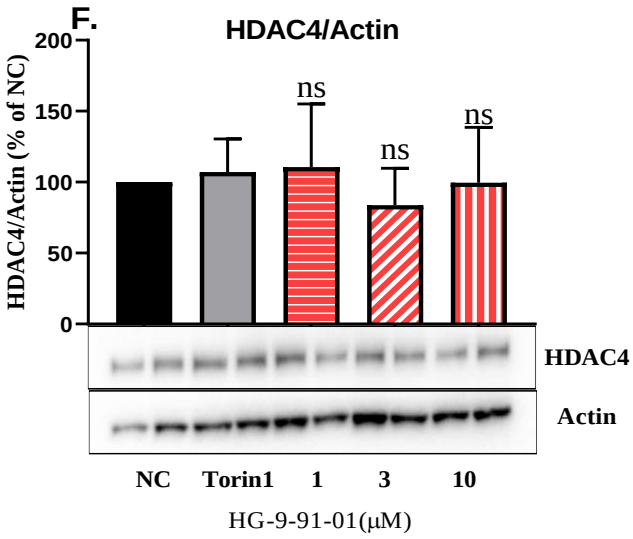
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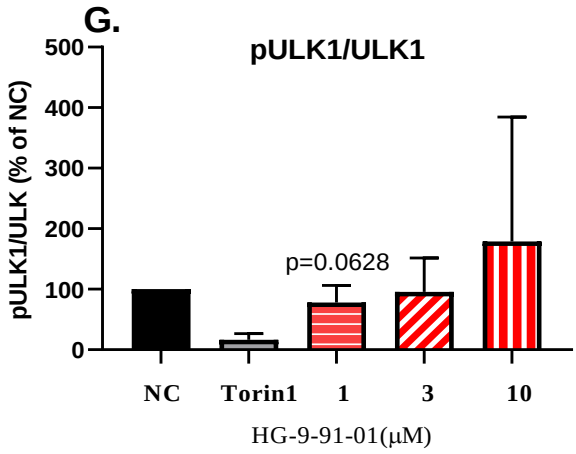
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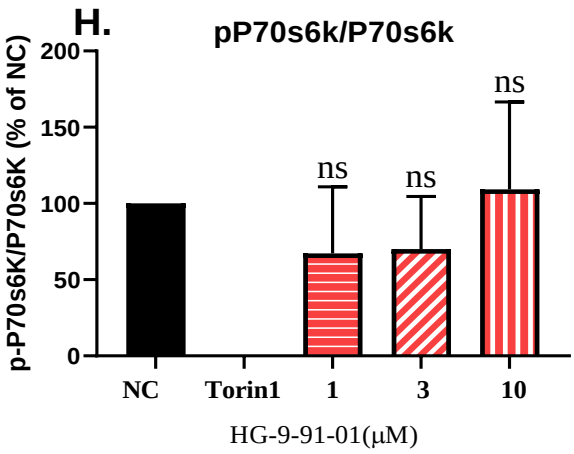
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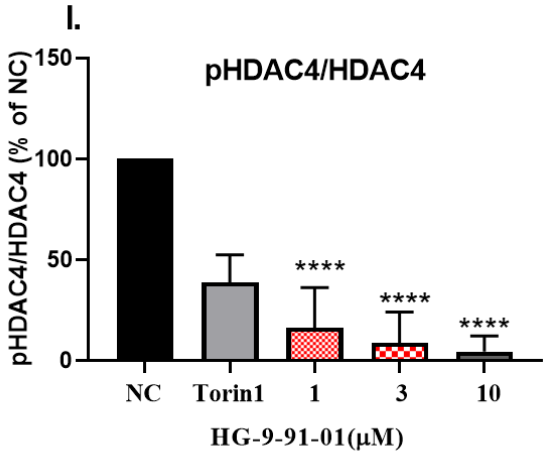


Figure 3 HG-9-91-01 treatment in 3T3-L1 adipocytes results in reduced ULK1 and P70S6K protein levels

Differentiated 3T3-L1 adipocytes were treated with different doses of HG-9-91-01(A-I) for one hour. Graphs represent quantification of ULK1(A, B, G), P70s6K (C, D, H) and HDAC4 (E, F, I) normalized to actin with a representative western blot below. All results are expressed as percentage of the negative control and shown as mean ± SD (n=3-7 independent experiments). Statistical analysis was performed by one-way ANOVA with Holm -Sidak's multiple comparison test (A, B, C, E, F,H,I) and two tailed student t-test (D,G) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001).

Figure 3A depicts pULK1 levels normalized to actin, compared to non-treated cells after one-hour treatment with HG-9-91-01. The total pULK1 levels were significantly decreased at all concentrations of HG-9-91-01, compared to the negative control, showing that inhibition of SIK2 may have inhibited mTORC1. Treatment with Torin1 (positive control), a well-established inhibitor of mTOR, resulted in reduced levels of pULK1 compared to the negative control, demonstrating inhibition of mTORC1 **[49]**. As shown in **Fig 3B,** protein levels of ULK1 were not significantly different between the negative control and 3 μM or 10 μM HG-9-91-01, however, there was a trend towards a reduced ULK1 level with 10 μM, the p-value being 0.054. HG-9-91-01 did not affect the specific phosphorylation of ULK1 (pULK1/ULK1) (**Fig 3G**), although 1 μM of HG-9-91-01 resulted in a reduction which was marginally non-significant (p-value 0.0628). Rate of phosphorylation, also known as specific phosphorylation, was analyzed because this is important to find out the amount of ULK1 proteins which are phosphorylated with respect to total

protein level of ULK1, thereby giving us evidence if there is an actual reduction in kinase activity of mTORC1. For Torin1 (positive control), on the other hand, the reduction of total phosphorylation of ULK1 was maintained when normalizing to total ULK1, indicating inhibition of mTOR.

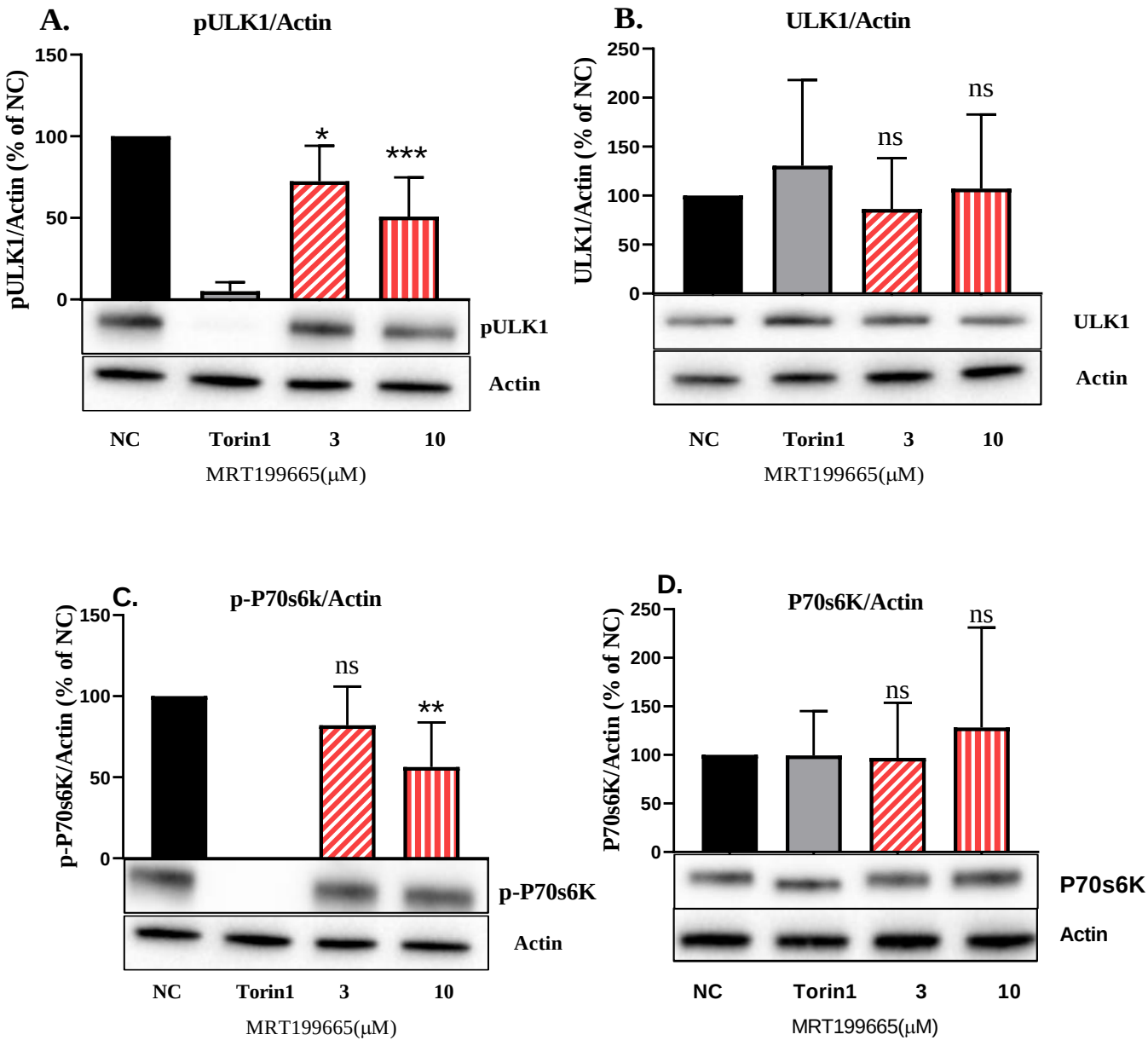
To monitor mTORC1 activity, total (normalized to actin) and specific (normalized to P706sK) phosphorylation levels of P70s6K was also analyzed. As shown in **Fig 3C,** total phosphorylation levels of P70s6K levels were significantly reduced compared to negative control after 1 μM and 3 μM HG-9-91-01, whereas with 10 μM it is not significantly reduced. Total phosphorylation of P70s6K levels was reduced under torin1 treatment confirming mTORC1 inhibition. Protein levels of P70s6K compared to negative control are represented in **Fig 3D.** Treatment with 10 μM HG-9-91-01 resulted in significantly reduced P70s6K levels compared to the negative control. Torin1 treatment did not affect P70s6K levels, however, a gel shift was observed, most likely due to reduced phosphorylation of P70s6K. With regard to the specific phosphorylation rate of P70s6K under HG-9-91-01 treatment, there is no significant variation between negative control and treated samples. The significant reduction in total phosphorylation levels was lost when calculating the specific phosphorylation (**Fig 3H)**.

HDAC4 phosphorylation levels were analyzed to confirm SIK2 inhibition by HG-9-91-01. Phosphorylation of HDAC4 in all treatment concentrations is significantly reduced, showing inhibition of SIK2 compared to a negative control (**Fig 3E)**. Interestingly, torin1 treatment also resulted in a significant reduction in pHDAC4. **Fig 3F** shows HDAC4 protein values under SIK2 inhibition and no significant variation between negative control and treatment was observed. This shows that there is no change in HDAC4 levels after HG treatment. Torin1 treatment may have caused a gel shift, indicating reduced HDAC4 phosphorylation. Specific phosphorylation of HDAC4 was reduced at all HG-9-91-01 concentrations (**Fig 3I).**

2. MRT199665 treatment in 3T3-L1 adipocytes results in mTOR inhibition

To investigate the effect of inhibiting SIKs and their relatives in the AMPK family, we analyzed cell lysates from differentiated 3T3-L1 adipocytes after treating the cells with MRT199665. MRT199665 is an inhibitor of AMPK, SIK1-3, MARK (microtubule affinity regulating kinase)

and other AMPK-related kinases. We analyzed total and specific phosphorylation of ULK1 and P70s6K levels by western blotting by preparing cell lysates from differentiated 3T3-L1 cells after one hour of treatment with MRT.



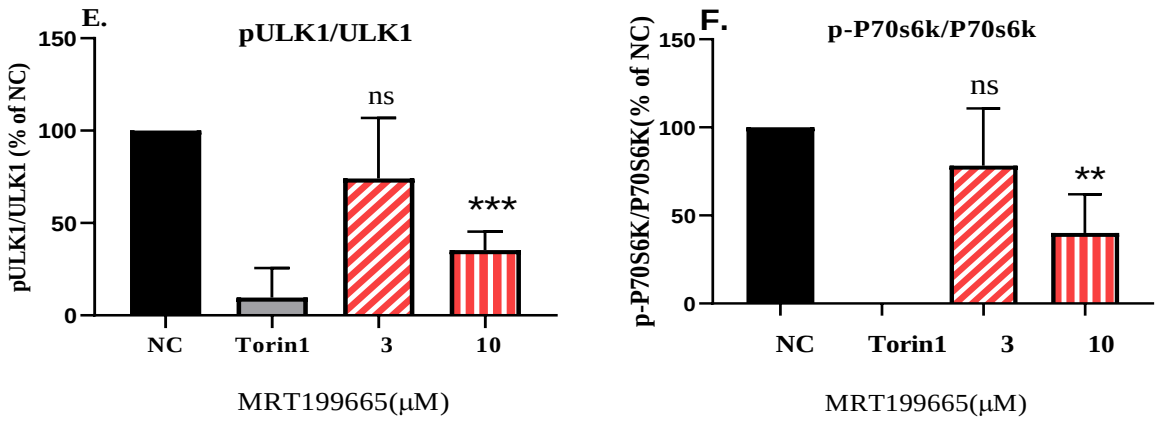


Figure 4 MRT19665 treatment in 3T3-L1 adipocytes results in inhibition of mTOR
Differentiated 3T3-L1 adipocytes were treated with different doses of MRT199665 (A-F) for one hour. Graphs shows quantification of ULK1 (A, B, E) and P70s6K (C, D, F) normalized to actin with the relevant western blot shown below. All results are expressed as a percentage of negative control and shown as mean ± SD (n=5-7 independent experiments). Statistical analysis was performed by one-way ANOVA with Holm -Sidak's multiple comparison test (A-F) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001).

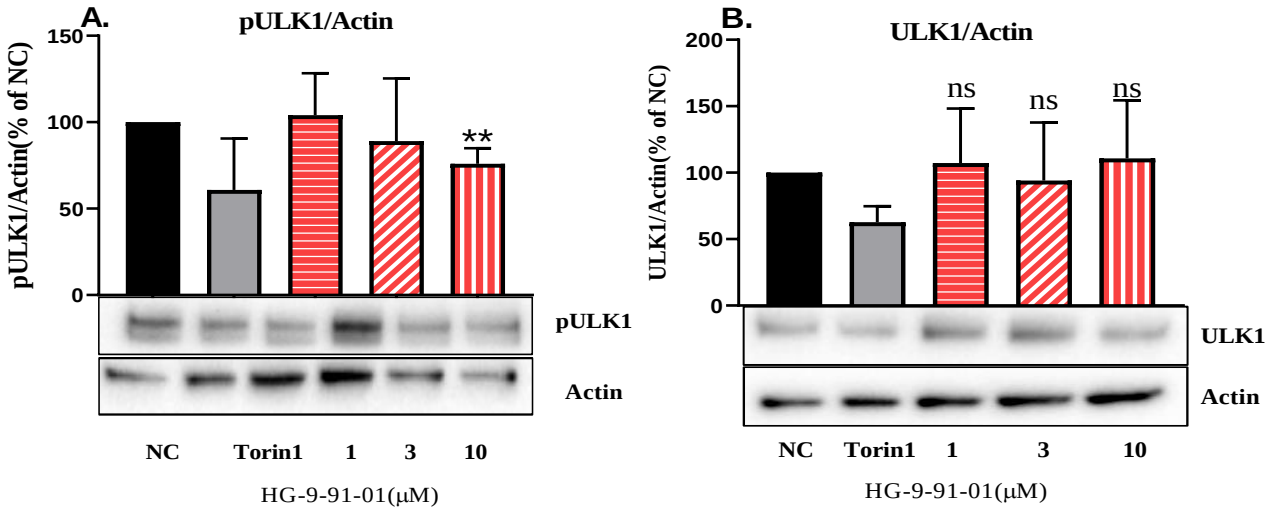
We observed a significant decrease in the total phosphorylation of ULK1 after both 3 μM and 10 μM of MRT199665 compared to the negative control (**Fig 4A)**. This may be due to inhibition of SIKs/AMPK, which may have caused inhibition of mTOR, resulting in decreased pULK1 levels. Torin1, as expected, inhibits mTOR, which was seen as a marked reduction in phosphorylation of ULK1. Concerning ULK1 protein levels (**Fig 4B),** there is no significant difference between the treated cells and the negative control, hence there is no change in the protein levels of ULK1 after inhibition of AMPK and AMPK-related kinases by MRT199665. **Fig 4E** shows the specific phosphorylation of ULK1 and there is a significant reduction with 10 μM MRT199665 compared to negative control whereas no significant reduction is seen with 3 μM.

Post inhibition of SIKs, AMPK and other AMPK-related kinases by MRT199665, P70s6K phosphorylation is reduced significantly at a higher concentration of MRT199665, as seen in **Fig 4C**. There is no significant difference between 3 μM MRT and negative control but there is a

significant reduction between 10 μM and negative control. Torin1 has inhibited mTOR since there is no phosphorylation of P70s6K. **Fig 4D** shows no significant reduction in P70s6K protein after MRT199665 treatment. In the case of torin1, there is a gel shift in the band which indicates reduced phosphorylation of the protein, supporting mTOR inhibition. Specific phosphorylation of P70s6k under MRT199665 exposure shows significant reduction with the highest concentration, 10μM, of the inhibitor (**Fig 4F)** and no significant difference between 3 μM of MRT199665 and control was seen in specific phosphorylation of P70s6K. This shows that inhibition of SIK/AMPK and other AMPK-related kinases after MRT199665 treatment has led to inhibition of mTOR. Torin1 treatment shows significantly reduced specific phosphorylation levels of P70s6K further indicating mTOR inhibition. Hence, under MRT199665 treatment SIKs/AMPK/MARKs for example along with other AMPK-related kinases are inactivated, which has led to inactivation of mTOR, which is shown by significantly reduced phosphorylation of ULK1 and P70s6K.

3. Human adipocytes after HG-9-91-01 treatment showed no mean significant difference in specific phosphorylation of ULK1 and P70s6k.

In order to confirm the effects obtained in 3T3-L1 adipocytes, it is important to investigate the signalling pathways in human adipocytes hence we investigated the role of SIK2 in the regulation of mTOR activity by western blotting in primary adipocytes after isolating them from human adipose tissue.



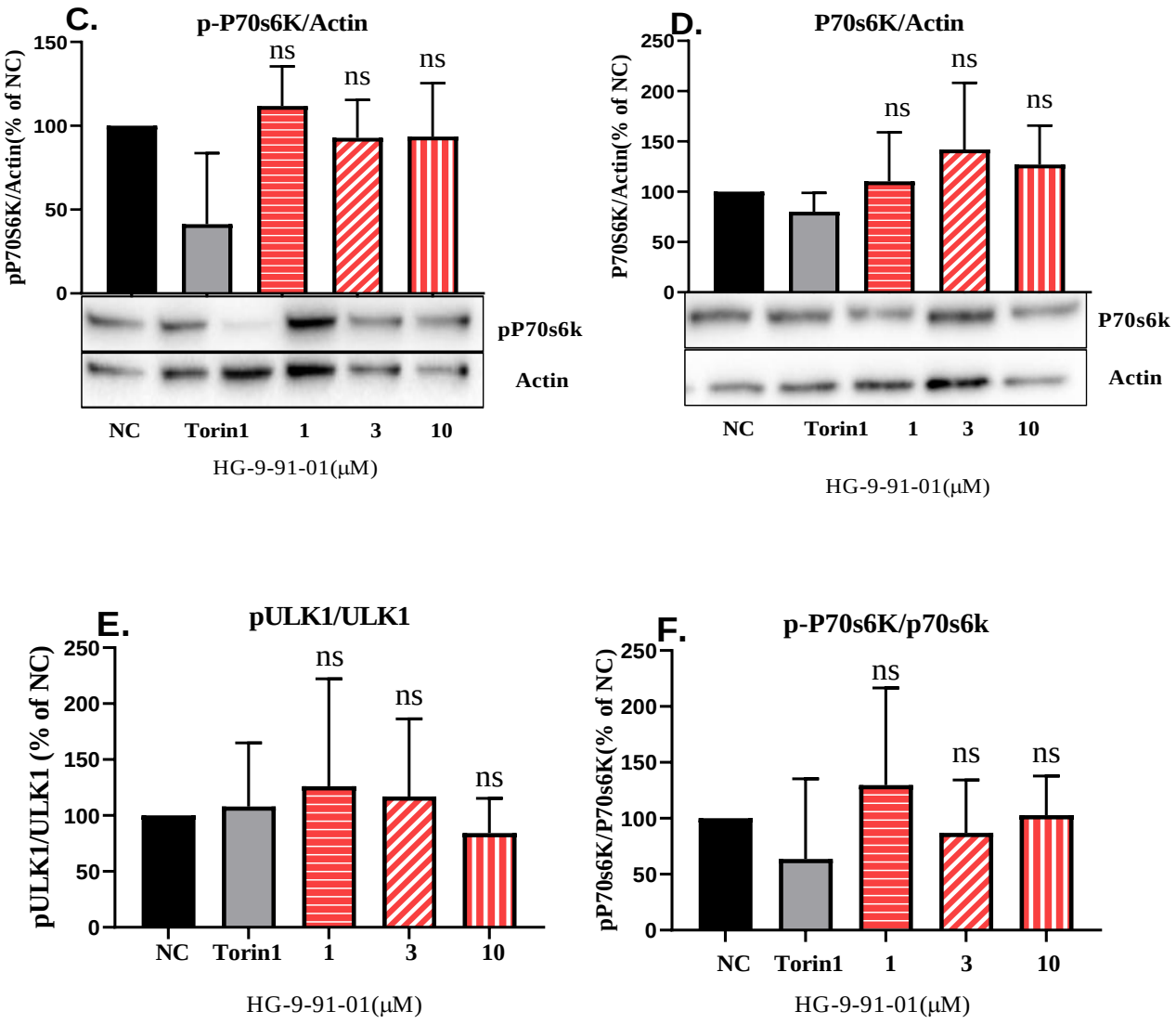


Figure 5 HG-9-91-01 treatment in human adipocytes

Human adipocytes were treated with different doses of HG-9-91-01 (A-F) for one hour. Graphs represent quantification of ULK1 (A, B, E) and P70s6K (C, D, F) normalizd to actin with a representative western blot shown below. All results are expressed as a percentage of the negative control and shown as mean ± SD (n=4 independent experiments). Statistical analysis was performed by one-way ANOVA with Holm -Sidak's multiple comparison test (B-F) or two tailed student's t-test (A) (*p < 0.05, **p < 0.01, ***p < 0.001, ****p<0.0001).

Fig 5A shows pULK1 after HG treatment in primary human adipocytes and there is a significant reduction after 10 µM. The protein levels of ULK1 **(Fig 5B)** show no significant reduction between control and treatment, which could be due to no actual reduction or due to high variation between the independent experiments. Specific phosphorylation of ULK1 (**Fig 5E)** was not significantly different between negative control and HG-9-91-01 treatment. This could be due to high variation and more experiments are required to confirm if there is a change in specific phosphorylation of ULK1 after HG treatment. With respect to P70s6K, the total phosphorylation (**Fig 5C)** and the protein levels (**Fig 5D)** show no significant mean difference compared to the negative control. Furthermore, there is no significant reduction in the specific phosphorylation of P70s6K (**Fig 5E)** between the negative control and treatment. This indicates that HG treatment has not resulted in mTOR inhibition. However, more experiments are required to confirm these results.

DISCUSSION

Our results suggest that AMPK-related kinases have a role to play in maintaining the activity of mTOR which is evident from the results from MRT199665. The results show reduced mTOR activity upon inhibition of AMPK and AMPK-related kinases, whereas when specific AMPK-related kinases were inhibited, that is the SIK family, the same results were not obtained, rather there was a decrease in the protein levels of ULK1 and P70s6k. To complement the results obtained in 3T3-L1 adipocytes, human adipocytes were also treated with HG-9-9-01. Inhibition of SIKs did on average not affect the specific phosphorylation of ULK1 and P70s6k in these cells.

The main reasoning behind choosing AMPK and the AMPK-related family, especially SIK family, as a focus of our investigation, was because recent studies in our laboratory showed that SIK2 is required for autophagy and that inhibition of SIK2 resulted in reduced TFEB expression as well as in TFEB dephosphorylation **[36]**. This dephosphorylation of TFEB observed after SIK inhibition could be due to SIK2 acting on novel substrates which in turn phosphorylate TFEB. A candidate for this novel substrate would be mTOR or one of its upstream regulators since mTOR is a known upstream kinase of TFEB [**23,33,34**]. Moreover, studies have shown that SIK2 is required for the proper metabolism of glucose and lipid **[50].** Furthermore, SIK2 expression is reduced in adipocytes from obese and insulin-resistant individuals and inhibition of SIK2 pharmacologically or genetically resulted in impaired glucose uptake and signalling of insulin **[51].**

Our results suggest that inhibition of the whole AMPK family by MRT199665 resulted in inhibition of mTOR activity. This contrasts with what would we expect from inhibiting AMPK alone since previous studies have shown that, usage of an AMPK inhibitor yielded increased activity of mTOR **[52,53].** Consequently, we believe that the results obtained after MRT19965 treatment concerning mTOR was not due to AMPK inhibition but rather inhibition of AMPK-related kinases, like SIKs or MARKs for example **[54].** We speculate that these AMPK-related kinases can be involved in mTOR inhibition.

To specifically test whether the SIK family is involved in mTOR regulation, we used a chemical inhibitor HG-9-91-01 **[47]**, which blocks all three SIK isoforms. Our results suggest decreased protein levels of ULK1 and P70s6K rather than decreased mTOR activity.To complement our work in cultured murine cells, we replicated the experiments in human adipocytes, but these results did not give any significant results. More experiments were not performed due to the unavailability of human adipose tissue.

The decrease in the protein levels of ULK1 and P70s6K observed with HG-9-91-01 could be due to reduced regulation of ULK1 and P70s6K translation. It is unlikely for reduced protein levels to be a consequence of transcriptional changes since the treatment time was only for an hour. For a transcriptional modification to be the likely scenario, longer periods would probably have been required. Hence, we could hypothesize that it could be a post-translation modification. Our hypothesis of this post-translation modification would be via proteasomal degradation of ULK1 and P70s6k upon HG-9-91-01 treatment. From our results, one could speculate that SIK2 inhibition by HG-9-91-01 has resulted in proteasomal degradation and that SIK2 under normal conditions is protective of ULK1 and P70s6K. The underlying mechanism could be that SIK2 regulates other proteins or enzymes, such as ubiquitin ligases, which are responsible for tagging ULK1 and P70s6K for ubiquitylation. More experiments will have to be performed to confirm these decreased protein levels and to describe the mechanisms. Torin1 was used as a positive control since it is known as a potent inhibitor of mTOR **[55]**. Indeed, from the results, we can see that there was a marked reduction in ULK1 and P70s6K specific phosphorylation and a molecular weight band shift in the case of P70s6K after torin1 treatment. We could also observe that there were no decreased protein levels of ULK1 and P70s6K, unlike HG-9-91-01. These observations further support the conclusion that HG-9-91-01 does not inhibit mTOR activity.

Changes in phosphorylation of HDAC4 was used as a control for successful inhibition of SIKs by HG-9-91-01. However, we also observed reduced phosphorylation of HDAC4 upon torin1 treatment, which could indicate the mTOR regulation of HDAC4. This is in line with studies showing that mTOR, especially mTORC2, promotes inhibitory phosphorylation of class IIa HDAC's **[56]**

Future studies could include testing the hypothesis that proteasomal degradation of ULK1 and P70s6K mediates the effect of HG-9-91-01 treatment on ULK1 and P70s6K protein levels. This could be done by inhibiting the proteasome machinery with inhibitors like MG132 **[57]**. Using other inhibitors of SIKs, such as ARN-3236, YKL-05-099 and YKL-05-093, can also be interesting. The ARN-3236 have a different structure concerning HG-9-91-01 hence it would be interesting to see their effect on mTOR activity **[35]**. Using other techniques such as RNA interference to silence SIK2 can also be employed to complement the results. With MRT199665 treatment we can assume that AMPK-related kinases are involved in controlling mTOR activity, and in future studies, we can use other AMPK-related kinase inhibitors besides HG-9-91-01. AMPK-related kinases may be used as a tool to modify autophagy which in the end can provide us with a new target to treat diabetes and obesity.

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