mTORC1 Activates SREBP-2 through Maintenance of Endosomal Cycling and Suppression of Autophagy

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A thesis submitted to the Faculty of Graduate and Postdoctoral Studies in partial fulfillment of the requirements for the PhD degree in Biochemistry

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ABSTRACT

The mammalian target of rapamycin complex 1 (mTORC1) is known to regulate lipogenesis through sterol regulatory element binding proteins (SREBPs), master regulators of cholesterol and fatty acid synthesis. Through an incompletely understood mechanism, mTORC1 triggers translocation of SREBPs, an endoplasmic reticulum (ER) resident protein, to the Golgi, where mature SREBP is proteolytically produced to activate transcription of lipogenic genes. Low ER cholesterol is a well-known trigger for SREBPs activation, which includes translocation, maturation, and transcriptional activation. The study investigated whether mTORC1 activates SREBP by limiting cholesterol delivery to the ER. The findings indicate an increase in mTORC1 activity is accompanied by lower ER cholesterol and by SREBP-2 activation, a transcription factor primarily responsible for cholesterol synthesis. A decrease in mTORC1 activity, on another hand, coincides with higher ER cholesterol and lower SERBP-2 activity. I further report that this ER cholesterol is of lysosomal origin, as blocking the exit of cholesterol from lysosomes by U18666A or NPC1 siRNA prevents ER cholesterol from rising and, consequently, SREBP-2 is activated without mTORC1 activation. I identified two membrane trafficking processes, triggered by low mTORC1 activity, supply the lysosomes with cholesterol: autophagy and re-routing of endosomes to lysosomes. Indeed, a dual blockade by $Atg5^{-/-}$ and rab5 kept the ER cholesterol low even when mTORC1 activity was low, and resulted in SREBP-2 activation. Conversely, over-expressing Atg7, which forces autophagy, raises the ER cholesterol and suppresses SREBP-2 activity even when mTORC1 activity is high. Thus, it can be concluded that mTORC1 actively suppresses the formation of autophagosomes and promotes endosomal recycling, both of which prevents cholesterol to reach the lysosomes, thereby reducing cholesterol levels in the ER and activating SREBP-2.

ACKNOWLEDGEMENTS

In the name of Allah, most gracious most merciful, the completion of this work wouldn't have been possible without the help and support of many people. I would like to express my sincere thanks to Dr. Xiaohui Zha, to whom I owe an immense debt of gratitude. She has shared her knowledge, supervision and time open-heartedly. Without her, this work wouldn't be possible.

I would like to thank my thesis advisory committee, Dr. Robin Parks and Dr. Thomas Lagace, for their sound advice and comments and to Dr. Alexander Sorisky and Dr. AnneMarie Gagnon for their excellent support, advice and critiques during the study. Many thanks to my lab members: Dr. Kristin Dauner and Dr. Kevin Courtney for their enormous assistance, discussion and support.

I would also like to extend my acknowledgement to King Abdullah International Medical Research Center (KAIMRC), and the government of Saudi Arabia, for giving me the opportunity and supporting me with a scholarship to pursue my PhD studies.

To all who made this journey memorable and enjoyable, I express my love and gratitude, particularly to my parents and siblings for their endless love and support and also to my mother-in-law, Miriam Falconi, for her help in my time of need.

Finally, but not the least, this thesis is dedicated to my husband, Juan Sebastian Arteaga, son, Elias Mateo Arteaga, for their unconditional love and the joy they give me. They are my inspiration to work hard, particularly during difficult times.

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LIST OF ABBREVIATIONS

25-OHCHOL	25-hydroxycholesterol
4E-BPs	Eukaryotic translation initiation factor 4E binding proteins
ABCA1	ATP Binding Cassette Subfamily A Member 1
ACAT	Acyl-CoA: cholesterol acyltransferase
ACC	Acetyl-CoA carboxylase
ADP	Adenosine diphosphate
Akt	Protein kinase B
AMP	Adenosine monophosphate
AMPK	AMP-activated protein kinase
ANOVA	Analysis of variance
ATG	Autophagy-related genes
BHLH-Zip	Basic helix-loop helix leucine zipper
CA-Akt	Constantly active Akt
CE	Cholesteryl ester
CO2	Carbon dioxide
COPII	Coat Protein II
Cys	Cysteine
DAPI	4',6-Diamidino-2-Phenylindole, Dihydrochloride
DEPTOR	DEP domain-containing mTOR-interacting protein
DGAT1	Diacylglycerol acyltransferase 1
DM	Diabetes mellitus
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic acid
DN-Akt	Dominant negative Akt

eIF4E	Eukaryotic translation initiation factor 4E
ER	Endoplasmic reticulum
ERK	Extracellular regulated kinases
ESCRT-0	Endosomal sorting complexes required for transport-0
FA	Fatty acid
FAS	Fatty Acid synthase
FBS	Fetal bovine serum
FDPS	Farnesyl pyrophosphate synthase
Fe	Ferrous
FXR	Farnesoid X receptors
GAP	GTPase-activating protein
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GEF	Nucleotide exchange factor
GFP	Green fluorescent protein
GPAT	Glycerol-3-phosphate acyltransferases
HCl	Hydrochloride
HEK293T	Human embryonic kidney cells
HFD	High fat diet
HIF-1α	Hypoxia inducible factor 1α
His	Histidine
HMGCoA-R	Hydroxymethylglutaryl-CoA reductase
HMGCoA-S	Hydroxymethylglutaryl-CoA synthase
HRP	Horseradish peroxidase
Hrs	Growth factor-regulated tyrosine kinase substrate
IGF-1	Insulin-like growth factor-1

IgG	Immunoglobulin G
INSIG	Insulin-induced gene
IRS1	Insulin receptor substrate 1
KO	Knock out
LAMP1/2	Lysosomal membrane proteins 1 and 2
LC3-I	Microtubule-associated protein 1 light chain 3
LC3-II	Microtubule-associated protein 2 light chain 3
LD	Lipid droplets
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LXR	Nuclear hormone receptor liver X receptor
MCD	Methyl-β-cyclodextrin
MEF	Mouse embryonic fibroblasts
mlsT8	Mammalian lethal with sEC13 protein 8
M-MLV	Moloney Murine Leukemia Virus
moi	Multiplicity of infection
mRNA	Messenger Ribonucleic acid
mSin1	Mammalian stress-activated protein kinase-interacting protein 1
mTOR	Mammalian target of rapamycin
mTOR	Mammalian target of rapamycin complex 2
mTORC1	Mammalian target of rapamycin complex 1
NaCl	Sodium chloride
NaOH	Sodium Hydroxide
NAPDH	Nicotinamide adenine dinucleotide phosphate
NPC1	Niemann-Pick C1

NPC2	Niemann-Pick C2
Ob	Obese
OSBP	Oxysterol-binding protein
ORP	OSBP-related proteins
PBS	Phosphate-buffered saline
PCSK9	Protein convertase subtilisin/kexin type-9
PDK1	Phosphoinositide-dependent kinase 1
PDK2	Phosphoinositide-dependent kinase 2
PE	Phosphatidylethanolamine
PGC1	Peroxisome proliferator activator receptor –γ co-activator 1α
pH	Potential of hydrogen
PI3K	Phosphatidylinositol 3-kinase
PIP2	Phosphatidylinositol-4,5-bisphosphate
PIP3	Phosphatidylinositol-3,4,5-trisphosphate
PKC-α	protein kinase C-α
PP2A	Protein Phosphatase 2A
PPARγ	Peroxisome proliferator activator receptor –γ
PRAS40	Protein-rich Akt substrate of 40-kDa
PUFAS	Poly unsaturated fatty acids
PVDF	Polyvinylidene difluoride
Rags	Ras small GTPase
RAPTOR	Regulatory-associated protein of mTOR
Reddl1	Regulated in development and DNA damage responses1
Rheb	Small GTPase Ras homolog enriched in brain
Rictor	Rapamycin-insensitive companion of mTOR

RNA	Ribonucleic acid
Rom2	Exchange protein 2
ROS	Reactive oxygen species
RPMI	Roswell Park Memorial Institute medium
RT-PCR	Real-time polymerase chain reaction
S1P	Site-1 protease
S2P	Site-2 proteases
s6	Ribosomal protein S6
S6K	Ribosomal protein S6 kinase
SCAP	SREBP cleavage activating protein
SCD1	Stearoyl-CoA desaturase
SDS	Sodium dodecyl sulfate
Ser	Serine
SGK	Glucocorticoid-induced protein kinase 1
siRNA	Small interfering RNA
SRE	Sterol regulatory element
SREBPs	Sterol regulatory element binding proteins
StAR	Steroidogenic acute regulatory protein
START	Steroidogenic acute regulatory protein related
TBS-T	Tris buffered saline-Tween
TCA	Trichloroacetic acid
TF	Transferrin
TFEB	Transcription factor EB
TF-Fe	Di-ferric transferrin
TFR	Transferrin receptor

TG	Triglyceride
TLC	Thin layer chromatography
TMD	Transmembrane domain
TSC1	Tuberous sclerosis complex protein 1
TSC2	Tuberous sclerosis complex protein 2
ULK1	Unc-51 like autophagy activating kinase 1
VAP	Vesicle-associated membrane protein-associated protein

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1 INTRODUCTION

1.1 Cholesterol Homeostasis in Mammalian Cells

Cholesterol, a sterol, is an essential component of mammalian cells. It makes up 30–40 percent of the lipid components in mammalian cell plasma membranes and plays an important role in maintaining membrane fluidity and organelle integrity (Gumí-Audenis et al. 2016). It also facilitates membrane curvature and fusion (Simons and Vaz 2004; Churchward et al. 2005). In addition, it serves as a precursor for bile acids, vitamin D, oxysterols and hormones (Yokoyama 2000). Cholesterol is synthesized by all cells, but the liver is responsible for 10 percent of cholesterol biosynthesis of the whole body (Dietschy, Turley, and Spady 1993). In general, cells can obtain cholesterol from two sources: endogenous biosynthesis by the mevalonate pathway in the endoplasmic reticulum (ER) and uptake of exogenous low-density lipoprotein (LDL) from the circulation through receptor-mediated endocytosis (Goldstein, DeBose-Boyd, and Brown 2006).

Despite cholesterol's importance, its toxic in excess and, in modern society, this imposes a major risk on the populations for the development of pathological conditions such as atherosclerosis, Type 2 diabetes mellitus (DM) and the metabolic syndrome (R. Sato 2010; Goedeke and Fernández-Hernando 2012). Thus, it is important to maintain cellular cholesterol homeostasis through balancing the internal and external supply while avoiding either a cholesterol shortage or an over-accumulation (R. Sato 2010). Consequently, cells have acquired complex mechanisms to control cholesterol homeostasis (Goedeke and Fernández-Hernando 2012). In cells, excess cholesterol is converted by the ER resident enzyme, Acyl-CoA – cholesterol acyltransferase (ACAT) to cholesteryl ester (CE). This temporarily removes the excess cholesterol to storage in lipid droplets (LD) (Rogers et al.

2015). ACAT is an ER resident protein and its enzymatic activity is primarily controlled by cholesterol availability in the ER membrane (Chang et al. 2006). As such, whole cell ACAT activity is commonly used to monitor ER cholesterol level in live cells (X. Xu and Tabas 1991). Conversely, in the absence of sufficient cholesterol supply, cells activate both cholesterol biosynthesis and lipoprotein uptake, which are regulated through a negative feedback mechanism governed by sterol regulatory element binding proteins (SREBPs) **(**see **Figure 1)** (R. Sato 2010).

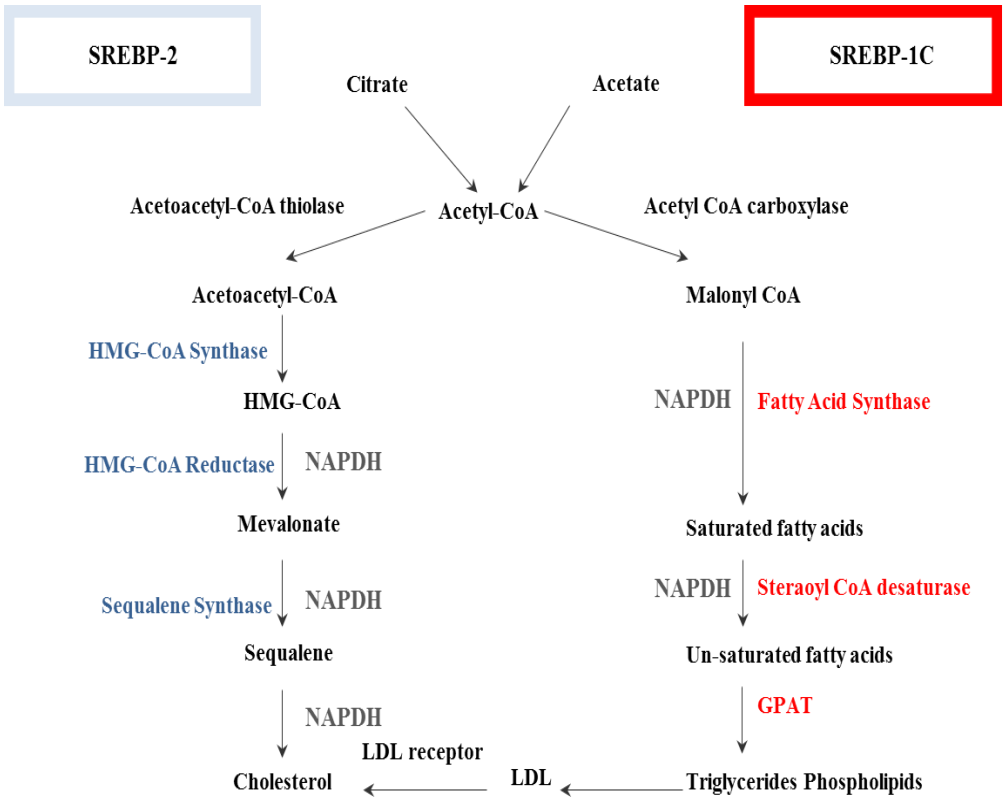


Figure 1: Biosynthesis pathways of cholesterol and fatty acid. The major metabolic intermediates in the pathways for synthesis of cholesterol and fatty acids. SREBP-2 activates genes of cholesterol metabolism, whereas SREBP-1c activates genes of fatty acid metabolism, Hydroxymethylglutaryl-CoA synthase (HMG-CoA), Fatty Acid synthase (FAS), Glycerol-3-phosphate acyltransferases (GPAT), Nicotinamide adenine dinucleotide phosphate (NAPDH).

1.2 Sterol Responsive Element Binding Proteins (SREBPs)

SREBPs are the precursors of transcriptional factors that regulate the expression of genes encoding enzymes and receptors involved in cellular lipogenesis (R. Sato 2010). They belong to the basic helix-loop helix leucine zipper (BHLH-Zip) family and have three isoforms, SREBP-1a, SREBP-1c, which are transcribed from alternative promoters on *SREBF1*, and SREBP-2, from *SREBF2* (J D Horton 2002; Goedeke and Fernández-Hernando 2012).

Each isoform of SREBP has a specific tissue distribution. SREBP-1a is mainly expressed in highly proliferative tissues including cultured cells, while SREBP-1c is highly expressed in the liver, skeletal muscle and adipose tissue. SREBP-2, on the other hand, is expressed ubiquitously and shares only 45 percent homology with SREBP-1a (Goedeke and Fernández-Hernando 2012; J D Horton 2002). SREBP-2 is primarily involved in the expression of genes for cholesterol biosynthesis, as well as the expression of LDL receptors responsible for exogenous LDL uptake (Jay D Horton, Goldstein, and Brown 2002). SREBP-1a and 1c primarily regulate the expression of genes involved in fatty acid synthesis, although there are considerable functional overlap with SREBP-2 (Jay D Horton, Goldstein, and Brown 2002).

1.2.1 Activating and processing SREBPs

SREBPs are synthesized as inactive ER membrane protein that require proteolytic processing in the Golgi to activate their transcription activity (Eberlé et al. 2004). In the ER, SREBPs form a complex with the SREBP cleavage activating protein (SCAP) (R. Sato 2010), a cholesterol sensor. The SCAP/SREBPs complex is then anchored to the ER by the insulin-induced gene (INSIG) (Radhakrishnan et al. 2007; Sun et al. 2007). When the ER

cholesterol level exceeds five percent of the total ER lipids, INSIG binds to the sterol-sensing domain on the SCAP and prevent the complex from exiting the ER (Radhakrishnan et al. 2008; Radhakrishnan et al. 2007; Sun et al. 2007). If the cholesterol level are low (i.e. below the five percent threshold), SCAP undergoes a conformational change such that INSIG can no longer bind to the SCAP (Osborne 2000; Goldstein, DeBose-Boyd, and Brown 2006; Radhakrishnan et al. 2008). This releases the SCAP/SREBP complex to exit the ER through a COPII-mediated secretory pathway (Goldstein, DeBose-Boyd, and Brown 2006; Osborne 2000). Once in the Golgi, SREBPs are cleaved sequentially by site-1 (S1P) and site-2 (S2P) proteases to release the N-terminal fragments (Jay D Horton, Goldstein, and Brown 2002). These fragments, or mature SREBPs, are transcription factors that enter the nucleus, bind to promoters with sterol regulatory elements (SRE) and activate the expression of lipogenic genes (Espenshade 2006; Goldstein, DeBose-Boyd, and Brown 2006; Osborne 2000). High cholesterol level, on another hand, cause the retention of the SCAP/SREBP by INSIG in the ER, therefore preventing SREBP translocation and proteolytic processing. Thus, SREBPs remain in inactive or premature forms (see **Figure 2**) (Espenshade, Li, and Yabe 2002).

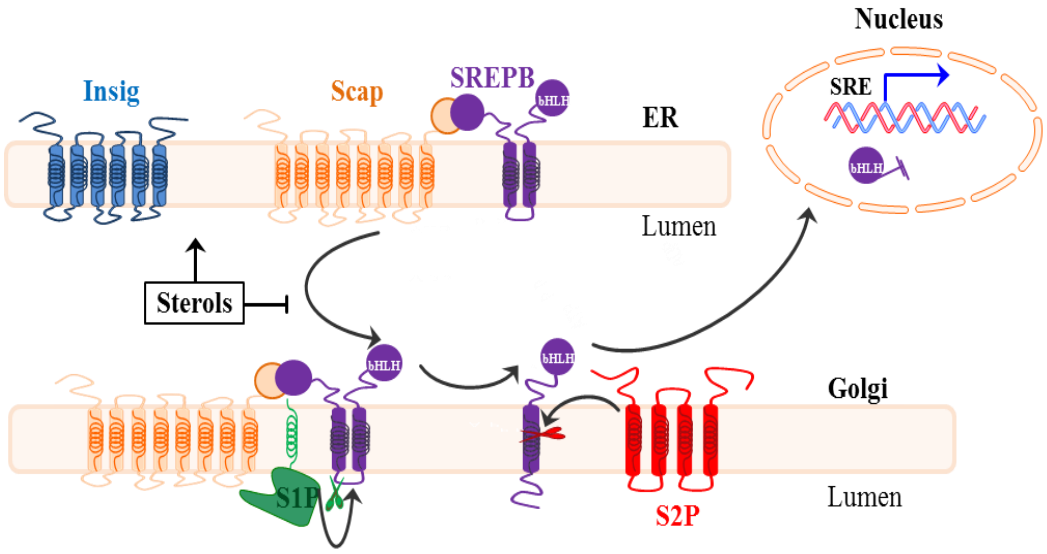


Figure 2: SREBP pathway. When cells are low in cholesterol, ER membrane bound SREBPs are escorted by SCAP to the Golgi. The two proteases S1P and S2P located on Golgi then cleave SREBP, releasing the active transcription factor, which is imported into the nucleus and activates genes involved in fatty acid, cholesterol synthesis and uptake. High cholesterol levels triggers SCAP to bind to Insig, retaining SREBP to the ER and inhibiting/blocking transport to the Golgi and subsequent transcriptional activation.

In addition, the SREBP genes also have SRE in their promoter regions. This allows mature SREBPs to upregulate their own expression by a positive feedback mechanism (Shao and Espenshade 2012). Furthermore, microRNAs-33a/b which is located within the intron of SREBP is co-transcribed with mature SREBP-1a/2, which negatively regulate cholesterol export by down-regulating the expression of ATP-binding cassette transporter A1 (ABCA1), further maintaining cellular cholesterol homeostasis (Gerin et al. 2010; Ono 2016; Moore et al. 2011). Moreover, SREBP-2 promotes the expression of the protein convertase subtilisin/kexin type-9, or PCSK9. PCSK9 mediates the degradation of the LDL receptor (LDLR) in the liver thus increasing the circulating LDL (Jeong et al. 2008). Loss-of-function mutations of PCSK9 result in higher levels of the LDL receptor in the liver, which lowers LDL cholesterol levels in the plasma and thus offers protection from coronary heart disease (Cohen et al. 2006). PCSK9 is currently a drug target for cholesterol-lowering in humans.

In addition to cholesterol, various cholesterol derivatives also interfere with SREBP pathways. Excess cholesterol, for example, promotes the accumulation of oxysterol derivatives such as 25-hydroxycholesterol, which independently bind to INSIG and promotes SREBP/SCAP complex retention in the ER (Osborne and Espenshade 2009). Oxysterols also stabilize the INSIG gene and further inhibit ER-to-Golgi transport of SREBPs (Goldstein, DeBose-Boyd, and Brown 2006).

Moreover, oxysterols activate the nuclear hormone liver X receptor (LXR), which stimulates the transcription of SREBP-1c by directly binding to the *SREBF1* promoter and promote fatty acid synthesis (Goldstein, DeBose-Boyd, and Brown 2006).

1.2.2 Other SREBP pathway regulators

For decades, cholesterol and its oxysterol derivatives, were thought to be the only effectors of the SREBP/SCAP pathway (Daemen, Kutmon, and Evelo 2013). Recent studies, however, have shown much more complex regulations. Environmental stressors and nutritional/hormonal status, for example, can affect the SREBP activities (Daemen, Kutmon, and Evelo 2013). For example, the exit of SREBPs from the ER is promoted under ER stress by inducing INSIG1 degradation (J. N. Lee and Ye 2004; Schuchman and Wasserstein 2015; Ye and DeBose-Boyd 2011). In addition, thyroid hormones directly auto-regulate and stimulate SREBP2 genes by effecting mRNA and nuclear protein levels (Shin and Osborne 2003). Moreover, hepatic SREBP-1c, which plays a major role in the upregulation of fatty acid synthesis in response to insulin (Osborne and Espenshade 2009), is activated by LXR (Shao and Espenshade 2012).

Insulin is a major stimulus of SREBP activation in the liver. Interestingly, insulin exhibited two major effects: reducing gluconeogenesis and increasing lipogenesis that is mediated by SREBPs (S. Li, Brown, and Goldstein 2010). In the case of obesity, which frequently leads to insulin resistance and eventually to type 2 diabetes, insulin fails to reduce gluconeogenesis but retains its ability to stimulate lipogenesis (Reaven 2005; Matsumoto et al. 2006). This results in hypertriglyceridemia due to SREBP 1c hyper-activation, in addition to hyperglycemia (Michael S. Brown and Goldstein 2008). Thus, the insulin signaling pathway is bifurcated at some point to cause this selective insulin resistance (S. Li, Brown, and Goldstein 2010).

1.2.3 SREBP regulation by intracellular signaling pathways

The phosphatidylinositol 3-kinase (PI3K)/Akt (protein kinase B) pathway is known to regulate SREBPs (Jeon and Osborne 2012). PI3k/Akt are generally involved in regulating cellular events including metabolism, inflammation, cell survival, motility, and cancer progression (Krycer et al. 2010). For example, growth factors activate tyrosine kinase receptors on the plasma membrane, which in turn activate PI3K through direct binding or through tyrosine phosphorylation of scaffolding adaptors, such as insulin receptor substrate 1 (IRS1). PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP3). PIP3 then recruits Akt to the plasma membrane where it is activated by phosphorylation by phosphoinositide-dependent kinase (PDK1 and PDK2) (Krycer et al. 2010).

In the case of SREBPs, SREBP-1a/c target genes are activated in sebocytes by insulin-like growth factor-1 (IGF-1) in a PI3K-dependent manner (Smith et al. 2008). SREBP1a/c can also be activated by platelet-derived growth factors in human fibroblasts (Demoulin et al. 2004). Moreover, constitutively active Akt (CA-Akt) resulted in increased SREBP activity, evidenced by increased promoter activities of the LDL receptor and FAS in human micro-vascular endothelial cells. Conversely, a dominant negative Akt (DN-Akt) inhibited SREBP activation. In addition, ectopic expression of myristoylated-Akt, which is constitutively activated by tethering to the plasma membrane (P. Li et al. 2010), resulted in the upregulation of HMG-CoA synthase, an SREBP-2 target gene (Zhou et al. 2004).

Furthermore, the PI3K/Akt pathway mediates the regulation of SREBPs via insulin signaling. Through Akt/PKB-dependent phosphorylation of SREBP1-c, insulin facilitates the transport of the SREBP/SCAP complex from the ER to the Golgi by increasing the affinity of the complex for COPII proteins and, at the same time decreases its binding to INSIG (Yellaturu et al. 2009).

Interestingly, Akt is hyper-activated in obesity and type II DM, as is another major metabolic regulator, mammalian target of rapamycin complex 1 (mTORC1) (Ždychová and Komers 2005). It is believed that the Akt arm of insulin signaling is disturbed in these pathological conditions resulting in mTORC1 hyper-activation and hence, SREBP hyper-activity. This leads to hypertriglyceridemia (S. Li, Brown, and Goldstein 2010; Ždychová and Komers 2005). How insulin and Akt regulate hepatic SREBP activation through mTORC1 is not well established (S. Li, Brown, and Goldstein 2010).

1.3 The Mammalian Target of Rapamycin (mTOR)

mTOR is a conserved 300 kD serine/threonine kinase that belongs to the phosphoinositide 3-kinase (PI3K)-related kinase family. mTOR signaling plays a central role in the regulation of several biological processes such as cell growth, differentiation, proliferation, survival, immune response and autophagy (Wataya-Kaneda 2015). Overall, mTOR signaling is activated by sensing cellular metabolic status, ranging from energy, hormones and growth factors to nutrients. Its deregulation gives rise to human diseases such as cancer and type 2 diabetes (Laplante and Sabatini 2010; Wataya-Kaneda 2015). As the name implies, mTOR is identified as a target of rapamycin, an antifungal produced by the bacterium Streptomyces hygroscopicus (K. Singh, Sun, and Vezina 1979).

mTOR is the catalytic unit in two structurally and functionally distinct interacting complexes: mTOR complex 1 (mTORC1) and complex 2 (mTORC2). Each complex differs in its upstream signals and downstream targets, thereby regulating distinct cellular processes (Efeyan, Zoncu, and Sabatini 2012; Laplante and Sabatini 2010; Wataya-Kaneda 2015). mTORC1 consists of mTOR, a regulatory-associated protein of mTOR (RAPTOR), and protein-rich Akt substrate of 40-kDa (PRAS40) (D. H. Kim et al. 2002; Oshiro et al. 2007; Laplante and Sabatini 2010). mTORC2, on another hand, is composed of mTOR, a rapamycin-insensitive companion of mTOR (Rictor), and mammalian stress-activated protein kinase-interacting protein 1 (mSin1) (Wataya-Kaneda 2015). Both complexes share mammalian lethal with SEC13 protein 8 (mlsT8; also known as Gβl) and DEP domain-containing mTOR-interacting protein (DEPTOR) (Loewith et al. 2002; Peterson et al. 2009). For details, see **Figure 3**.

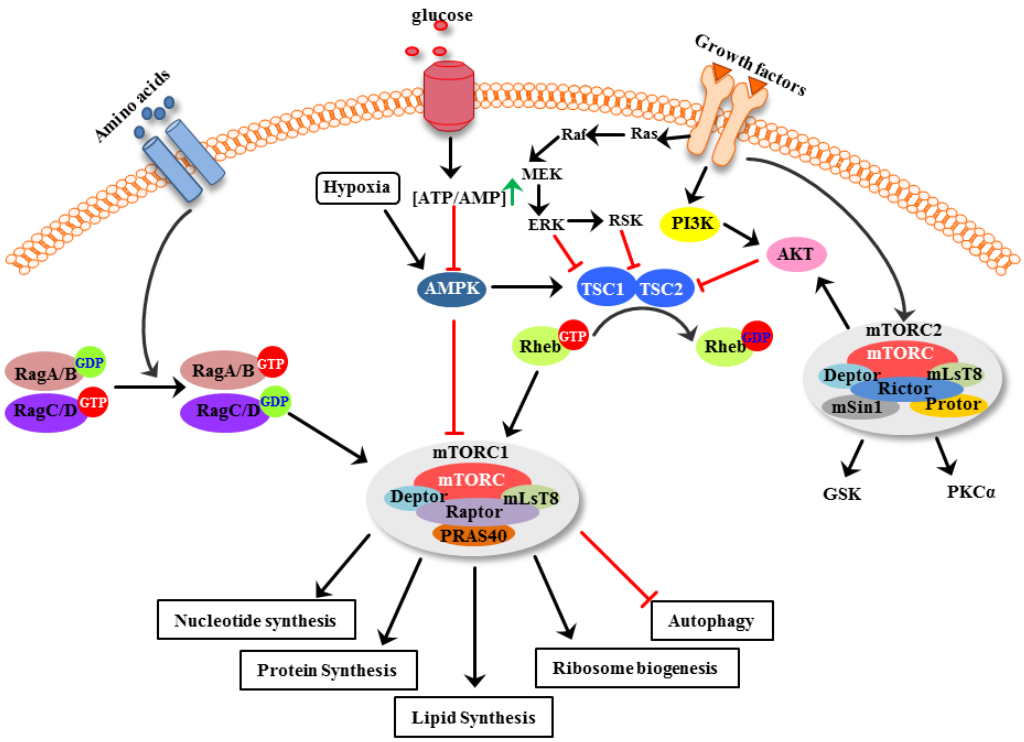


Figure 3: The mTOR signaling pathways. The composition of mTORC1 & 2 and the main signaling inputs are indicated. Growth factors, amino acids, cellular energy status, and stress are integrated into mTORC1. This signal integration occurs at the level of the TSC1–TSC2 complex. Akt and extracellular regulated kinases (ERK) phosphorylate TSC2, thus inhibiting the GAP activity of TSC1–TSC2 towards Rheb. In contrast, AMPK phosphorylate TSC2 activates the TSC1/2 complex and inhibits mTORC1 activity. In response to amino acid Rag GTPases carries the second level of integration by recruiting mTORC1 to the lysosome, allowing it to interact with GTP-bound Rheb and thus activating mTORC1 kinase activity. When mTORC1 is active, it plays a major role in promoting anabolic processes such as protein, lipid, and nucleotide synthesis and ribosome biogenesis, and by inhibiting catabolic processes such as autophagy. On another hand, mTORC2 is regulated by growth factors but, unlike mTORC1, it does not respond to other upstream signals derived from nutrients or stress. mTORC2 phosphorylates several proteins such as serum- and glucocorticoid-inducible kinase (SGK), and protein kinase C (PKC). It also phosphorylates AKT as a feedback into the pathway.

mTORC1 mainly phosphorylates ribosomal protein S6 kinase (S6K) and eukaryotic translation initiation factor 4E (eIF4E) binding proteins (4E-BPs), which are the major regulators of protein synthesis (Ma and Blenis 2004). mTORC1 also upregulates transcription factors involved in ribosome biogenesis (Mayer et al. 2004), lipid synthesis (Porstmann et al. 2009; Huffman, Mothe-satney, and Lawrence 2002; J. E. Kim and Chen 2004), mitochondrial metabolism/biogenesis (Cunningham et al. 2007; Schieke et al. 2006) , and nutrient uptake (Shimobayashi and Hall 2014). At the same time, mTORC1 suppresses catabolic processes such as autophagy (Codogno and Meijer 2005).

mTORC2, on the other hand, is primarily involved in cell survival, metabolism and proliferation through phosphorylation of multiple family kinases (Gaubitz et al. 2016), including protein kinase B (Akt/PKB), serum and glucocorticoid-induced protein kinase 1 (SGK), and protein kinase C-α (PKC-α) (García-Martínez and Alessi 2008; Guertin et al. 2006; Ikenoue et al. 2008; D. H. Kim et al. 2002). It also regulates actin cytoskeleton organization and cell polarization by directly phosphorylating regulators such as Rho1 GDP–GTP exchange protein 2 (Rom2) and the kinase ypk2 (Kamada et al. 2005; Schmidt et al. 1997).

1.3.1 mTORC1 activation

Akt once activated by growth factors, Akt phosphorylates two distinct substrates that have an inhibitory role on mTORC1 activity. The first substrate is the tuberous sclerosis complex protein 2 (TSC2) within the TSC1-TSC2 complex, which functions as a GTPase-activating protein (GAP) for the small GTPase Ras homolog enriched in the brain (Rheb). When TSC is phosphorylated by Akt, it loses its inhibitory GAP activity over Rheb, promoting GTP-bound Rheb, an activator of mTORC1. Furthermore, Rheb is located on

lysosomes and interacts with Raptor, mLST8 and the kinase domain of mTOR (T. Sato et al. 2009; Long et al. 2005). The second substrate of Akt is PRAS40, which inhibits mTORC1 by binding to Raptor and preventing mTORC1 activation by Rheb-GTP. Akt phosphorylates PRAS40 and relieves its binding to mTORC1, thereby activating mTORC1 (see **Figure 3**) (Sancak et al. 2007; Haar et al. 2007).

mTORC1 activity is also be regulated by additional cellular metabolic statuses such as energy level. High adenosine diphosphate (ADP) and monophosphate (AMP) levels that manifest under low energetic states activate AMP-activated protein kinase (AMPK), which directly phosphorylates TSC2 on Thr1227 and Ser1345 residues, thereby stimulating its GAP activity and inhibiting Rheb. This leads to mTORC1 inactivation (Xiao et al. 2011; Inoki, Zhu, and Guan 2003). AMPK also inhibits mTORC1 activity by directly phosphorylating Raptor, the substrate-binding subunit of mTORC1, at residues Ser722 and Ser792 (Gwinn et al. 2008).

Deoxyribonucleic acid (DNA) damage is implicated in mTORC1 activation. In response to DNA damage, p53, a tumor suppressor transcription factor, activates AMPK and TSC2 expression, resulting in mTORC1 inhibition (Feng et al. 2007). In addition, under low oxygen conditions (hypoxia), the transcription hypoxia inducible factor 1α (HIF-1α) induces the expression of Reddl1 (Regulated in development and DNA damage responses 1), which in turn activates TSC2 and hence inhibits mTORC1 (Brugarolas et al. 2004).

On the other hand, nutrients, especially amino acids, activate mTORC1 independent of the TSC complex (Shimobayashi and Hall 2016). Ras small GTPase (Rags) were identified as essential components in amino acid sensing upstream of mTORC1 activation (Bond

2016). Mammalian Rags consist of four Rag proteins – A, B, C & D – which are present in two complexes, Rag A/B and Rag C/D that form a heterodimer. The activation of Rag GTPase is reflected by their guanine nucleotide binding state (i.e. RagA/B are GTP bound and RagC/D are GDP bound). When amino acids are available, the inactive RagA/B.GDP-RagC/D.GTP complex that binds to the lysosome surface through the Ragulator complex, a nucleotide exchange factor (GEF). This switches the Rag heterodimer into an active complex (RagA/B.GTP–RagC/D.GDP) (Bar-peled et al. 2012). Active Rag then interacts with Raptor to recruit mTORC1 to the surface of the lysosomes. This allows mTORC1 to physically interact with Rheb and be activated (see **Figure 4**) (Bar-Peled and Sabatini 2014).

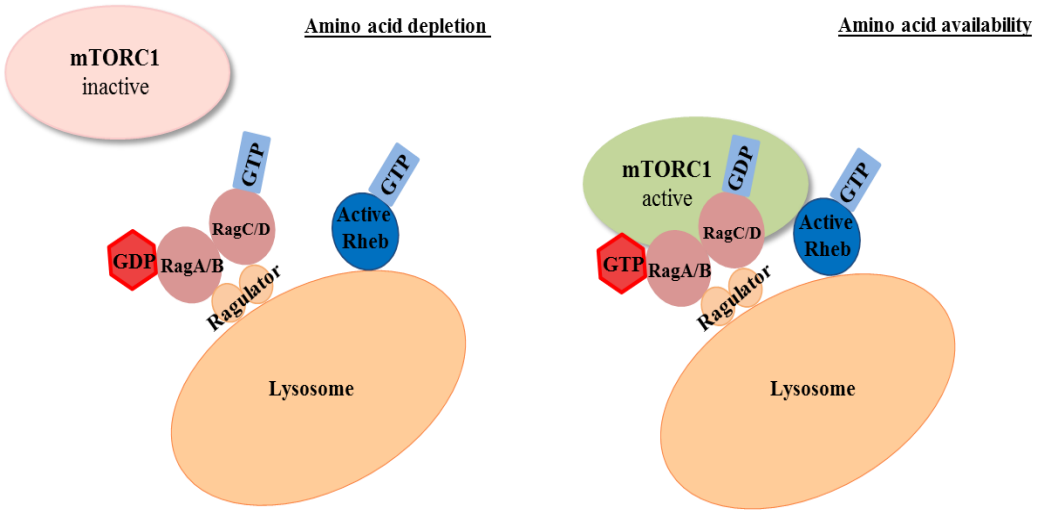


Figure 4: Rag proteins mediate the activation of mTORC1 in response to amino acids. The RagA/B–RagC/D heterodimer is anchored to the surface of the lysosome by Ragulator. In cases when amino acids are low (left), the Rag heterodimer is inactive (RagA/B–GDP and RagC/D–GTP). This prevents it from recruiting mTORC1 to the lysosomal surface. When amino acids are available (right), the Rag heterodimer is active (RagA/B exchanges GDP for GTP and RagC/D converts its GTP to GDP). This change in conformation binds to, and thereby recruits, mTORC1. Once recruited to the surface of the lysosome, mTORC1 interacts with a GTP-Rheb and becomes activated.

1.3.2 mTORC1 regulation of lipogenesis

In recent years, it has become increasingly clear that mTORC1 plays a central role in promoting lipid biogenesis by upregulating the expression of lipogenic genes via SREBPs (Shao and Espenshade 2012). The activation mechanism includes SREBP translocation from the ER to the Golgi, proteolytic processing in the Golgi, and binding of the SRE elements in the promoters of genes in the nucleus. Furthermore, Akt activates SREBPs by enhancing mTORC1 activity. Several studies have used mTOR inhibitor rapamycin as a test for mTORC1 dependence. For example, rapamycin blocked the nuclear accumulation of the mature SREBP-1a and expression of SREBP-1a target genes (Porstmann et al. 2008).

In addition, rapamycin blocked insulin induction of SREBP-1c activation in rat hepatocytes (S. Li, Brown, and Goldstein 2010). However, as mentioned earlier, rapamycin broadly binds mTOR, which exert inhibition to both mTORC1 and mTORC2 to various extents. This led to the development of Torin-1, an mTOR catalytic site ATP competitive inhibitor, a more potent and more selective inhibitor of mTORC1. Torin-1 was found to also potently block SREBP activation in mouse fibroblasts (Peterson et al. 2011). In addition to mTORC1 inhibitors, silencing Raptor and thus inactivating mTORC1 prevented the processing of SREBP-1a and the expression of lipogenic genes (Porstmann et al. 2009). On the other hand, when mTORC1 was hyper-activated in an obese mouse model, SREBPs were also hyper-activated, leading to steatosis (Moon et al. 2012). Interestingly, deletion of hepatic SCAP prevented steatosis in this obesity mouse model (Moon et al. 2012). As SCAP directly senses the cholesterol level in ER prior to escorting SREBP to the Golgi for proteolytic processing, this was the first indication that the ER cholesterol level may participate in mTORC1-induced SREBP activation.

It was suggested that mTORC1 regulates SREBPs through lipin-1, a phosphatidic acid phosphatase involved in triacylglycerol biosynthesis that can also function as a transcriptional coactivator in lipogenic gene expression (Reue 2009). When mTORC1 is active, lipin-1 is sequestered in the cytosol in a hyper-phosphorylated state that prevents its nuclear entry. On the other hand, inactivation of mTORC1 results in the dephosphorylation of lipin-1 and its nuclear import. Nuclear lipin-1 promotes mature SREBPs to associate with the nuclear matrix and impairs their ability to bind SREs (Peterson et al. 2011). Indeed, constitutive dephosphorylated lipin-1 significantly impairs SREBP function and conveys resistance to hepatic steatosis and hypercholesterolemia, induced by high-fat and high-cholesterol diets (Peterson et al. 2011).

Nevertheless, it should be noted that lipin-1 primarily interferes with mature SREBPs in the nucleus. It therefore does not offer a full explanation of how SREBPs mature and enter the nucleus, a step necessary for SREBP activation by mTORC1 (see above SCAP KO study). Thus, it remains to be determined how mTORC1 promotes SREBP translocation from ER and proteolytic processing in the Golgi. As a well characterized trigger for SREBP activation is ER cholesterol (Sun et al. 2007), it is plausible that mTORC1 may influence ER cholesterol by regulating membrane trafficking. One of the best-known membrane trafficking events regulated by mTORC1 is autophagy.

1.4 Autophagy

Autophagy, or self-eating, is an ancient and highly conserved process (King 2012). Essentially, under condition of nutrient limitation, organisms are forced to consume their own non-essential cellular components and adapt to less energy-consuming metabolic pathways in order to survive (Gallagher, Williamson, and Chan 2016; Yin, Pascual, and Klionsky 2016). Macroautophgy, referred to hereafter as autophagy, is a cellular process in which a double membrane structure (phagophore) is formed under energy and nutrient stress conditions. Phagophores then elongate and engulf a portion of the cytoplasm containing protein aggregates, lipid droplets, organelles or foreign substances (Mizushima 2007; Glick, Barth, and Macleod 2010; Gallagher, Williamson, and Chan 2016). Phagophores are then enclosed to form double bilayer membrane vesicles, known as autophagosome, which undergo further maturation to eventually fuse with the late endosomes/lysosomes (Gallagher, Williamson, and Chan 2016; Mizushima 2007). This enables the lysosomal degradation of the contents of the autophagosomes leading to the release of amino acid and membrane components, such as fatty acids and cholesterol, to be re-used by the cell (Gallagher, Williamson, and Chan 2016; Mizushima 2007).Autophagy was first described in yeast, where approximately 30 autophagy-related (ATG) genes were identified. Their mammalian genetic homologues were discovered thereafter (Thumma et al. 1994; Tsukada 1993).

In mammalian cells, starvation induces the initiation of autophagy at a phagophore assembly site (PAS), mediated by the action of unc-51 like autophagy activating kinase 1 (ULK1) complex. ULK1 then transduces cellular signals downstream autophagy to promote phagophore membrane elongation and autophagosome formation (Kaur and Debnath 2015). The origin of autophagosomes in mammalian cells has been traced to a wide range of cellular

organelles, including the ER (Axe et al. 2008), mitochondria (Hailey et al. 2010), endosomes, the Golgi (Puri et al. 2013) and plasma membrane (Ravikumar et al. 2008). Such plasticity of autophagosome initiation may reflect the ancient as well as membrane contact sites in the mitochondria and ER primitive/adoptive nature of this process. Regardless, the elongation and closure of the isolation membrane requires ubiquitin-like protein complexes in two conjugation reactions (Ohsumi and Mizushima 2004). First, Atg5 and Atg12 are conjugated to each other forming an Atg5–Atg12 complex on the isolation membrane. This complex then facilitates the recruitment of LC3-I (microtubule-associated protein 1 light chain 3) and subsequently its conversion to LC3-II by covalent conjugation with phosphatidylethanolamine (PE), resulting in membrane bound LC3-II (Ichimura et al. 2000). PE conjugation to LC3-I is required for expansion of the autophagic membrane, its ability to recognize cargo, and the fusion of the autophagosome to the lysosome (Kaur and Debnath 2015). Usually, LC3-II from the outer surface is recycled back to LC3-I by a Atg4-mediated mechanism and returned to the cytosol. LC3-II on the inner surface is degraded through the fusion with the lysosomes (Tanida, Ueno, and Kominami 2004). As such, the amount of LC3-II is commonly used as a marker for autophagy to estimate the rate of autophagy, or autophagic flux (Mizushima 2007).

The final step in the autophagic process is the fusion of the autophagosome with endosomal compartments (amphisomes) or the lysosome to form an autolysosme, eventually delivering the cargo to be degraded by low pH lysosomal acid hydrolases. This last fusion step is mediated by the small GTPase Rab7 and lysosomal membrane proteins LAMP1/2 (see **Figure 5)** (Bj et al. 2009).

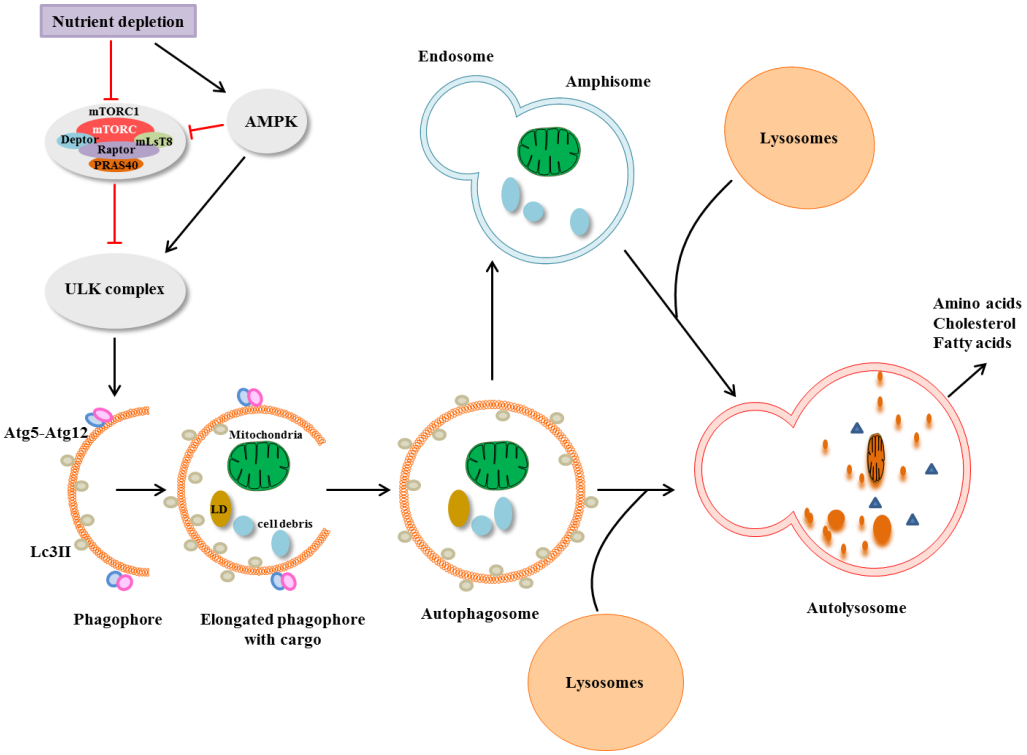


Figure 5: The autophagic pathway. Upon nutrient deprivation, mTORC1 is inhibited while AMPK is activated. This leads to the activation of the ULK complex that promotes phagophore/autophagosome formation. To expand the autophagosome membrane two "ubiquitin-like conjugation systems" are required to catalyze the formation of the ATG5–ATG12 conjugate and phosphatidylethanolamine (PE)-conjugated LC3 (LC3-II) and direct its proper incorporation into the phagophore membrane. The elongated phagophore then closes, marking the formation of a mature autophagosome. Some autophagosome fuses with endosomes forming an amphisome. The complete autophagosome and amphisomes then fuse with a lysosome and its cargo is degraded for the recycling of nutrients and metabolites.

1.4.1 Autophagy regulation by mTORC1

A large body of evidence demonstrates that mTORC1 is a primary regulator of autophagy, which includes both autophagy machinery and lysosomal biogenesis (J. Kim et al. 2011). Autophagy, activated by acute nutrient deprivation, has been widely studied in the context of mTORC1 suppression (Gallagher, Williamson, and Chan 2016). Under starvation conditions, mTORC1 is inactivated. ULK1 is phosphorylated at Ser317 and Ser777 by AMPK, which induces autophagy (Nixon 2013; J. Kim et al. 2011). AMPK also inhibits mTORC1 directly in two mechanisms – phosphorylation of TSC2, and Raptor (Gwinn et al. 2008; Inoki, Zhu, and Guan 2003). On the other hand, under nutrient-rich conditions, mTORC1 phosphorylates ULK1 at multiple sites including Ser637 and Ser757 to inhibit autophagy.

As autophagy requires lysosomal degradation activity to produce nutrients, lysosome biogenesis is also intimately linked to autophagy and mTORC1 activity. For example, transcription factor EB (TFEB) is required for the expression of genes involved in autophagy, such as Atg5, and lysosomal biogenesis. mTORC1 phosphorylates TFEB to prevent it from entering the nucleus. Dephosphorylation by nutrient starvation and mTORC1 inhibition enables TFEB to enter the nucleus and activate the transcription of genes involved in autophagy and lysosome biogenesis (Martina et al. 2017).

1.4.2 Autophagy and lipid metabolism

The notion that autophagy plays a significant role in lipid metabolism has gained much support in recent years. Autophagy is known to mobilize cellular stores of lipids, carbohydrates and ferritin to supply metabolites during normal and stressed conditions (Kaur and Debnath 2015). In nutrient-starved hepatocytes, autophagy selectively targets lipid storage in a process called macrolipophagy (lipophagy), wherein lipid droplets are captured and delivered by autophagosomes to the lysosomes to release fatty acids for mitochondrial oxidation. Accordingly, genetically inactivating autophagy causes the accumulation of triglycerides (R. Singh et al. 2009). Excess cellular lipids, on the other hand, negatively affect autophagy in cultured cells and animals, leading to further deterioration in already damaged metabolic conditions. This interference by autophagy is due to diminishing autophagosome-endolysosomal fusion and/or suppressing the acidic and hydrolytic activity of the lysosome (Koga, Kaushik, and Cuervo 2017; Las et al. 2011). Indeed, defective autophagy in a mouse's liver leads to excessive LD accumulation (R. Singh et al. 2009), a common consequence of SREBP-1c hyper-activation. Similarly, Atg7 haplo-insufficient mice have reduced lipophagy with lipids accumulation, resulting in the progression from obesity to diabetes (Lim et al. 2014).

During lipophagy, lipids delivered by autophagosomes are broken down by the acidic lipases in lysosomes. These lipases are transcriptionally upregulated by the HLH-30 transcription factor in response to starvation in Caenorhabditis elegans (Rourke and Ruvkun 2014). The mammalian orthologue of HLH-30, TFEB, also responds to starvation by inducing the expression of autophagy and lysosomal genes (Settembre et al. 2011). Depletion of TFEB in the mouse liver by a 24-hour fast caused lipid droplet accumulation due to

defective autophagy and lipid degradation. In addition, TEFB activates peroxisome proliferator activator receptor –γ (PPARγ) co-activator 1α (PGC1) to promote lipid catabolism (Settembre et al. 2013). In contrast, nuclear farnesoid X receptors (FXR) binds to the promoter regions of autophagy genes in fed cells, thereby competing with PPARγ and inhibiting lipophagy (J. M. Lee et al. 2014).

Interestingly, genetic ob/ob and high-fat fed obese mice were found to be defective in autophagy, due primarily to the degradation of autophagy proteins such as Atg7 and Atg5 (Yang et al. 2010). In these mouse models, defective autophagy was also accompanied by hyper-activated SREBP-1c and lipogenesis (Moon 2017). Significantly, such SREBP-1c activation is mainly due to elevated proteolytic processing. Furthermore, in a non-insulin-dependent diabetic animal model, SREBP-2, as well its target gene HMGCOA reductase, was upregulated and accompanied by lower liver ACAT activity (Jiao et al. 1991). It is also noteworthy that autophagy is down-regulated in aging animals, which may make them more susceptible to environmental nutrient challenges to develop fatty liver, hypertriglyceridemia and overall dyslipidemia (Foster and Fingar 2010). Thus, autophagy may present a mechanism to counter metabolic deregulation, particularly when mTORC1 and SREBP are hyper-activated.

1.5 The Endocytic Pathway and mTORC1

In addition to autophagy, another major membrane trafficking event regulated by mTORC1 is endocytosis (Dauner et al. 2017). In cultured and highly proliferating cells, endocytosis is defined as the uptake of extracellular material in vesicles invaginated from the plasma membrane. Those vesicles, or early endosomes, undergo acidification to develop into sorting endosomes, where membrane and membrane-bound receptors are separated from the lumen cargo. Bulk membranes and receptors then form recycling endosomes and return to the plasma membrane for another run of the delivery. The endosomes containing the bulk lumen cargo, or late endosomes, then mature into lysosomes (see **Figure 6**) (Mayor, Presley, and Maxfield 1993).

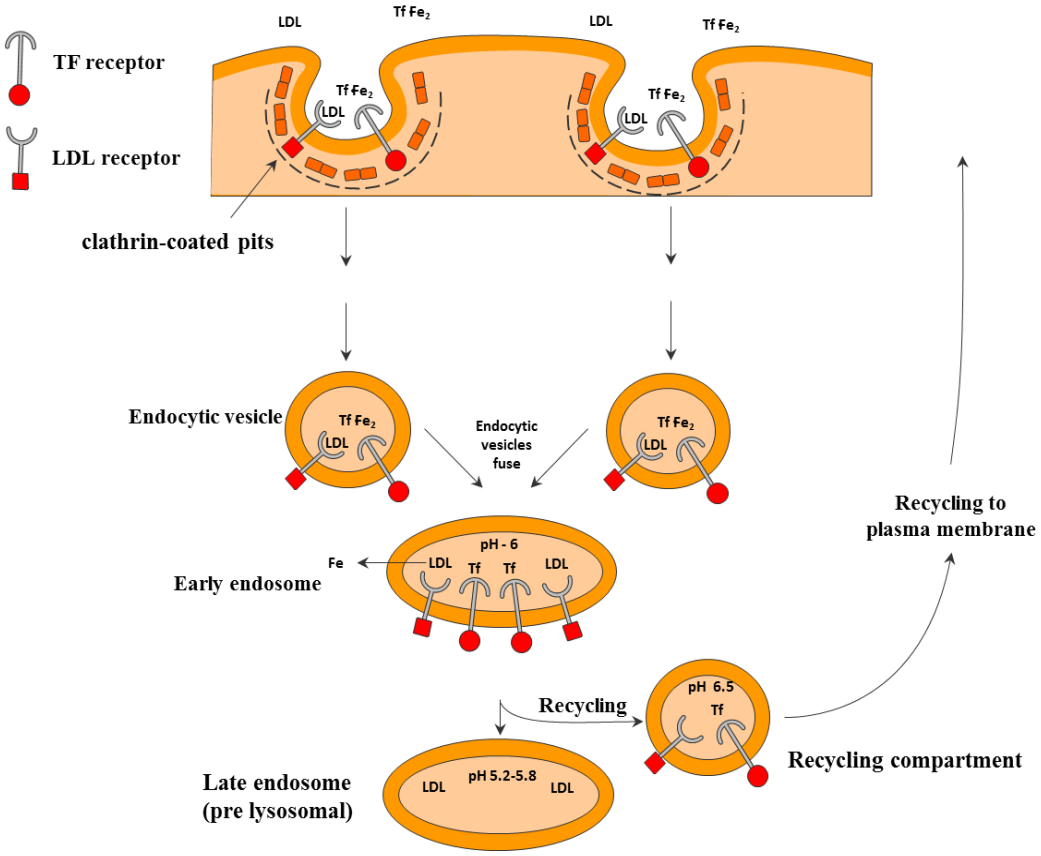


Figure 6: Transferrin and LDL-mediated endocytosis.

LDL receptor (LDLR) and transferrin receptor (TFR) mediate uptake and internalization of LDL and di-ferric transferrin (TF-Fe) respectively. The LDLR and TFR are endocytosed in clathrin-coated pits. They deliver LDL and Fe into endocytic vesicles that fuse with early endosomes. The acidic milieu of early endosomes dissociates most receptor–Fe complexes and Fe exits these compartments. LDLRs and TFRs are recycled from the recycling compartments back to the cell surface. LDL is sorted into late endosomes and lysosomes, where it is degraded while iron-free TF remains bound to the TFR. Upon return to the cell surface, iron-free TF dissociates from the receptor.

However, a recent discovery from our laboratory revealed that, in cells without sufficient nutrients in the medium (starved), endosomal membrane and receptors were no longer recycling or recycling is reduced. Instead, they were directly targeted the lysosomes. This resulted in rapid degradation of membrane remnants and receptors (Dauner et al. 2017). Importantly, this process is entirely governed by mTORC1: both starvation and pharmacological mTORC1 inhibition results in identical lysososomal targeting of endosomes, which includes transferrin receptors, LDL receptors and bulk plasma membrane sphingomyelin. Furthermore, this altered targeting was found independent of autophagy: autophagy defective $Atg5^{-/-}$ mouse embryonic fibroblasts (MEFs) also had targeting of endosomes to lysosomes identical to wild type cells. It was further established that this effect is mediated by hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), a component of ESCRT-0 (endosomal sorting complexes required for transport-0), although detailed molecular interactions remain to be determined (Dauner et al. 2017).

Perhaps strikingly, this work is a reminder that current knowledge of endocytosis and related membrane trafficking may be limited to these rapidly proliferating cells where mTORC1 is constitutively high). An understanding of how cells react under various metabolic conditions and also in various developmental stages, particularly quiescent and terminally differentiated cells, is just beginning. Nevertheless, cultured cells have provided a vast amount of knowledge over the years. Many regulators of membrane trafficking and membrane fusion, although primarily identified and characterized in cultured cells, could be involved in cells growing under more physiological conditions or *in vivo*. Rab small GTPases are likely some of these regulators (X. Li, Garrity, and Xu 2013).

1.5.1 Rab family of small GTPase

Rab proteins are a large family of small GTPases that regulate membrane trafficking at multiple sites and stages. In humans, there are more than 60 members of the Rab family (Stenmark et al. 1994). Rab GTPases primarily function by recruiting effector proteins onto membrane vesicles to facilitate the movement and the fusion of vesicles to their target membranes (Stenmark et al. 1994). Localization of Rab GTPases is highly compartmentalized, which provides the organelle specificity for its function (Stenmark 2009). For example, Rab1, located at ER exit sites and the pre-Golgi intermediate compartment (IC), mediates ER–Golgi trafficking. In addition, Rab7, associated with the late endosomes, mediates maturation of late endosomes and their fusion with lysosomes. Rab5, on the other hand, is localized to early endosomes, phagosomes and the plasma membrane (Stenmark 2009).

Rab5, one of the most broadly studied GTPases, plays a key role in early endosome maturation and trafficking. Overexpression of Rab5 Q79l, a constitutively active mutant (CA-Rab5), increased endocytosis and formed enlarged endosomes and inhibited transferrin recycling. On the other hand, expression of Rab5 S34N, a dominant negative mutation (DN-Rab5), resulted in inhibition of endocytosis and recycling (Stenmark et al. 1994).

Interestingly, plasma membrane is the most cholesterol rich membrane (40 percent) among other cellular membranes and it constantly undergoes endocytosis and exocytosis through recycling and secretion. Cholesterol in the endosomal membranes is directly involved in the sorting and transport of endocytic vesicles (Gruenberg 2001). Conversely, the perturbation to membrane trafficking may broadly influence intracellular cholesterol distribution and homeostasis.

1.6 Intracellular Cholesterol Trafficking and Homeostasis

Intracellular membranes are known to have vastly different levels of cholesterol. For example, the ER membrane is low in cholesterol (approximately five percent) in comparison to other cellular membranes, particularly the plasma membrane (40 percent) (Ikonen 2008). This is primarily due to the preference of cholesterol for saturated hydrocarbon acyl chains of phospholipids in the membrane (Simons and Vaz 2004). The sphingolipid and saturated phospholipid content of cellular membranes thus influences cholesterol distribution, making it enriched in the plasma membrane, the endocytic recycling compartment, and the trans-Golgi (TGN) (Mukherjee et al. 1998).

Cholesterol, a highly lipophilic amphiphile, is insoluble in water and rapidly incorporated into cellular membranes. The transport of cholesterol inside cells is mediated by vesicular and non-vesicular mechanisms involving carrier proteins at membrane contact sites (Ikonen 2008). For example, oxysterol-binding protein (OSBP) moves cholesterol and other oxygenated derivatives between adjacent membranes, such as ER and Golgi apparatus (Lagace et al. 1997). Steroidogenic acute regulatory protein (StAR) and StAR-related (START) proteins can also bind cholesterol and enable its rapid movement between membrane compartments (Alpy and Tomasetto 2005).

Mammalian cells can acquire cholesterol either by de novo synthesis in the ER or by receptor mediated endocytosis of LDL. LDL receptors bind extracellular LDL and internalize LDL by coated-pits by receptor-mediated endocytosis (Storch and Cheruku 2005; Ikonen 2008). LDL is then delivered through early endosome and sorting endosomes to the lysosomes for hydrolysis while the LDL receptor recycles back to the plasma membrane (Ikonen 2008). Acidic lipases in the lysosomes degrade LDL and produce fatty acid and

cholesterol. This lysosomal cholesterol then depends on two lysosomal proteins, Niemann-Pick C1 (NPC1) and NPC2 proteins, for export from the lysosomes (Liscums and Faust 1989). NPC2 is a soluble protein in the lysosome lumen that binds cholesterol and delivers it to NPC1, an integral protein in the lysosomal membrane. NPC1 then transports cholesterol across the lysosomal membrane and releases it to the cytoplasm (Infante et al. 2008; Xie et al. 2011). Newly released cholesterol is rapidly incorporated into cellular membranes, including the plasma membrane and ER membrane. However, the mechanism of cholesterol transport from late endosomes/lysosome (LEL) to the ER is poorly characterized. Soluble transport proteins such as OSBP and OSBP-related proteins (ORP) were implicated in such process. For example, ORP1L was shown to be involved in cholesterol egress from the LELs to the ER by a mechanism that requires a complex of sterol-, phospholipid-, and vesicle-associated membrane protein-associated protein (VAP)-binding activities (Zhao and Ridgway 2017).

ER membrane is cholesterol poor (five percent), which makes it sensitive to small changes in its cholesterol levels. Indeed, all mammalian cells seem to use the ER membrane to sense cholesterol and make decisions as to whether to synthesize cholesterol from acetyl CoA through the mevalonate pathway (Ikonen 2008). The ER harbors most of the enzymes for cholesterol synthesis (Ikonen 2008). There, cholesterol in the ER can exert two actions. First, when ER cholesterol is below five percent, SREBP-2 translocation is activated by proteolytic cleavage in the Golgi. Once mature SREBP-2 enters the nucleus, it stimulates cholesterol synthesis and LDL uptake (Jay D Horton, Goldstein, and Brown 2002). Secondly, when ER cholesterol is over the five percent threshold, it activates the cholesterol export and storage mechanisms (Radhakrishnan et al. 2008). Excess cellular cholesterol, for

example, can be oxidized to oxysterol in the mitochondria, a ligand for the transcription factor LXR (Chen et al. 2007). Activation of LXR by oxysterols up-regulates genes that are involved in the efflux of excess cholesterol from the cell, such as ABCA1 (Im and Osborne 2011). The rise in the ER cholesterol level also activates the ER resident enzyme ACAT which esterifies and stores excess cholesterol as CE in the lipid droplets (LD) (Greenberg et al. 2011). ACAT belongs to a family of small enzymes comprising three homologous members: ACAT 1 and 2 and acyl-coenzyme A: diacylglycerol acyltransferase 1 (DGAT1). They are primarily involved in vivo neutral lipid synthesis (Liu and Guo 2009). ACAT1 is ubiquitously expressed in all tissues and involved in the regulation of cholesterol homeostasis. ACAT2 is expressed primarily in the liver and small intestine, but still present at a lower level than ACAT1 in all tissues (Rogers et al. 2015). In intestines, ACAT2 function in providing CE for lipoprotein assembly. Both ACATs catalyzes the synthesis of cholesteryl esters using long-chain fatty acyl-coenzyme A and free cholesterol as the major sterol substrate in an allosteric manner. This reaction forms an ester linkage between the 3β-OH moiety in cholesterol and the carboxyl group of a long-chain fatty acid from the long-chain fatty acyl coenzyme A (Huang et al. 2015). As a polypeptide containing 550 amino acids, ACAT1 is an integral ER membrane protein. A homotetramer in vitro and in intact cells, it consist of nine transmembrane domains (TMDs), with five loops located at the cytoplasmic side of the ER and three at the lumen side (Huang et al. 2015). ACAT1 has two active sites: histidine (His-460) located in a membrane sealed region at the luminal end of a long hydrophobic peptide region of TMD7, and asparagine located within a long hydrophilic peptide region within the fourth large cytoplasmic loop (Rogers et al. 2015). All of the ACAT enzymes contain several cysteine (Cys) residues in which a disulfide bond between two cysteines in the C-terminal is formed. This bond contribute to ACAT stability (Liu and

Guo 2009). It was reported that ACAT2 is stabilized by cholesterol and FA as they induce reactive oxygen species (ROS), which oxidize Cys277 of ACAT2 resulting in the protection of the protein from degradation. ACAT2 then further converts cholesterol and FAs to CEs, thereby preventing the lipotoxicity (Wang et al. 2017).

As previously discussed, mTORC1 is known to activate SREBP to promote lipogenesis, including inducing many genes for cholesterol and fatty acid biosynthesis (K. Du et al. 2010). Also, in patients with primary breast cancer, mTORC1 is hyper-activated, which is accompanied by a high expression of SREBP target genes (Ricoult, Yecies, and Manning 2015). mTORC1's potential activates SREBP translocation from ER-to-Golgi as hepatic SCAP deletion resulted in SREBP inhibition and prevented steatosis in the obese mouse model, even though mTORC1 was hyper-activated (Moon et al. 2012).

On the other hand, NPC1 haplo-insufficient mice are susceptible to obesity and insulin resistance, likely due to mTORC1 hyper-activation (Jelinek et al. 2010). Cells without NPC1 would be deficient in cholesterol exit from the lysosomes. This could limit cholesterol levels in the ER membrane. Indeed, these animals were also susceptible to fatty liver due to hyper-activation of SREBP-1c (Castillo et al. 2017). Thus, it is possible that cholesterol trafficking through the lysosome plays a major role in regulating SREBP activity.

1.7 Thesis Rationale

Recent evidence has demonstrated the important role of mTORC1 in regulating hepatic lipogenesis by activating SREBPs. Obesity and over-nutrition results in hyper-activation of mTORC1, which causes a persistent activation of SREBP-1c in the liver (Sengupta et al. 2010). This leads to over-production of lipids and hence, hepatic steatosis. Furthermore, constitutively activated mTORC1 greatly elevates de novo lipid synthesis in cell culture (K. Du et al. 2010). This effect is not limited to hepatic cells; mTORC1 activates SREBP-2 in cultured fibroblasts (Peterson et al. 2011). Significantly, mTORC1 promotes SREBPs translocation from the ER to the Golgi where its proteolytically cleaved to translocate to the nucleus as a transcription factor to activate target gene expression (Peterson et al. 2011). However, the key question remains: how does mTORC1 promote the translocation and activation of SREBPs? SREBP translocation is well-established to be under the control of ER cholesterol levels (Michael S Brown and Goldstein 1997). We thus asked how mTORC1 may influence ER cholesterol.

mTORC1 is known to play a key regulatory role in two membrane trafficking events. Low mTORC1 activity triggers autophagy (J. Kim and Guan 2017) and also re-routes cholesterol-rich endosomes, which are normally recycled (Hao et al. 2002), to lysosomes (Dauner et al. 2017). From there, amino acids and membrane components, possibly cholesterol, can be released for re-use. This thus raises the possibility that low mTORC1 activity could increase ER cholesterol by releasing it from the lysosomes, thereby supressing SREBP activation.

I therefore hypothesize that mTORC1 activates SREBPs in mammalian cells by suppressing cholesterol trafficking to the lysosomes. This suppression is achieved by

inhibiting the autophagic process when nutrients are abundant and by facilitating endosomal recycling.

Specific Aims

Aim 1: Characterize the relationship between mTORC1/autophagy and ER cholesterol.

> *Aim 1a*: Measure mTORC1 activity under amino acid starvation and re-feeding conditions.
>
> *Aim 1b:* Measure ACAT activity to estimate ER cholesterol under amino acid starvation and re-feeding conditions.

Aim 2: Characterize the relationship between autophagy and SREBP activity.

2 MATERIAL AND METHODS

2.1 Cell Culture

Wild type, $Atg5^{-/-}$ and Atg5 (Tet-Off) mouse embryonic fibroblasts (MEF) were generously provided by Dr. Mizushima (The University of Tokyo, Tokyo, Japan). $TSC1/2^{-/-}$ MEFs were provided by Dr. Guan (University of California, San Diego, USA) and human embryonic kidney cells (HEK293T) by Dr. Bell (University of Ottawa, Ottawa, Canada). All cell lines were grown and maintained in DMEM (Fisher, 12800-017) supplemented with one percent antibiotics (100 units/mL penicillin and 100 µg/mL streptomycin, life technologies, 15140-122) and 10 percent fetal bovine serum (FBS) (Wisent, 080-150) at 37°C in a five percent CO_2 incubator. Amino acid starvation was performed using RPMI 1640 modified without L-glutamine, without amino acids, without glucose (Usbiological, R9010-01) supplemented with 25 mM glucose and one percent penicillin/streptomycin. Re-feeding was performed using regular RPMI1640 medium (Life Technologies, 31800-022).

2.2 Materials and Reagents

Reagents were purchased from the indicated vendors: QuantiTect SYBR® Green PCR Kit (Qiagen, 204143), [1,2-3H(N)]-cholesterol (Perkinelmer, NET139005MC), [9,10-^{3}H(N)]-Oleic Acid (Perkinelmer, NET289005MC), oleic acid (Sigma, O1383-5G), Torin-1 (Cedarlane laboratory LTD, 4247), cholesterol (Sigma, C8667-5G), U-18666A (Cayman, 10009085), chloroquine (Sigma, C6628), LysoTracker® Red DND-99 and DAPI (4',6-Diamidino-2-Phenylindole, Dihydrochloride) (Fisher, L7528 & D1306), doxycycline hydrochloride (VWR, CAAAJ60422-06), ChemiBLOCKER (Merck Millipore, 2170), ATP (Sigma, A2383), coenzyme A hydrate (Sigma, C4282). Antibodies were purchased from the following vendors: SREBP-2 (Santa Cruz, sc-13552), p70 S6 Kinase rabbit mAb (49D7) and

phospho-p70 S6 Kinase (Thr389) (Cell Signaling, 2708S and 9205S), phospho-S6 Ribosomal Protein (Ser235/236) and S6 Ribosomal Protein (54D2) Mouse mAb (Cell Signaling, 2211S and 2317S,), LC3B (Cell Signaling 2775s), anti-actin (Santa Cruz, sc-1616), HSP-70 (BD Biosciences, 554243), anti-Atg5 (New England Biolabs, 8540P), anti-Atg7 (MJS Biolynx, ABGAP1813C), anti-Niemann Pick C1 (Abcam, ab36983), GAPDH (Cell Signaling, 2118), GFP (Cell Signaling, 2555S), Peroxidase-AffiniPure Donkey Anti-Rabbit IgG and Peroxidase-AffiniPure Sheep Anti-Mouse IgG (Cedarlane Laboratories).

Dominant negative Atg5 cDNA and CA-Atg7 adenovirus were generously provided by Dr. Hotamisligi (Harvard University, Massachusetts, USA). DN-rab5 DNA was kindly provided by Dr. Zerial (Max Planck Institute, Germany). Lalistat-1 was kindly provided by Dr. Yves L. Marcel (University of Ottawa, Canada).

2.3 Gene Expression Analysis

Total RNA was isolated using RNeasy Mini Kit (Qiagen, 74106) and then reverse-transcripted using M-MLV reverse transcriptase (Life Technologies, 28025013) according to the manufacturer's instructions. The synthesized cDNA was then quantified by real-time PCR in a 7500-fast real time PCR system (Applied Biosystem). All data were normalized to endogenous 18S RNA and expressed as fold change to the starvation condition as indicated.

2.4 siRNA Knockdown

Cells were grown for 24 hours to 40–60 percent confluence and then transfected using siRNA Transfection Medium and reagent (Santa Cruz, sc-36868 & sc-29528). Each transfection contained 60 pmols of siRNA [Control siRNA-A (Santa Cruz, sc-37007), NPC1 siRNA (m) (Santa Cruz, sc-41589)]. The transfection was performed according to the manufacturer's instructions.

2.5 Protein Degradation Assay

The long-lived protein degradation assay was performed using previously described methodes (Ogier-denis et al. 1996; Pattingre, Petiot, and Codogno 2004). Briefly, cells were incubated for two days at 37°C with 0.2 μCi/ml [^{14} C]-L-valine (PerkinElmer, NEC291EU050UC). Cells were washed with PBS to remove unincorporated radioisotopes and incubated for 60 minutes with fresh medium containing 0.1 percent bovine serum albumin and cold 10 mM valine (Sigma, A4503) to allow the short-lived proteins to be degraded. The medium was then removed and replaced with fresh growth medium containing 10 percent FBS or starvation medium for four hours. The medium was then collected and precipitated with 10 percent trichloroacetic acid (TCA) (Sigma, T6399) at 4°C, and TCA-soluble radioactivity was measured using a scintillation counter. The cells were then washed with 10 percent TCA and dissolved using 0.5 ml of 0.2 M sodium hydroxide (Sigma, S8045) and radioactivity was measured by liquid scintillation counting. The rate of protein degradation was calculated from the ratio of radioactivity in the TCA soluble fraction versus cell-associated radioactivity. Parallel cell samples were treated with lysosome inhibition reagent chloroquine (30 μM) (Sigma, C6628) to distinguish the autophagic versus non-autophagic protein degradation, and were then subtracted from the total.

2.6 Western Blot Analysis

Cells were washed twice with cold PBS and then lysed with 200 μl of SDS buffer (50 mM Tris-Cl pH 6.8, 100 mM dithiothreitol, 2% SDS, 10% glycerol, and 1 tablet each protease and phosphatase inhibitor per 10 mL buffer). Lysates were sonicated for 10–15 seconds to shear DNA and then heated to 75°C for five minutes. Lysates were then centrifuged for one minute at 13,000 g and protein contents in the supernatant were

quantified using DC™ Protein Assay Kit II (Bio-Rad, 5000112). For protein separation**,** samples were prepared by mixing 4× SDS loading buffer (200 mM Tris HCl, pH 6.8, 400 mM dithiothreitol, 8% SDS, 40% glycerol, 0.4% bromophenol blue) with 10–25 µg of cell lysates and separated using SDS-PAGE 10–15 percent gels. Proteins were then transferred to PVDF membranes and blocked with TBS-T (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20) containing one percent milk powder for 30 minutes and then incubated with primary antibody overnight at 4°C. Membranes were probed with primary antibodies and the next day membranes were gently agitated for two hours in secondary antibodies, in TBS-T with one percent milk. Chemiluminescence was detected via Immobilon Western Chemiluminescent HRP Substrate (Millipore, WBKLS0500) using UltraCruz® Autoradiography Film (Santa Cruz, sc-201697).

2.7 Whole-cell Cholesterol Esterification Assay (ACAT assay)

Cells were cultured for 24 hours in growth medium and then subjected to different treatments as indicated. During the last 30 minutes of the incubation, cells were pulsed at 37ºC with [^{3}H]oleate-albumin complex [^{3}H]oleate (10 mM with 1.2 mg/ml bovine serum albumin). Excess radioactivity was removed by washing twice with PBS. Cellular lipids were extracted using 3 ml of hexane:isopropanol (3:2, v:v), and cholesteryl ester was separated using thin-layer chromatography (EMD Millipore, 1057210001) and determined using a scintillation counter. The remaining cell debris after extraction was dissolved in 1 ml 1 N NaOH and assayed for protein content using Bio-Rad Protein Assay Dye Reagent (BioRad, 5000006).

2.8 Microsomal ACAT Assay

The assay was performed as described previously (Tabas, Boykow, and Tall 1987; Balasubramaniam, Mitropoulos, and Venkatesan 1978). Briefly, MEFs and HEK 293T cells were pre-incubated and treated as indicated in the figure legends. The cells were washed, scraped and collected in cold PBS by centrifugation and stored at -70ºC for next day use. Microsomal fractions were prepared by taking cell pellets and thawing them in 2 ml of 20 mM potassium phosphate (pH 7.4) containing 2 mM dithiothreitol. Cells were then homogenized at 4°C with 60 strokes using a tight fitted type A pestle homogenizer. The homogenate was then centrifuged at 800 g for 10 minutes. The post-nuclear supernatant was then centrifuged at 100,000 g for 1 hour. The pellet, containing microsomal fractions, was collected and re-suspended in 0.5 ml of 0.1 M potassium phosphate, pH 7.4, containing 2 mM dithiothreitol. For the ACAT assay, aliquots of 50 ug of protein were incubated for 15 minutes at 37ºC in 0.2 ml containing 100 mM potassium phosphate, PH 7.4, 2 mM dithiothreitol, 1.2 mg of fatty acid-free BSA, 2 mM ATP, 4mM MgCl2, 0.2 mM coenzyme A, 20 µg of cholesterol added in 2 µl acetone as were indicated. The reaction was started by adding [^{3}H] oleic acid (1mM) for 15 minutes at 37ºC. The reaction was stopped by adding 4 ml of chloroform/methanol (2/1, v/v). The cholesterol ester was separated from the mixture using thin layer chromatography and the radioactivity was measured by scintillation counter.

2.9 Cell Transfection

DNA transfections were performed using Effectene transfection reagent (Qiagen, 301425), 1 µg of DN-ATG5, DN-rab5 and control plasmid were used according to manufacturer's instructions.

2.10 Adenovirus production and infection

Adenoviruses were amplified in HEK293 cells and purified following the protocol described by Ross et al(Ross and Parks 2009). For infection, cells were seeded at a density of $1.4x10^9$ in a 6-well plate and 1 MOI was used to transduce the virus in the cells. The cells were then washed and a new fresh medium was added. The cells were then grown for three days before preforming the experiments.

2.11 Immunofluoresence Assays

Cells were cultured on glass-coverslip-bottom microscopy dishes to 50–70 percent confluency. The cells were then subjected to four hours of starvation followed by four hours of re-feeding as indicated in the result section. Cells were then washed with PBS, fixed with four percent paraformaldehyde in PBS for 10 minutes and permeabilized with 0.1 mg/ml saponin in PBS for 30 minutes. Cells were blocked with five percent calf serum and 50 mM NH_4Cl in PBS for 20 minutes. The primary SREBP2-specific antibody was then added at 1:500 dilution in a five percent calf serum/PBS for 30 minutes. After washing with PBS and incubating with five percent calf serum/PBS for 20 minutes, secondary antibody (Alexa Fluo 488 goat anti-rabbit IgG) then added at a concentration of 1:200 for 30 minutes, followed by a 45 minute incubation in five percent calf serum/PBS. The cellular localization of immunofluorescence was observed and recorded using a C1 confocal module on a Nikon TE2000-E inverted fluorescent microscope with a 60× objective.

2.12 Statistics

Data were analyzed using the analysis of variance (ANOVA) using PRISM software (GraphPad). The statistical significance of differences between groups was analyzed using Tukey tests. Differences were considered significant at a P-value < 0.05.

Table 1: Primer sequences used for real-time polymerase chain reaction.

Gene name	Sequences for forward and reverse primers (5'–3')		
		Forward	Reverse
HMGCOAS	Mouse	GCCGTGAACTGGGTCGAA	GCATATATAGCAATGTCTCCTGCAA
	Human	AGCAAGTTTCTTTTCATTTCGAGTATC	GATGTGCTGGACACCAACTTGT
HMGCOAR	Mouse	CTTGTGGAATGCCTTGTGAT	AGCCGAAGCAGCACATGAT
	Human	GGGAACCTCGGCCTAATGAA	CACCACGCTCATGAGTTTCCA
SREBP2	Mouse	GCGTTCTGGAGACCATGGA	ACAAAGTTGCTCTGAAAACAAATCA
	Human	AGGAGAACATGGTGCTGA	TAAAGGAGAGGCACAGGA
LDLR	Human	GTCTTGGCACTGGAACTCGT	CTGGAAATTGCGCTGGAC
Squalene synthase	Mouse	CCAACTCAATGGGTCTGTTCCT	TGGCTTAGCAAAGTCTTCCAACT
18S	Mouse	TGACTCAACACGGGAAACCT	AACCAGACAAATCCAC
	Human	AACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
FAS	Mouse	GCTGCGGAAACTTCAGGAAAT	AGAGACGTGTCACTCCTGGACTT
	Human	CGGGCCGCAAAGC	CGGCTCGCCCACCT
SCD1	Mouse	CCGGAGACCCCTTAGATCGA	TAGCCTGTAAAAGATTTCTGCAAACC
	Human	GCAGGACGATATCTCTAGC	GTCTCCAACTTATCTCCTCCATTC
ACC	Mouse	TGACAGACTGATCGCAGAGAAG	TGGAGAGCCCCACACACA
FDPS	Human	ATTGGAGATGGGCGAGTTCTTC	CCGACCTTTCCCGTCACA
GPAT	Mouse	CAACACCATCCCCGACATC	GTGACCTTCGATTATGCGATCA
SREBP-1	Mouse	AAGCAAATCACTGAAGGACCTGG	AAAGACAAGGGGCTACTCTGGGAG
	Human	TCAGCGAGGCGGCTTTGGAGCAG	CATGTCTTCGATGTCGGTCAG

3 RESULTS

3.1 Inactivation of mTORC1 by Starvation or Inhibitor Promotes Cholesterol Trafficking From the Lysosomes to the ER

3.1.1 Nutrient regulation of mTORC1 activity and the autophagic process

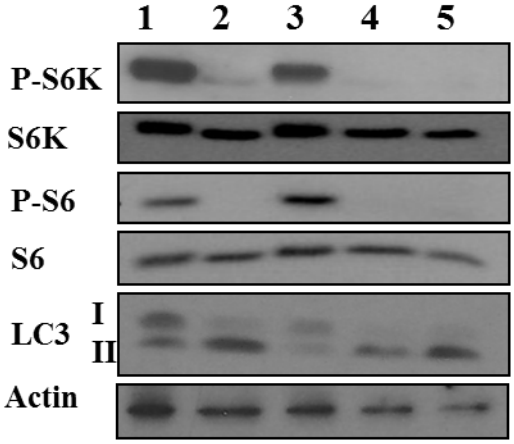
In order to understand the relationship among mTORC1, autophagy and cholesterol trafficking, I first established a cell culture system to assess mTORC1 activation/suppression and its relationship with autophagy. I observed that, in mouse embryonic fibroblasts (MEFs), mTORC1 activity, assessed by phosphorylation of its substrates, S6K1 and S6, is high in full growth medium with serum (see **Figure 7A, lane 1**) and in the presence of amino acids (re-feeding) (see **Figure 7A, lane 3**). mTORC1 activity is diminished in the presence of mTORC1 inhibitor Torin-1, a potent and selective mTORC1 inhibitor (see **Figure 7A, lane 4**), consistent with previous reports (Peterson et al. 2011). Furthermore, four hours of amino acid removal (starvation) abolishes mTORC1 activity, as shown by low p-S6K and p-S6 (see **Figure 7A, lane 2**). The occurrence of autophagy in starved cells is evident in the higher amounts of LC3-II, the processed form, relative to LC3-I, the precursor, in starved cells (see **Figure 7A, lane 2**), consistent with increased autophagic flux of LC3 using this common marker of autophagy (Mizushima 2007). This increase in LC3-II flux is inhibited by addition of serum/amino acid or amino acid to cells (see **Figure 7A, lanes 1&3**). Torin-1 promoted LC3 flux in the presence of amino acids (see **Figure 7A, lane 4**), again confirming the effect of mTORC1.

Consistent with the initiation of autophagy by starvation, the long-lived protein degradation is increased based on release of [^{14}C]-L-valine from degraded protein to the medium during 4-hour starvation (see **Figure 7B**) in comparison to that from cells in normal

medium (control). Importantly, [^{14} C]-L-valine release to the medium can be blocked with chloroquine that neutralizes the acidic pH in the lysosomes (Homewood et al. 1972). This confirms that acidic lysosomes are primarily responsible for enhanced protein degradation during starvation in these cells. Such protein degradation is a most reliable hallmark of autophagy (Ashford and Porter 1962; X. Du et al. 2012).

Thus, mTORC1 activity in MEFs is regulated by amino acid availability: high when amino acids are available and abolished during starvation. Autophagy, on the other hand, shows an opposite trend: activation by starvation and suppression in the presence of amino acids or serum plus amino acids. Lack of mTORC1 activity is likely the cause of starvation-induced autophagy: mTORC1 inhibitor Torin-1 induced autophagy even when amino acids are available.

A



Serum	+	-	-	-	-
Amino acid	+	-	+	+	-
Torin 1	-	-	-	+	-
U18666A	-	-	-	-	+

B

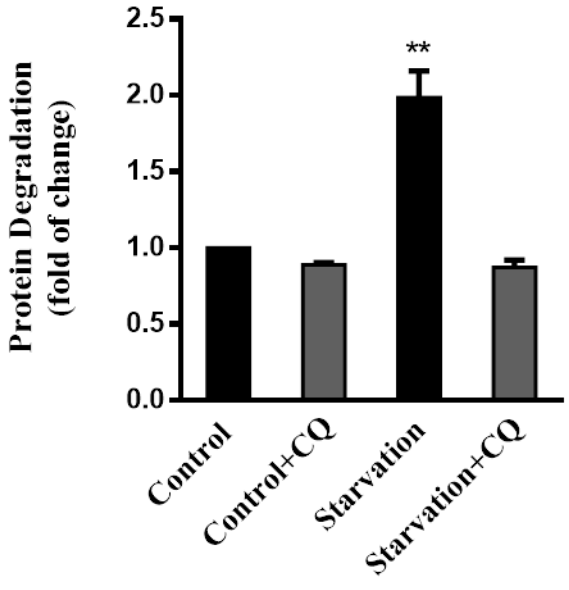


Figure 7: mTORC1 promotes autophagy in response to nutrient condition.

(A) MEF cells were incubated in serum-containing medium (control) for four hours (lane 1) or medium without serum and amino acids (starvation) for four hours (lane 2) or starvation medium plus 1 μM U18666A (lane 5). Some of the cells were in starvation medium for four hours and then switched to medium containing amino acids (re-feeding) for four hours, with or without Torin-1 (250 nM) (lane 3 and 4). Cells were treated with 30μM choloroquine in the last 30 min of the 4 hour treatments. Cells were then lysed, subjected to SDS-Page and immunoblotted with indicated antibodies. Data are representative of at least three experiments.

(B) MEF cells were grown in normal serum medium containing [^{14}C]-L-valine for three days and then shifted to fresh medium containing 0.1% bovine serum albumin for one hour. Cells were then subjected to four hours incubation with control or starvation medium for four hours, with or without chloroquine (30 μM). Medium was then collected and analyzed for TCA soluble [^{14}C]-L-valine as described in methods. Results are expressed as fold increase of cellular protein degraded in four hours in starvation medium, relative to that in control medium. Data are the average+/-SEM of three independent experiments.

P-value: *P<0.05, **P<0.005, ***P<0.000.

3.1.2 Autophagy promotes cholesterol trafficking from the lysosomes to the ER

Autophagy delivers membrane-rich organelles and cell debris to the lysosomes, where these membrane-rich materials are hydrolyzed. Autophagy thus releases amino acids and, inevitably but less noticeably, membrane lipids including cholesterol. This lysosome-derived cholesterol could be sensed by the ER membrane, along with other cellular membranes including the plasma membrane (M S Brown and Goldstein 1986). I thus used a whole cell ACAT assay to estimate ER cholesterol. As mentioned in the introduction, ACAT is an ER resident enzyme and its activity is mostly governed by cholesterol availability in the ER membrane (Suckling and Stange 1985). Whole cell ACAT activity is commonly used to monitor ER cholesterol level in live cells (Chang et al. 2006).

If free cholesterol is released by autophagy from the lysosomes, ACAT have more substrate and thus be more active. In the cells loaded with excess oleate acid ACAT activity estimated by the amount of the product cholesteryl ester (CE) should be proportional to cholesterol availability in the ER membrane.

I indeed observed that ACAT activity was enhanced by amino acid starvation (see **Figure 8A)**, when autophagy was triggered. This implicates a higher level of cholesterol in the ER membrane during autophagy (see **Figure 7A, lane 2**). On the other hand, cells with activated mTORC1 (i.e. in amino acid), containing medium (control) or in medium resupplied with amino acids after starvation (re-feed), exhibited lower ACAT activity. This is likely indicative of lower cholesterol levels in the ER membrane in the absence of autophagy. Moreover, if mTORC1 activity is blocked by Torin1, ACAT activity is elevated, even in the presence of amino acid (see **Figure 8A**). Autophagy was also activated under this

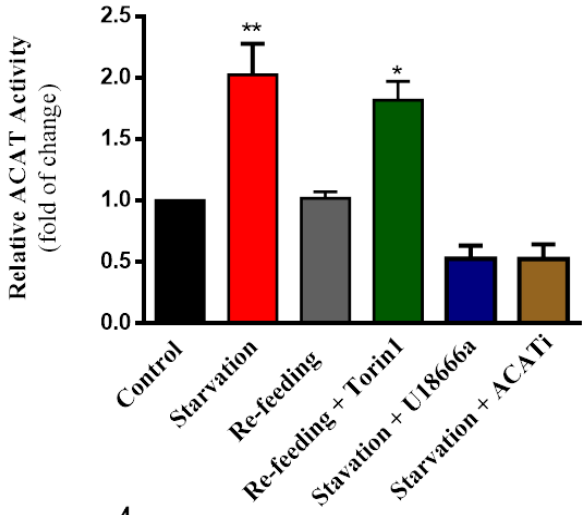
condition (see **Figure 7A, lane 4)**. Thus, it is most likely that autophagy elevates ER cholesterol when mTORC1 is low.

To confirm that the cholesterol that appeared in the ER during starvation was of lysosomal origin, I treated cells during starvation with U18666A. U18666A is known to target NPC1 and inhibit cholesterol efflux from the lysosomes (Lu et al. 2015). I observed that U18666A blocked the rise of ACAT activity during starvation (see **Figure 8A, blue bar**), even though mTORC1 is suppressed by starvation and autophagy occurs normally (see **Figure 7, lane 5**). As expected, CE formation during starvation is blocked by ACATi, a specific ACAT inhibitor, (see **Figure 8A, brown bar**). This verifies that ACAT is solely responsible for the CE formed during starvation.

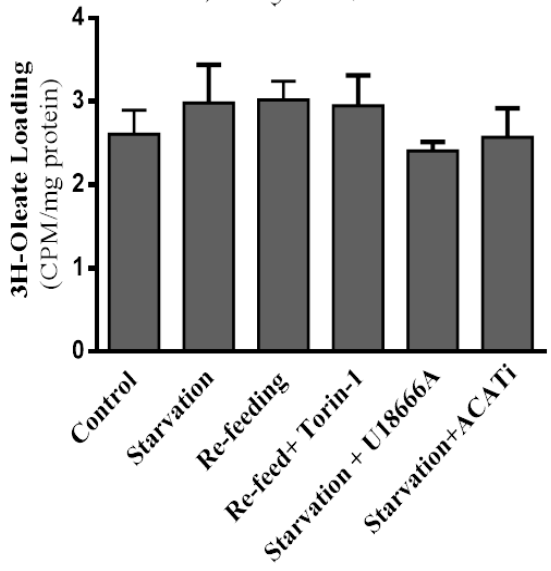
Additionally, the levels of cellular ^{3}H-oleate, which forms ^{3}H-CE with ER cholesterol in whole cell ACAT assay, are identical among cells treated under various nutrient conditions. Furthermore, for ACAT activities in isolated ER membranes (microsome fraction), the readout of ACAT protein contents and intrinsic enzymatic activities are not influenced by nutrients or U18666A (see **Figure 8B & Figure 8C**). Thus, the changes in whole cell ACAT activities most likely reflect changes in cholesterol content in the ER membrane by metabolic conditions. I was not able to use the incorporation of oleate into triglyceride (TG) measurement as part of the ACAT assay, which is frequently used as a measure of oleate loading and utilization not linked to ACAT. In our experiment models, we found that TG level responded in the same manner to nutrient conditions as CE level. This suggests that the DGAT1, which catalyzes the synthesis of TG from long-chain fatty acyl-coenzyme A and diacylglycerol, enzyme activity might also enhanced by starvation. Indeed, it was demonstrate in a recently published study that DGAT1 activity is enhanced during

nutrient deprivation to channel FAs into LDs as TG. This protects against lipotoxicity due to lack of nutrients (Nguyen et al. 2017).

A


Relative ACAT Activity
(fold of change)
2.5
2.0
1.5
1.0
0.5
0.0
**
*
Control
Starvation
Re-feeding
Re-feeding + Torin1
Stavation + U18666a
Starvation + ACATi

B


3H-Oleate Loading
(CPM/mg protein)
4
3
2
1
0
Control
Starvation
Re-feeding
Re-feed+ Torin-1
Starvation + U18666A
Starvation+ACATi

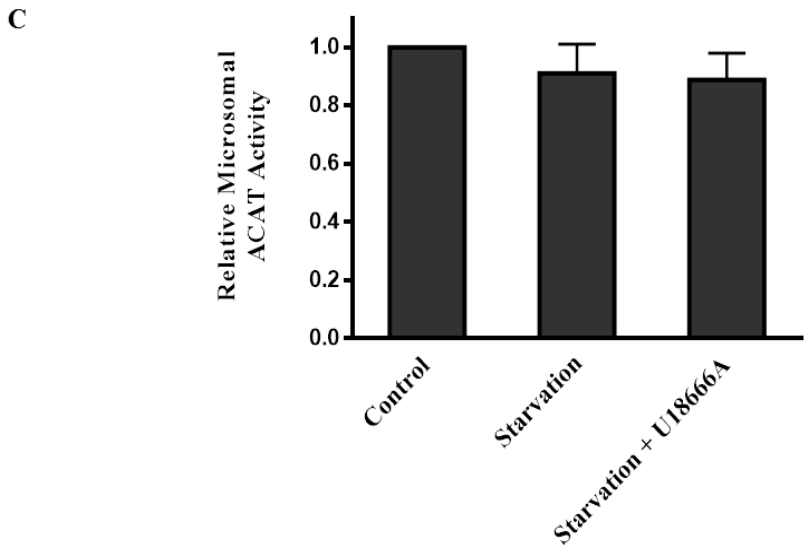


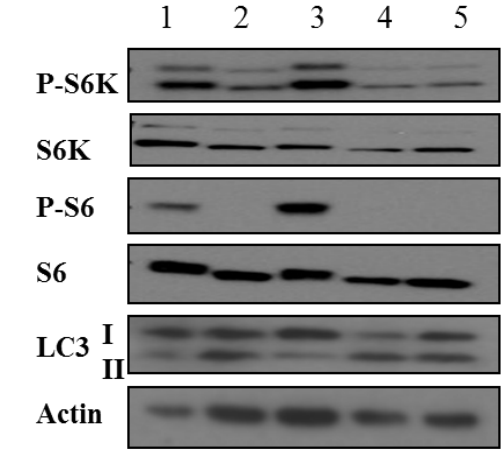
Figure 8: Autophagy promotes cholesterol trafficking from the lysosomes to the ER.

(A) MEF cells were subjected to control or starvation medium for four hours or starvation for four hours followed by four hours in re-feed medium, and treated with Torin-1 and U18666A as indicated. ^{3}H-oleate was added to cells during the last 30 minutes to measure ACAT activity. Results are represented as fold increase of CE formation, relative to cells in the control medium. Data are the average+/-SEM of three independent experiments.
(B) MEF cells were treated as in (A), ^{3}H-oleate loading was analyzed by measuring the radioactivity in whole cells lysate and normalized to the protein content. Data are represented as average and STD (n=3).
(C) Microsomal ACAT activity. MEFs were grown for two days then starved for four hours with or without U18666A (1 μM). Cells were then collected and microsomal fractions isolated. ACAT activity was measured as described in materials and method. Results represent the fold increase in enzyme activity relative to cells in control medium. Data are the average+/-STD (n=3).
P-value: *P<0.05, **P<0.005, ***P<0.0001.

Identical experiments were also performed in HEK293T cells, another mammalian cultured cell line responsive to nutrient conditions and inhibitors (Peterson et al. 2011). mTORC1 and autophagy was similarly regulated by nutrients (see **Figure 9A & Figure 9B)** as in MEFs. In addition, the cholesterol levels in the ER membranes, indicated by whole cell ACAT activity, are altered by autophagy and by Torin-1 and U18666A as was observed for MEFs (see **Figure 9A)**. Oleate loading and microsomal ACAT activities were unaffected by starvaiton conditions that affected ACAT activity (**see Figure 9D & Figure 9E**).

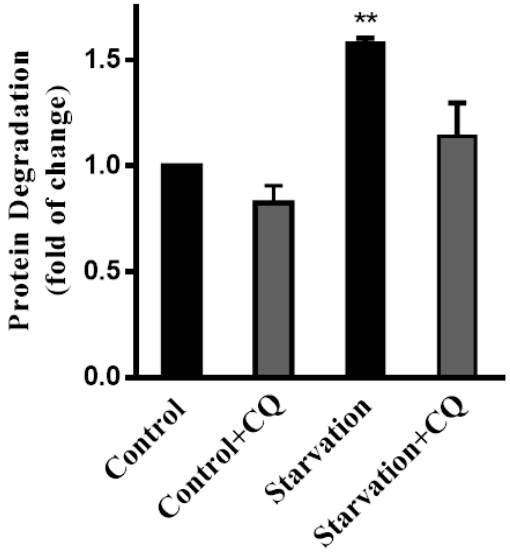
Taken together, I conclude that inactivation of mTORC1 by starvation or by pharmacological inhibition not only increases lysosomal protein degradation, but also increases cholesterol release from the lysosomes. leading to higher cholesterol levels in the ER.

A



Serum	+	-	-	-	-
Amino acid	+	-	+	+	-
Torin 1	-	-	-	+	-
U18666A	-	-	-	-	+

B



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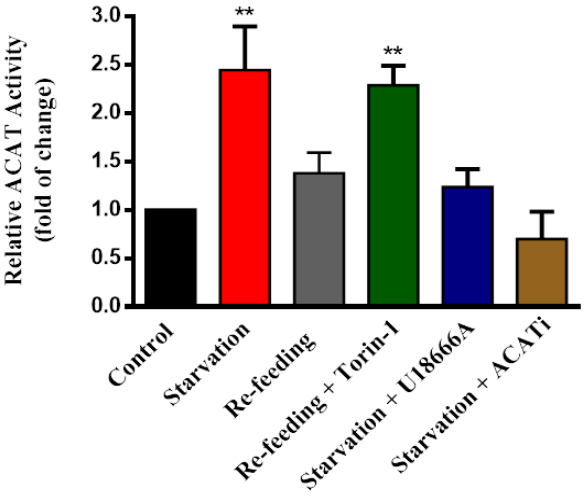
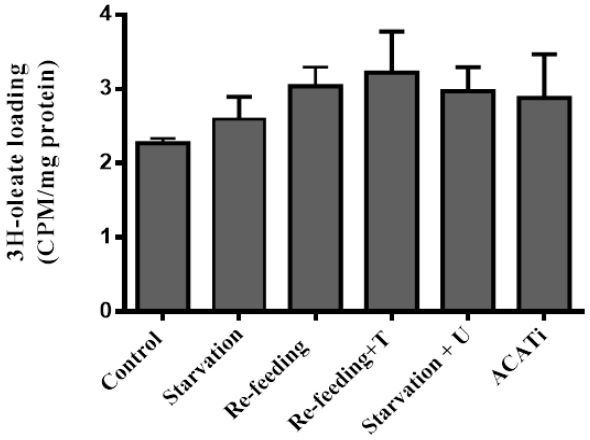

Relative ACAT Activity
(fold of change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Control
Starvation
Re-feeding
Re-feeding + Torin-1
Starvation + U18666A
Starvation + ACATi

D


3H-oleate loading
(CPM/mg protein)
4
3
2
1
0
Control
Starvation
Re-feeding
Re-feeding+T
Starvation + U
ACATi

E

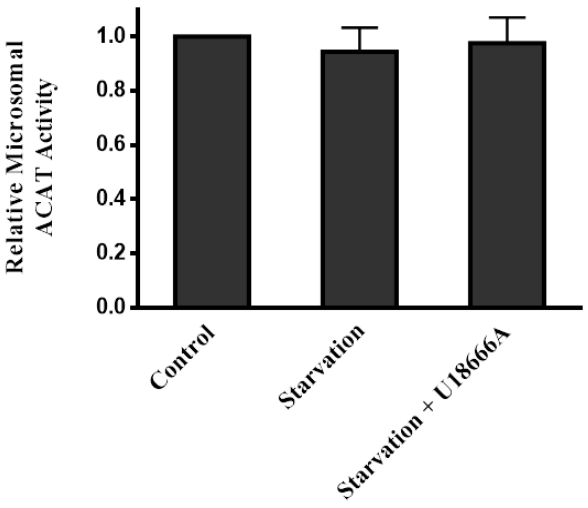

Relative Microsomal ACAT Activity
1.0
0.8
0.6
0.4
0.2
0.0
Control
Starvation
Starvation + U18666A

Figure 9: mTORC1 induces autophagy and promotes FC trafficking to the ER in HEK 293T cells.

(A) HEK 293T cells were incubated in with serum-containing medium (control) for four hours (lane 1) or medium without serum and amino acids (starvation) for four hours (lane 2) or starvation medium plus 1 μM U18666A (lane 5). Some cells were in starvation medium for four hours and then switched to medium containing amino acids (re-feeding) for four hours, with or without Torin-1 (250 nM) (lane 3 and 4). Cells were treated with 30μM of chloroquine in the last 30 min of the 4 hour treatments. Cells were then lysed, subjected to SDS-PAGE and immunoblotted with indicated antibodies. Data are representative of at least three experiments.
(B) HEK293T cells were grown in normal serum medium containing [^{14}C]-L-valine for three days and then shifted to fresh medium containing 0.1% bovine serum albumin for one hour. Cells were then subjected to four hours' incubation with control or starvation medium for four hours, with or without chloroquine (30 μM). Medium was then collected and analyzed for TCA soluble [^{14}C]-L-valine as described in methods. Results are expressed as fold increase of cellular protein degraded for four hours in starvation medium, relative to that in control medium. Data are the average+/-SEM of three independent experiments.
(C) HEK293T cells were subjected to control or starvation medium for four hours or starvation for four hours followed by four hours in re-feed medium, and treated with Torin-1 and U18666A as indicated. ^{3}H-oleate was added to cells during the last 30 minutes to measure ACAT activity. Results are represented as fold increase of CE formation, relative to cells in control medium. Data are the average+/-SEM of three independent experiments.
(D) HEK293T cells were treated as in (C). Cells in normal serum medium were treated with Torin-1 and U18666A, and ACAT activity was measured.
(E) HEK293T cells were treated as in (C), ^{3}H-oleate loading was analyzed by measuring the radioactivity in whole cells lysate and normalized to the protein content. Data are represented as average and STD (n=3).
(F***)*** Microsomal ACAT activity. HEKs were grown for two days then starved for four hours with or without U18666A (1 μM). Cells were then collected and microsomal fractions isolated. ACAT activity was measured as described in materials and method chapter. Results represent the fold increase in enzyme activity relative to cells in control medium. Data are the average+/-STD (n=3).
P-value: *P<0.05, **P<0.005, ***P<0.0001.

3.2 Starvation suppresses SREBP-2 activation by facilitating cholesterol trafficking from lysosome to the ER

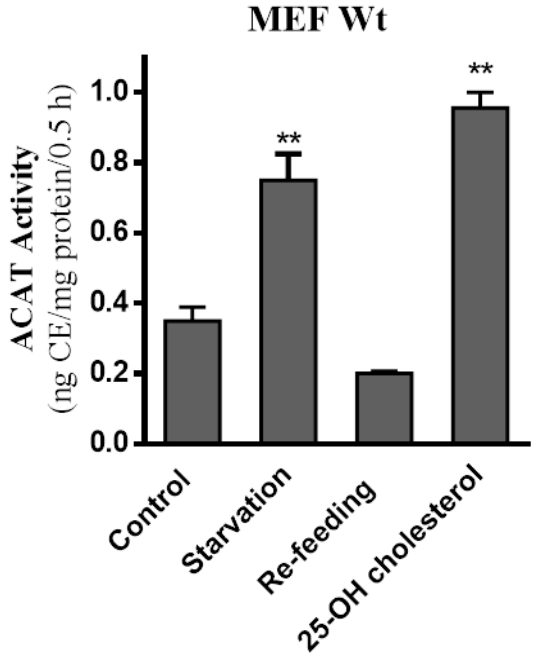
Having established above that starvation increase delivery of cholesterol to the ER membrane, I next wanted to establish whether this rise in ER cholesterol level is of sufficient magnitude to suppress SREBP-2, the key regulator of the cholesterol biosynthesis. For this, MEFs were treated with 25-hydroxycholesterol (25-OH cholesterol). 25-OH cholesterol is known to trigger intracellular cholesterol transport to the ER and activates cholesterol esterification by ACAT, as well as being a potent suppressor of the SREBP pathway (Adams et al. 2004). I observed that 25-OH cholesterol enhanced ACAT activity to a similar magnitude as starvation in both MEFs and HEK cells (see **Figure 10A *a*, second and fourth bars**). Significantly, 25-OH cholesterol was able to effectively suppress gene expression of SREBP-2 and HMG-CoA Reductase, a SREBP-2 target gene (see **Figure 10B, *a***). Thus, cholesterol elevation in the ER membrane, triggered by starvation, is likely able to suppress SREBP-2. Identical effects of 25-OH cholesterol were also observed in HEK293T cells (see **Figure 10A, *b* & Figure 10B, *b*)**.

Proteolytic processing of SREBPs triggered by low ER cholesterol are expected to enter the nucleus and bind SREs and initiate the transcription of genes involved in cholesterol and fatty acid synthesis (Michael S Brown and Goldstein 1997). I preformed immunofluorescence staining of SREBP-2 to test whether SREBP-2 activity could be reflected in its cellular localization (Michael S Brown and Goldstein 1997). The results show that SREBP-2 is predominantly in the nucleus in normal growth medium or after amino acid re-feeding (see **Figure 11, first and third columns)**, indicative of activation. After starvation, however, SREBP-2 is excluded from the nucleus, consistent with its inactivated

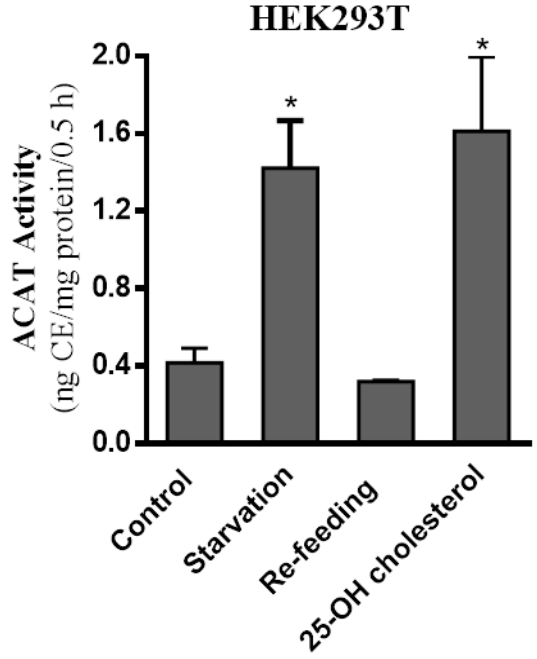
state (see **Figure 11, middle column)**. Thus, SREBP-2 cellular localization is consistent with its activity as a transcription factor as mentioned above.

A

a


MEF Wt
ACAT Activity
(ng CE/mg protein/0.5 h)
1.0
0.8
0.6
0.4
0.2
0.0
**
**
Control
Starvation
Re-feeding
25-OH cholesterol

b


HEK293T
ACAT Activity
(ng CE/mg protein/0.5 h)
2.0
1.6
1.2
0.8
0.4
0.0
*
*
Control
Starvation
Re-feeding
25-OH cholesterol

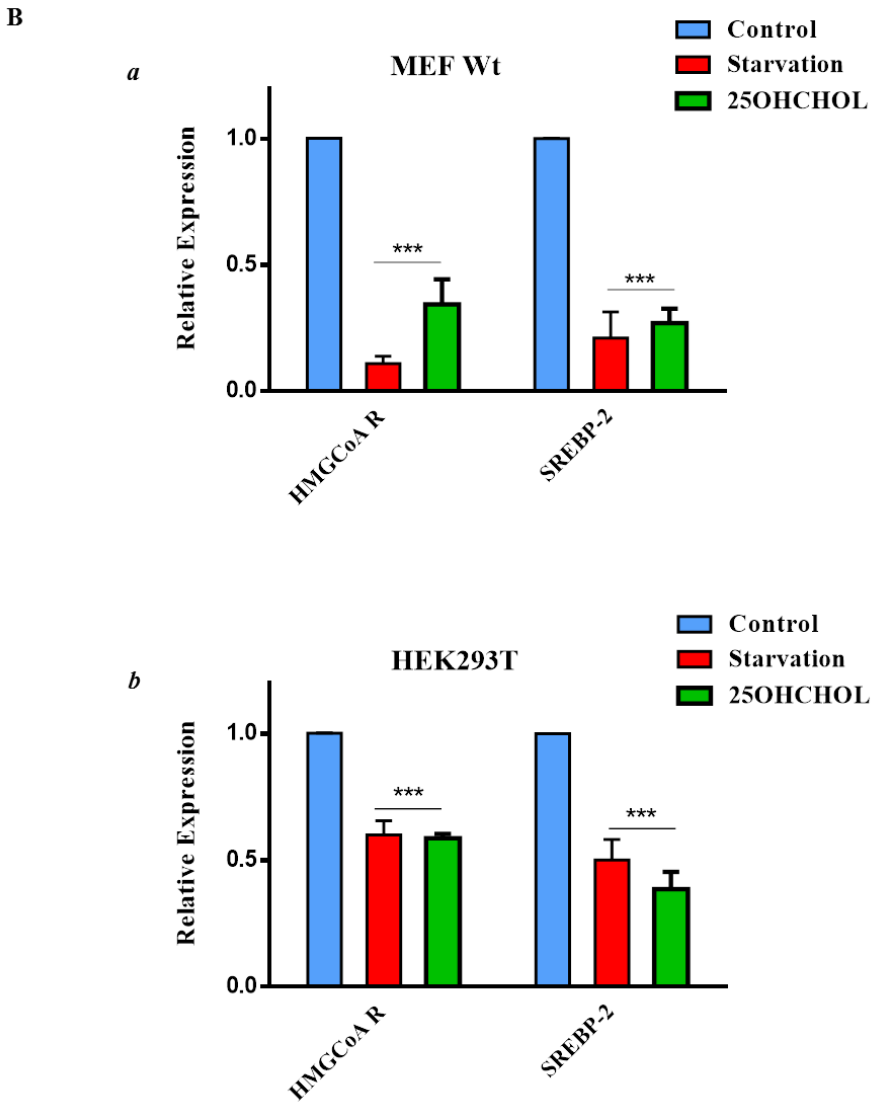


Figure 10: ER membrane cholesterol regulates SREBP-2 activity.

(A) ACAT activity in MEF (*a*) and HEK293T (*b*) cells treated with 25-hydroxyl-cholesterol (25-OH CH). Cells were incubated in starvation or starvation/refeeding medium. Parallel dishes were treated with control medium containing 25-OH CH (10 µM) for four hours. Data represent the average+/- STD (n=3).
(B) Relative expression of SREBP-2 target genes in cells treated as above. Data represent the average +/-STD (n=4).
P-value: *P<0.05, **P<0.005, ***P<0.0001.

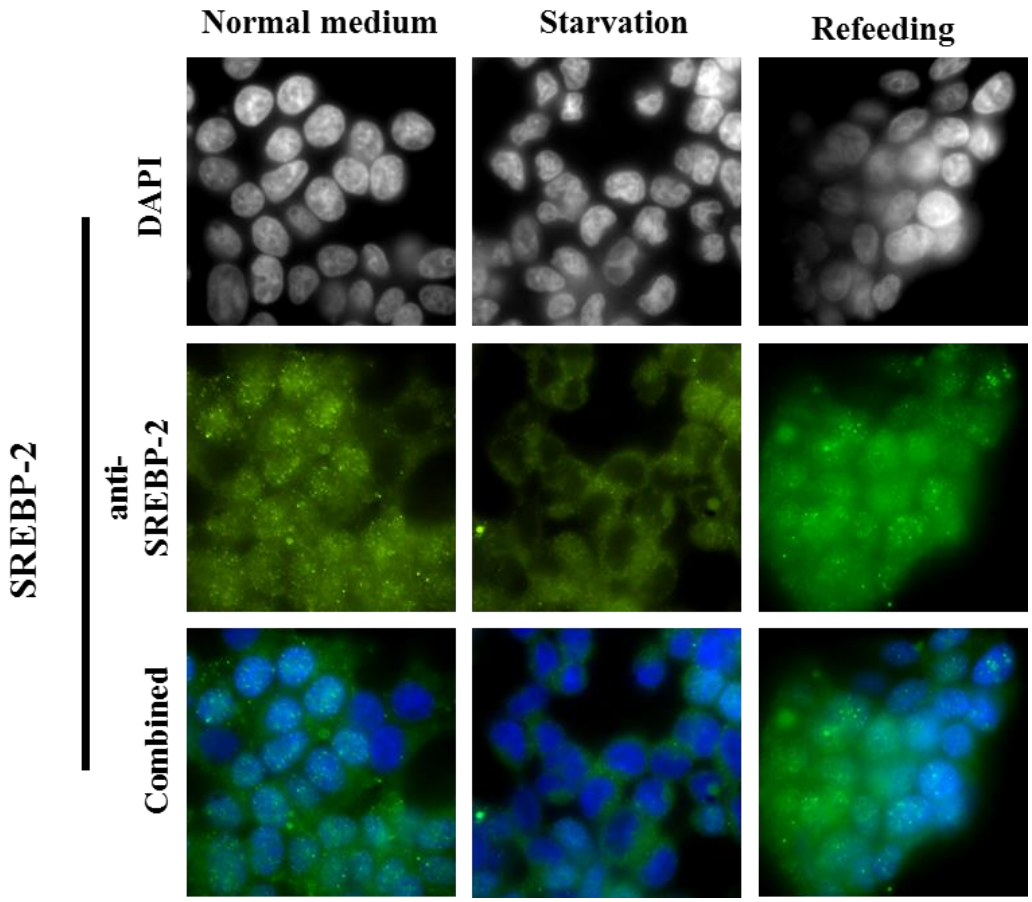


Figure 11: ER cholesterol impact on SREBP-2 processing.

Immunofluorescence staining of MEFs with anti-SREBP2 (green) and DAPI (blue) under indicated conditions.

I next tested the transcription of lipogenic genes involved in cholesterol and fatty acid metabolism that are controlled by SREB-2 and SREBP-1a respectively (K. Du et al. 2010). I first examined the SREBP-2 target gene expression in MEFs under various nutrient conditions as in **Figure 7**. I observed that transcriptional expression of HMG-CoA synthetase, HMG-CoA Reductase, Squalene synthase and SREBP2 are low during starvation (see **Figure 12, first bar**). This is the condition where mTORC1 activity is low and autophagy is high (see **Figure 7A, second lane**) and ACAT activity (thus ER cholesterol) is high (see **Figure 8A, second red bar**). Importantly, if U18666A is present during starvation (i.e. cholesterol efflux from the lysosomes is inhibited), SREBP-2 activity is high (see **Figure 12, fourth bar)** even with low mTORC1 activity. Thus, starvation is indeed capable of suppressing SREBP-2 activation, most likely through promoting cholesterol trafficking to the ER from the lysosomes.

Conversely, SREBP-2 suppression is relieved by amino acid replenishing (re-feeding) (see **Figure 12, second bar**) when mTORC1 activity is high, autophagy is low (see **Figure 7A, third lane)** and ACAT activity (thus ER cholesterol) is low (see **Figure 8A, gray bar)**. Again, treating cells with Torin-1 during re-feeding to inhibit mTROC1 suppressed SREBP-2 activity (see **Figure 12A, third bar**) was consistent with elevated ACAT activity by Torin-1 (see **Figure 8A, green bar**).

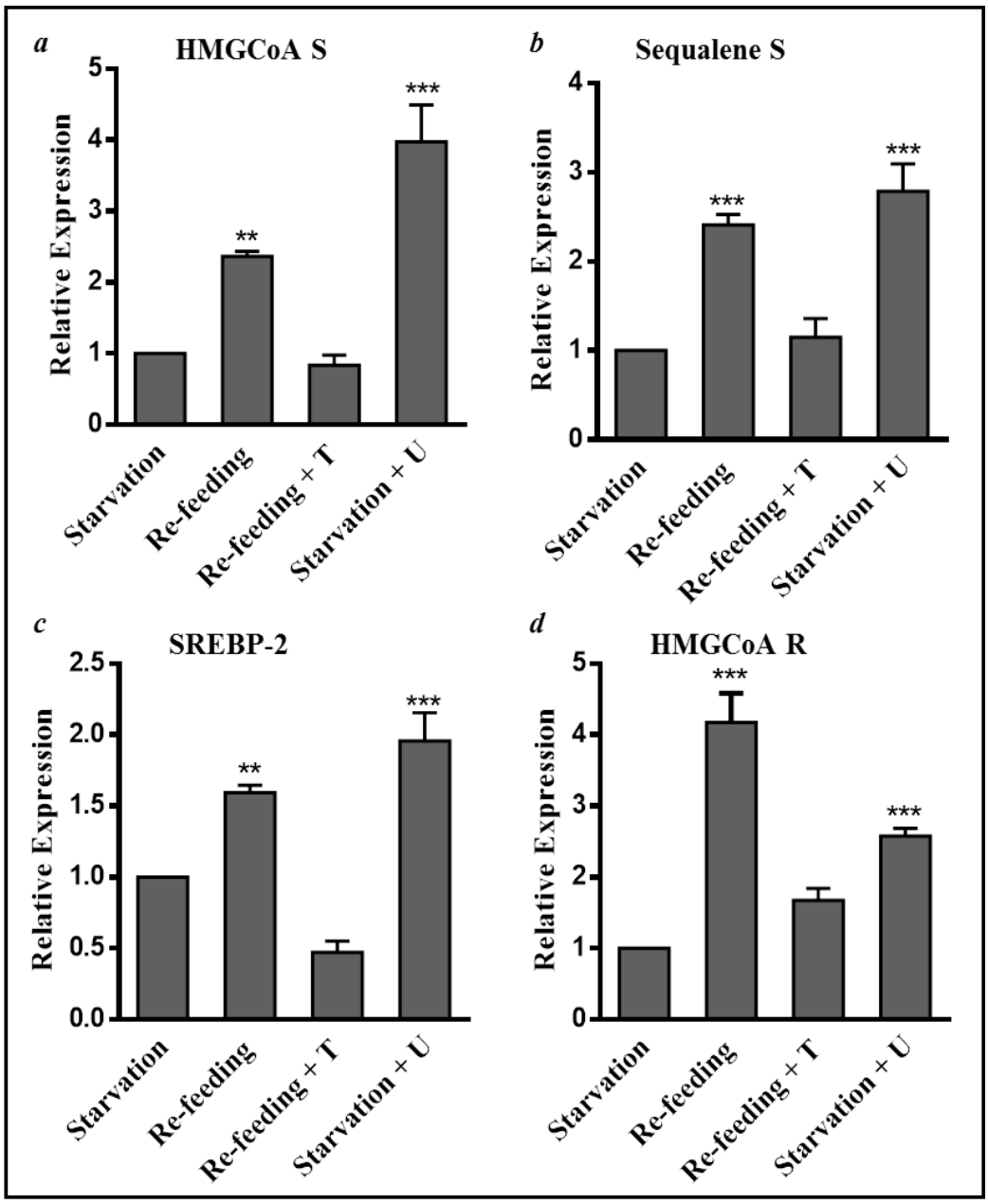


Figure 12: mTORC1 regulates SREPB-2 transcriptional activity though Cholesterol trafficking via lysosomes.

Relative expression of SREBP-2 target genes in MEF cells subjected to the conditions indicated in **Figure 7A**. mRNA levels were determined by real-time qPCR and normalized to 18S mRNA. Data are presented as the fold increase in gene expression relative to cells in starvation medium and represent the average+/- SEM of four independent experiments.

P-value: *P<0.05, **P<0.005, ***P<0.0001.

To further confirm the involvement of cholesterol efflux from the lysosomes, I silenced NPC1 (see **Figure 13A**), a protein necessary for cholesterol exit from lysosomes and the pharmacological target of U18666A (Infante et al. 2008; Lu et al. 2015). The results showed that silencing NPC1 produced identical effects as U18666A on SREBP-2 target gene expression: starvation no longer suppressed SREBP-2 targeted genes (see **Figure 13B, green bars**) and their expression levels are comparable to re-fed cells (see **Figure 13B, purple bars**).

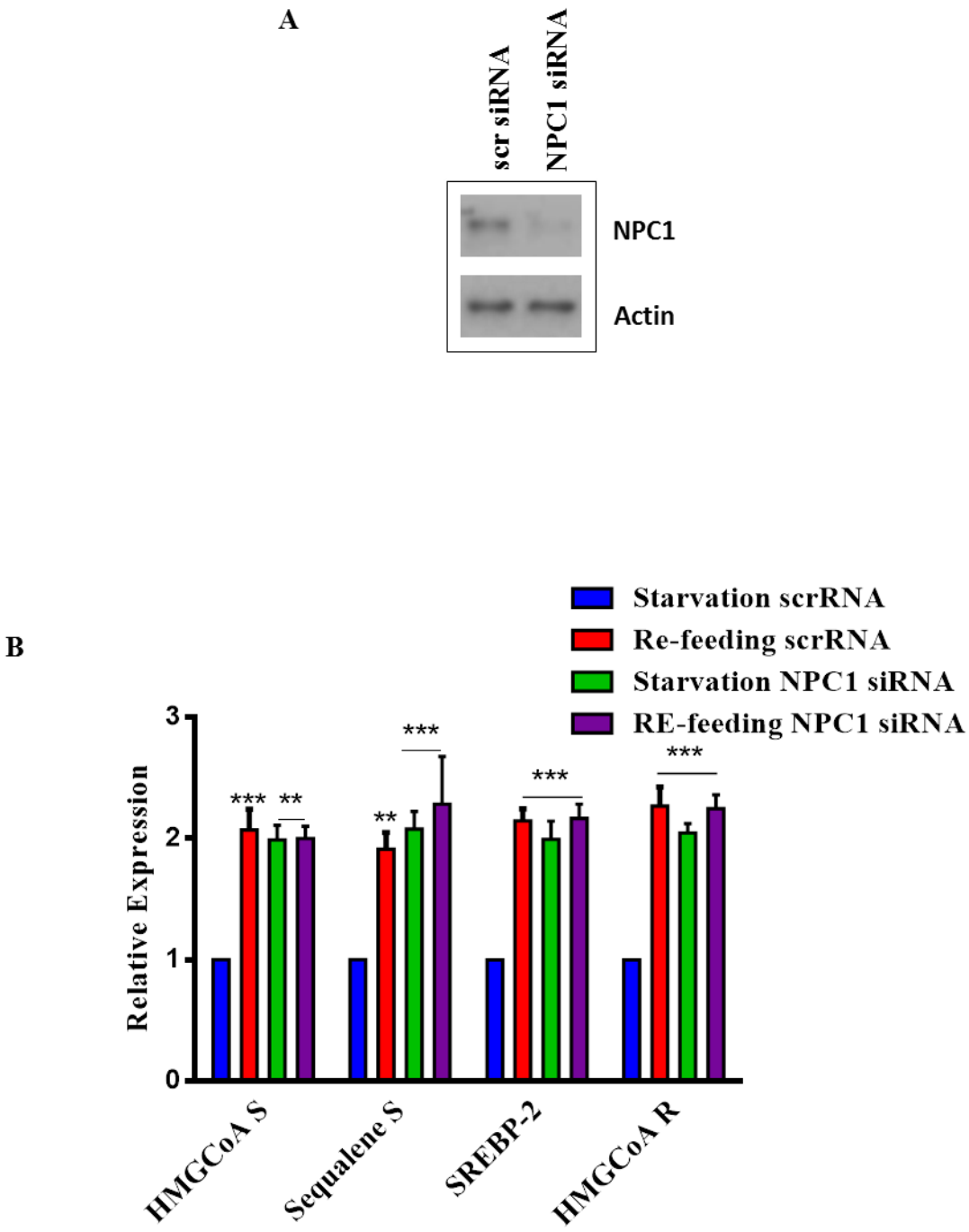
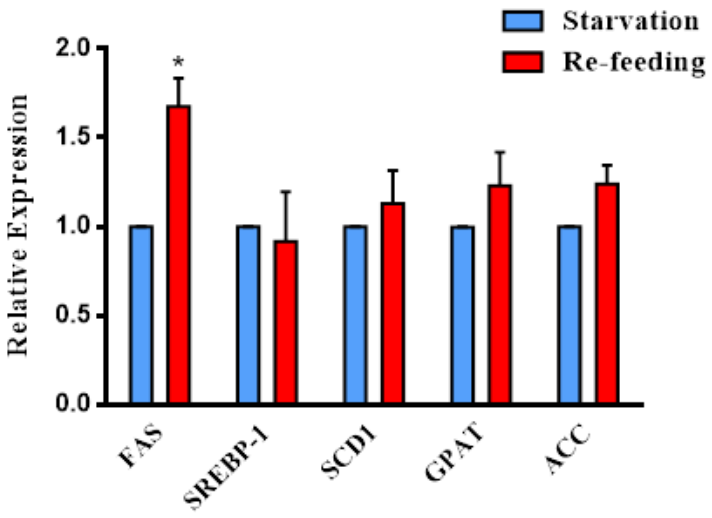


Figure 13: NPC1 silencing effect on mTORC1 mediated FC trafficking and SREBP2 genes expression. (A) Western blotting showing MEFs transfected with either scrambled or NPC1 siRNA. (B) SREBP2 genes expression, assessed by RT-PCR, in cells transfected with scrambled or NPC1 siRNA. Results show fold increases of transcriptional activity relative to starvation. Data are the average+/-SEM from four independent experiments.

P-value: *P<0.05, **P<0.005, ***P<0.0001.

I next probed the effect of nutrient conditions on SBEBP-1 target genes. With the exception of fatty acid synthase, changes in nutrient conditions do not generate similar pattern as seen with SREBP-2 (see **Figure 14**). It is likely that additional regulatory mechanisms are involved in SREBP-1 activation (MISEREZ et al. 1997). Interestingly, I observed that SREBP-1 translocation was triggered by full growth medium and refeeding, but suppressed by starvation (see **Figure 14B**), identical to that of SREBP-2 (see **Figure 11**).

A.


Starvation
Re-feeding
Relative Expression
2.0
1.5
1.0
0.5
0.0
*
FAS
SREBP-1
SCDI
GPAT
ACC

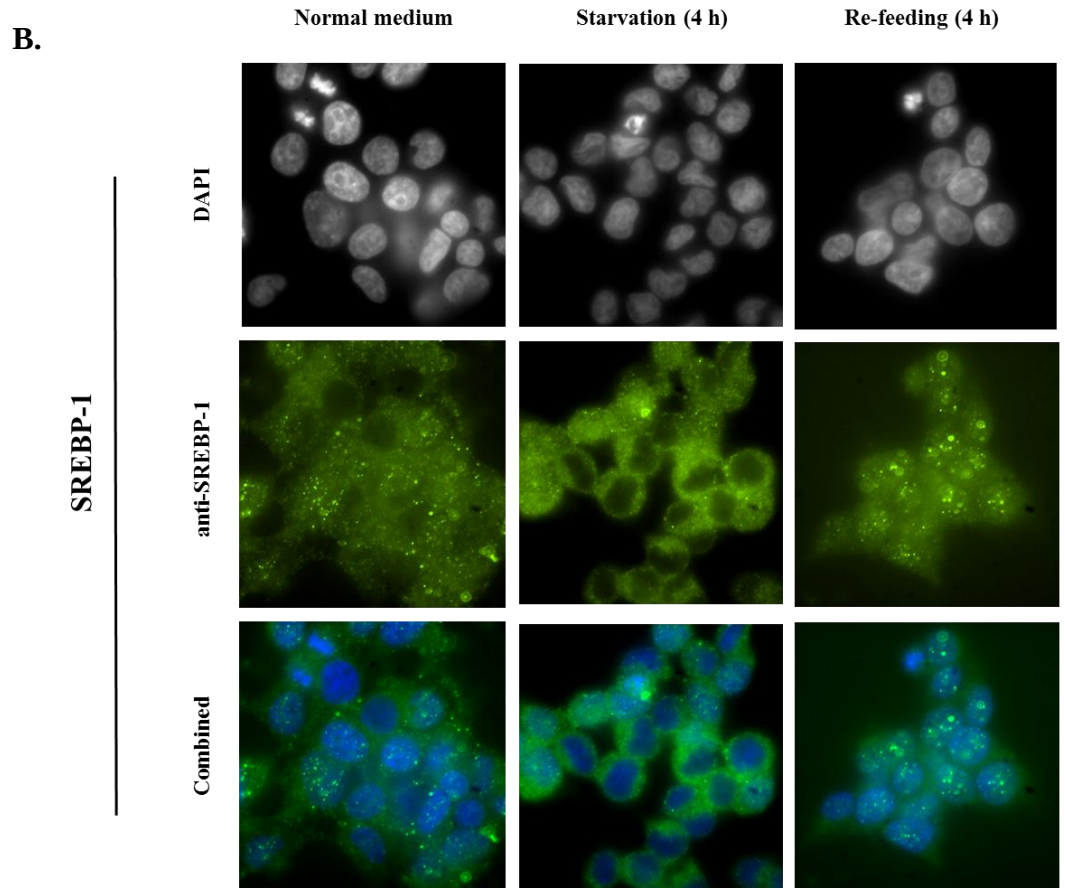


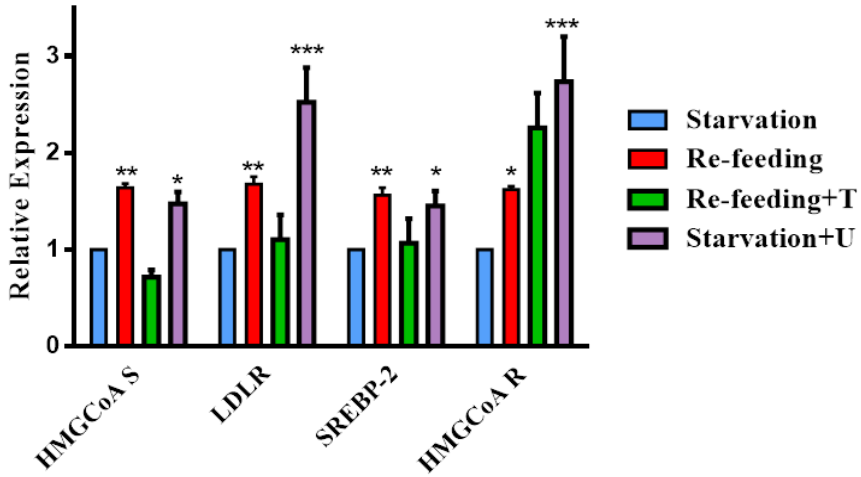
Figure 14: Starvation and refeeding effect on SREBP-1 activity.(A) Relative expression of SREBP-1 target genes in MEF cells subjected to starvation (4hr) and re-feeding (4hr) conditions. mRNA levels were determined by real-time qPCR and normalized to 18S mRNA. Data are presented as the fold increase in gene expression relative to cells in starvation medium and represent the average +/- SEM of four independent experiments. P-value: *P<0.05, **P<0.005, ***P<0.000.

(B**)** Immunofluorescence staining of MEFs with anti-SREBP1 (green) and DAPI (blue) under indicated conditions.

*SCD1: Stearoyl-CoA desaturas. ACC: Acetyl-CoA carboxylase.

Once again, in HEK cells, the expression of SREBP-2 targeted genes, responding to nutrient conditions were correlated positively with mTORC1 activity and negatively with ACAT activity (see **Figure 15A-B**), and completely mirrored the observations made in the MEFs.

A


Relative Expression
0
1
2
3
**
*
**
***
**
*
*
***
HMGCoA S
LDLR
SREBP-2
HMGCoA R
Starvation
Re-feeding
Re-feeding+T
Starvation+U

B

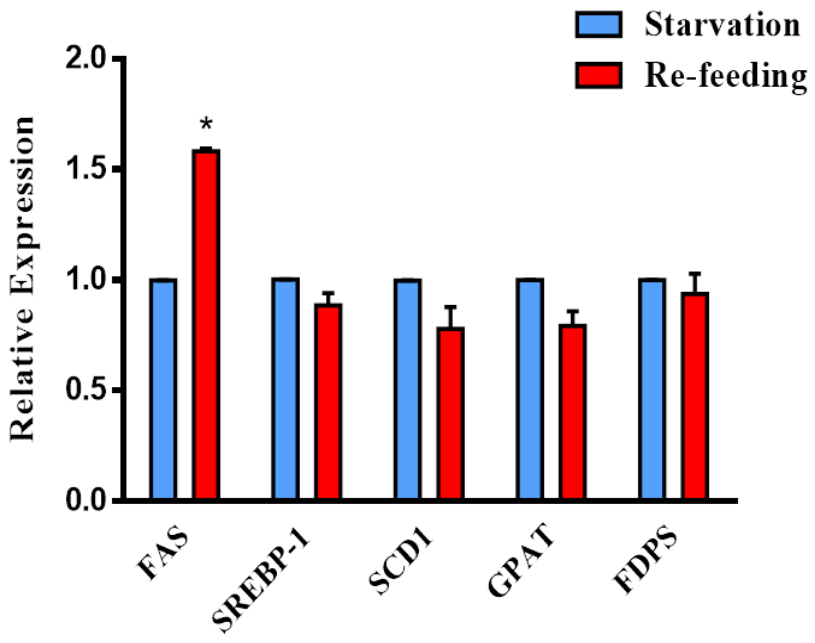

Starvation
Re-feeding
Relative Expression
0.0
0.5
1.0
1.5
2.0
*
FAS
SREBP-1
SCD1
GPAT
FDPS

Figure 15: Nutrient induced SREPB-1/2 transcriptional activity in HEK293T.

A) Relative expression of SREBP-2 target genes in HEK293T cells subjected to the conditions indicated in Figure 1A. mRNA levels were determined by real-time qPCR and normalized to 18S mRNA. Data are presented as the fold increase in gene expression relative to cells in starvation medium and represent the average +/- SEM of four independent experiments.
B) Relative expression of SREBP-1 target genes in HEK293T cells subjected to the same conditions as in (A). mRNA levels were determined by real-time qPCR and normalized to 18S mRNA. Data are presented as the fold increase in gene expression relative to cells in starvation medium and represent the average +/- SEM of four independent experiments. P-value: *P<0.05, **P<0.005, ***P<0.0001.

*FDPS: Farnesyl Diphosphate Synthase.

Taking these results together, I concluded that activation of mTORC1 coincides with low autophagy, low ER cholesterol, and activation of SREBP-2. Conversely, suppression of mTORC1, by starvation or by pharmacological inhibition, is accompanied by activated autophagy, high ER cholesterol due to increased cholesterol release from lysosomes. This suppresses the transcription of SREBP-2 target genes.

3.3 mTORC1 Suppresses Autophagy and Promotes Endosomal Recycling to Limit Cholesterol Supply to the Lysosomes, Thereby Activating SREBP-2.

3.3.1 Lipid droplets are not likely to be the source of cholesterol released from the lysosomes

Lysosomes are the primary digestive organelles that degrade macromolecules and cell debris. The products, such as amino acid and cholesterol, are then released for re-use. Given that mTORC1 controls cholesterol availability to ER through lysosomes, mTORC1 may regulate the delivery of cholesterol-containing material to lysosomes. I thus searched for potential sources of cholesterol that can reach lysosomes when mTORC1 activity is low.

One of the candidates is lipid droplets (LD), major cellular organelles for the storage of neutral lipids and a rich source of CE. Lipid droplets (LD) CE hydrolysis can be mediated by lipases in the lysosomes to produce cholesterol. This process should be sensitive to lalistat 1, a lysosomal acidic lipase inhibitor that prevents lysosomal digestion of CE to produce cholesterol (Rosenbaum et al. 2011). MEFs were treated with lalistat 1, and ACAT activities were measured under various nutrient conditions. The results showed that lalistat 1 had a limited impact on nutrient-dependent changes in ER cholesterol: ACAT activity increased similarly as untreated cells during starvation (see **Figure 16**). This suggested that acid lipases are not necessary to produce cholesterol in these cells. Therefore, LD could not be the

significant source of cholesterol during starvation. Instead, cholesterol-containing membranes were more likely to provide cholesterol during starvation.

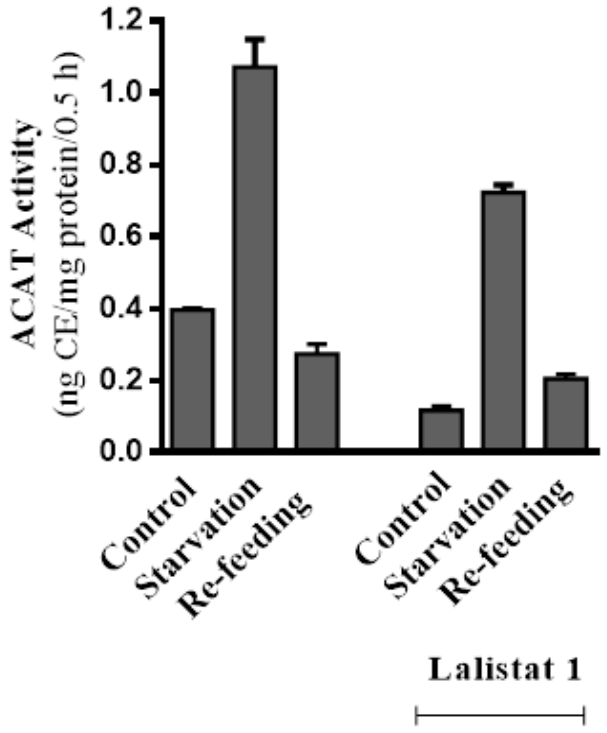


Figure 16: Lalistat-1 effect on mTORC1 mediated FC trafficking.

MEF Wt were incubated in control, starvation or starvation/re-feeding medium, with or without Lalistat-1 (10 µM) for 4 hours. ACAT activity was measured and is presented as average +/- STD (n=3).

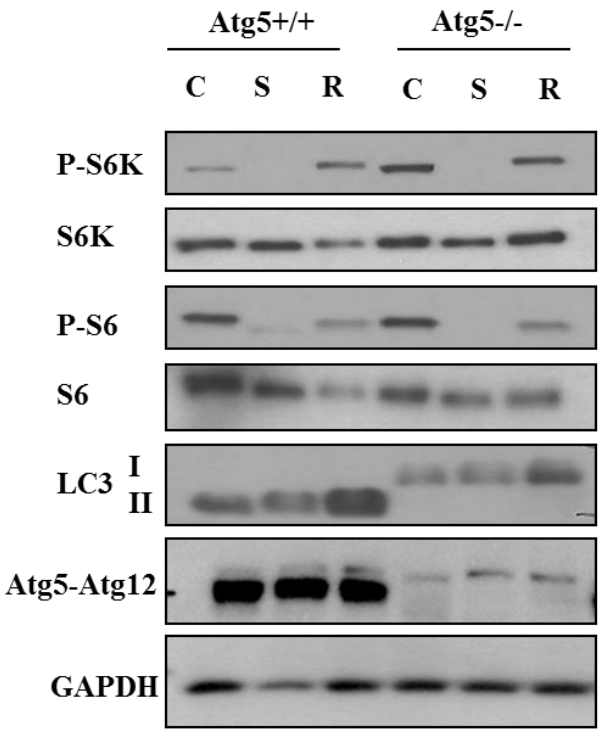
3.3.2 Defective autophagy fails to suppress cholesterol release from the lysosome to ER during starvation.

mTORC1 is known to suppress autophagy (J. Kim and Guan 2017). In the absence of mTORC1 activity, autophagy sends membrane-rich autophagosomes, a potential source of cholesterol, to the lysosomes. Hence, I first investigated MEFs lacking Atg5 ($Atg5^{-/-}$). These cells are defective in canonical autophagy (Nishida et al. 2009).

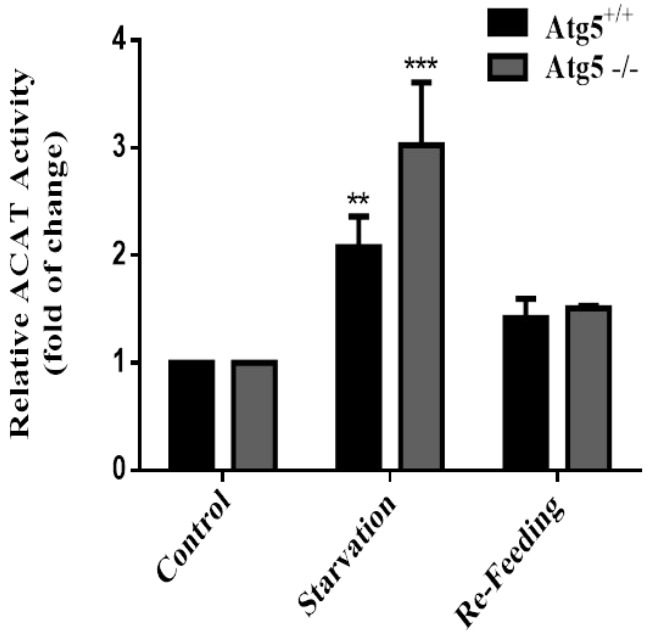
Although lacking LC3 defined autophagy (defective conversion from LC3I to LC3II) during starvation (see **Figure 17A**), $Atg5^{-/-}$ MEFs responded to starvation normally, with both a rise in ACAT activity (see **Figure 17B, second bar**), and protein degradation (see **Figure 17C)**. The enhancement of protein degradation by starvation in these KO cells was also observed previously although the reasons are unknown (Nishida et al. 2009).

I observed little alteration of SREBP-2 target gene expression pattern in $Atg5^{-/-}$ MEFs, relative to that in wt MEFs (see **Figure 17D**). I then considered that other entities, in addition to autophagosomes, might also deliver cholesterol-rich membranes to lysosomes during starvation when mTORC1 activity is low.

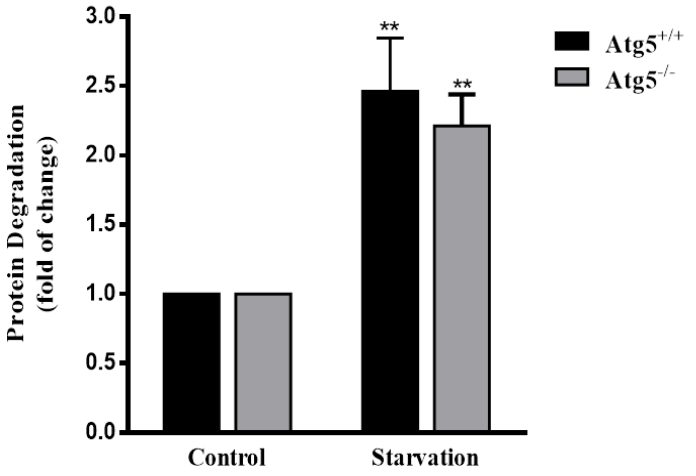
A


Atg5+/+
Atg5-/-
C S R C S R
P-S6K
S6K
P-S6
S6
LC3 I
II
Atg5-Atg12
GAPDH

B


Atg5+/+
Atg5 -/-
Relative ACAT Activity
(fold of change)
0
1
2
3
4
**
***
Control
Starvation
Re-Feeding

C


Protein Degradation
(fold of change)
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
**
Atg5+/+
Atg5-/-
Control
Starvation

D

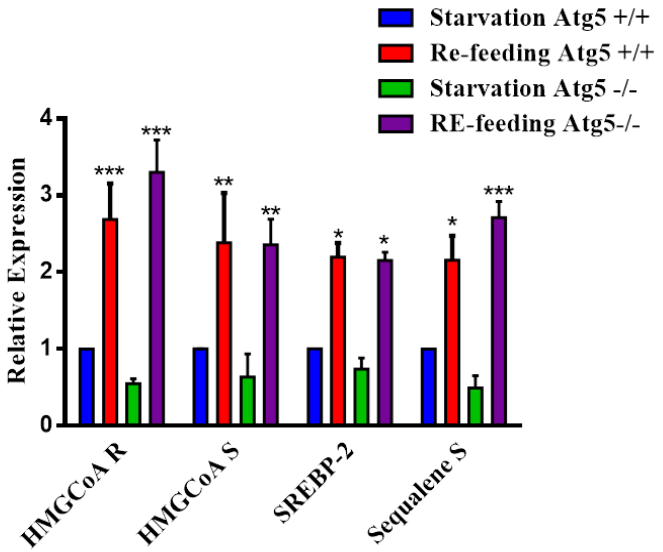

Starvation Atg5 +/+
Re-feeding Atg5 +/+
Starvation Atg5 -/-
RE-feeding Atg5-/-
Relative Expression
4
3
2
1
0
***
***
**
**
*
*
*
***
HMGCoA R
HMGCoA S
SREBP-2
Sequalene S

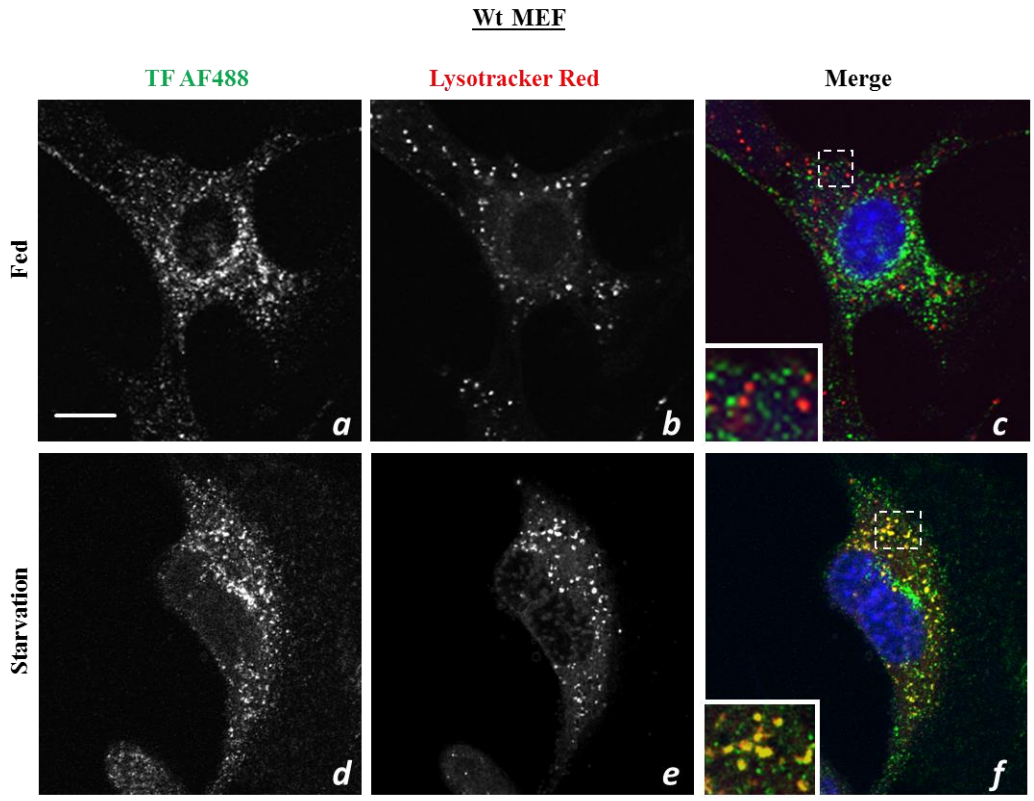
Figure 17: Defective canonical autophagy had no effect on ACAT activity and SREBP2 expression.

(A) $Atg5^{+/+}$ and $Atg5^{-/-}$ MEFs were incubated in control (C), starvation (S) and starvation/re-feeding (R) medium as in Figure 1A. Cells were then lysed, subjected to SDS-PAGE and western blotted with indicated antibodies.
(B) ACAT activity was measured in $Atg5^{+/+}$ and $Atg5^{-/-}$ MEFs. Results represent the fold increase of CE formation relative to cells in control medium. Data are the average +/- SEM of three independent experiments.
(C) Protein degradation in $Atg5^{+/+}$ and $Atg5^{-/-}$ MEFs. Data represent the average +/- SEM of three independent experiments.
(D) Relative expression of SREBP-2 target genes in cells treated as above. Data represent the average +/- STD (n=4).
P-value: *P<0.05, **P<0.005, ***P<0.0001.

3.3.3 Suppression of mTORC1 targets normally recycled endosomes to the lysosomes

Endosomes are derived from cholesterol-rich plasma membrane. In normal proliferating cultured cells, such as fibroblasts, endosomes deliver nutrients (LDL, iron, etc.) to lysosomes while efficiently recycling membrane and receptors back to the plasma membrane (Mayor, Presley, and Maxfield 1993). However, our laboratory recently discovered a significant re-routing of endosomes to lysosomes when mTORC1 activity is low (Dauner et al. 2017). It was shown that, in normal or re-feeding conditions, transferrin (Tf) and its receptor (TfR) efficiently recycle between the plasma membrane and sorting endosomes membrane and little appears in the lysosomes (see **Figure 18A, first row, Atg5$^{+/+}$**). However, during starvation or mTORC1 inhibition by Torin-1, Tf is targeted to the lysosomes (see **Figure 18A, second row, Atg5$^{+/+}$**), along with Tf receptors and plasma membrane sphingomyelin (not shown here). This raised the possibility that starvation, as well as mTORC1 inhibition, delivered cholesterol-rich endosomes to lysosomes, even when autophagosomes were blocked by Atg5 knockout (see **Figure 18B, second row, Atg5$^{-/-}$)**. This could lead to higher ER cholesterol and suppression of SREBP-2, even in the absence of Atg5 or conical autophagy.

A


Wt MEF
TF AF488
Lysotracker Red
Merge
Fed
Starvation
a
b
c
d
e
f

B

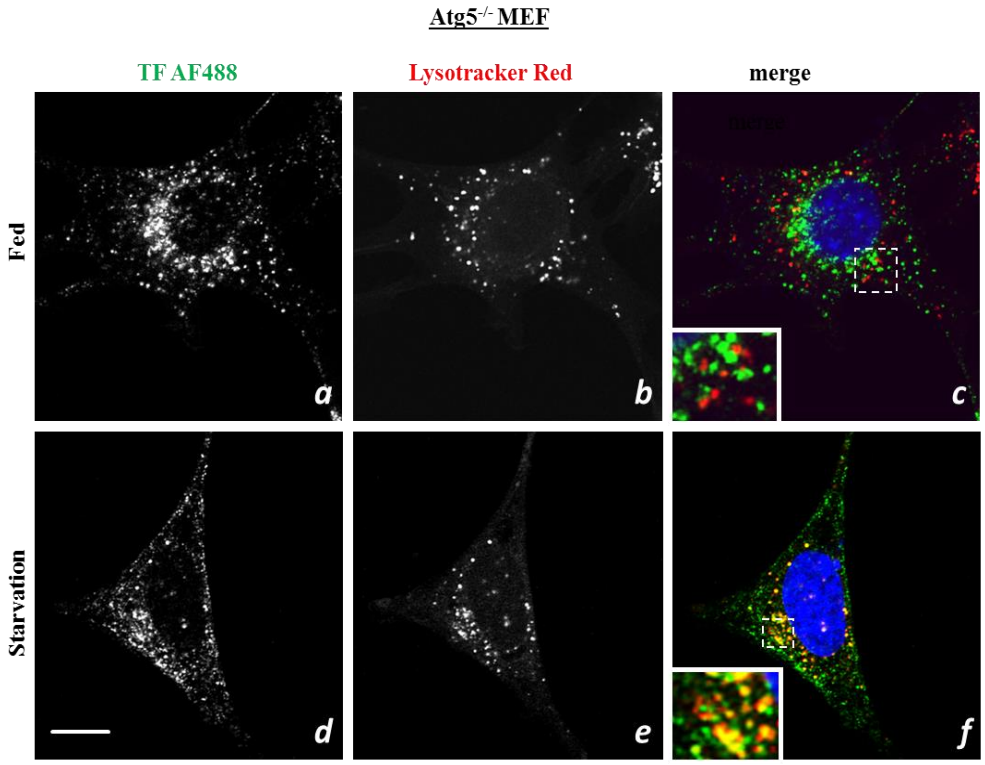


Figure 18: mTORC1 regulates endocytic recycling pathway under nutrient conditions.

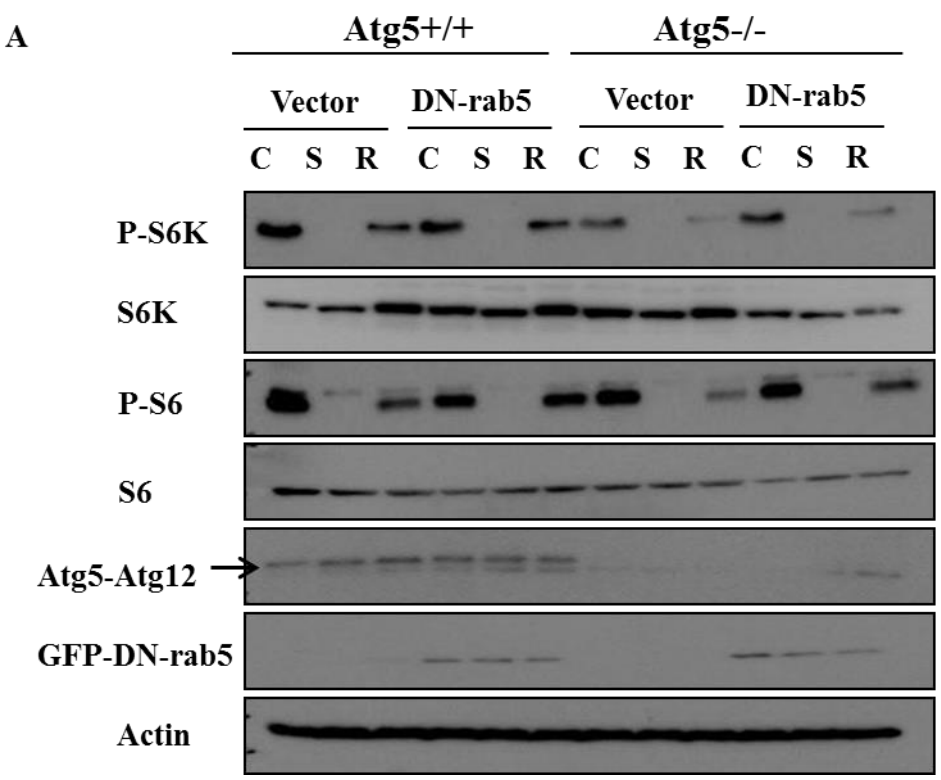
MEF, Atg5+/+ (A) and Atg5-/- (B) cells were incubated under indicated conditions. AF488-transferrin (Tf) and Lysotracker Red were added in the last 30 minutes. When incubated in full media, Tf is exclusively localized to the endosomal recycling system with none in lysosomes. In contrast, when subjected to amino acid starvation, Tf is rerouted to the lysosomes, indicated by an overlap of the AF488 Tf fluorescence with the Lysotracker Red staining. Scale bar 10 μm.

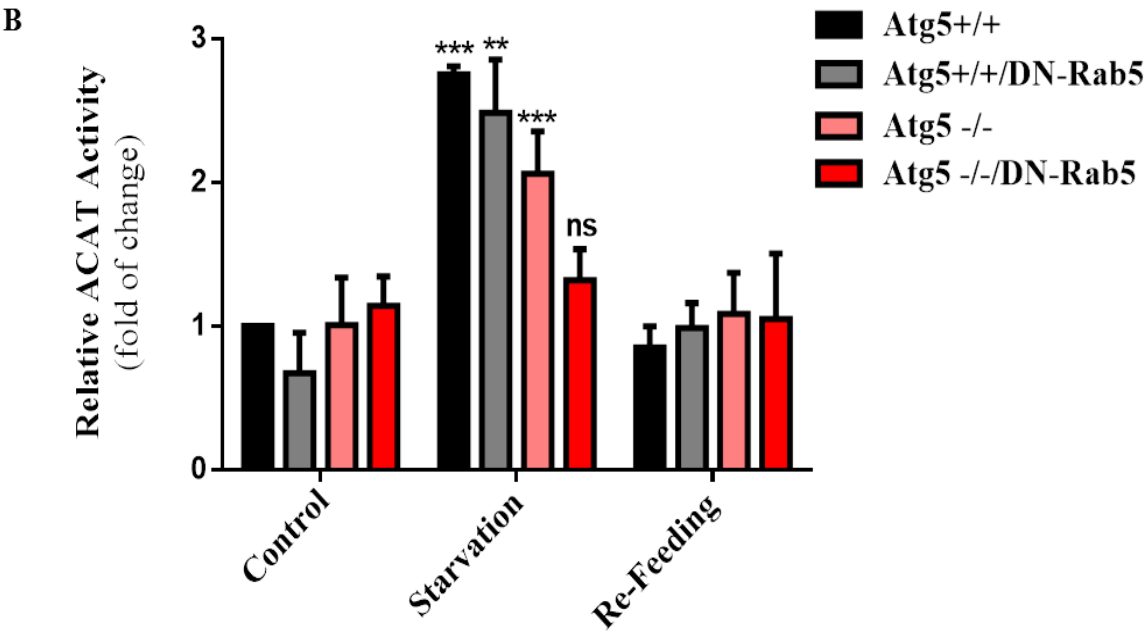
This research was originally published in the Journal of Biological Chemistry. Dauner, Kristin, Walaa Eid, Riya Raghupathy, John F. Presley, and Xiaohui Zha. 2017. "mTOR Complex 1 Activity Is Required to Maintain the Canonical Endocytic Recycling Pathway against Lysosomal." The Journal of Biological Chemistry 292 (14): 5737–47. © the American Society for Biochemistry and Molecular Biology.

To test this possibility, dominant negative (DN) rab5 was expressed in Atg5-/- MEFs. DN-rab5 is known to block endocytosis as well as the fusion of early endosomes (Bucci et al. 1992). DN-rab5 or Atg5-/- or in combination had little effect on mTORC1 activity upon nutrient conditions: phosphorylation of S6k and S6 is similar to wt MEFs (see **Figure 19A**). Also, Atg5-atg12 conjugate was present in $Atg5^{+/+}$ MEFs, an indication of active autophagy, but not in $Atg5^{-/-}$ MEFs (see **Figure 19A**).

The rise of ER cholesterol during starvation was minimally affected by blocking either pathway alone ($Atg5^{-/-}$ or $Atg5^{+/+}$+ DN-rab5), as shown by the increase in ACAT activity similar to non-transfected $Atg5^{+/+}$ cells (see **Figure 19B, grey, pink and black bars**). This correlated with suppressed SREBP-2 activity during starvation (see **Figure 19C, $Atg5^{-/-}$ or $Atg5^{+/+}$+ DN-rab5**).

However, when both pathways were blocked simultaneously (DN-rab5 expressing Atg5-/- MEFs), ER cholesterol failed to rise during starvation, again indicated by reduced ACAT activity (see **Figure 19B, red bars**). This correlated with high SREBP-2 activity during starvation (see **Figure 19C, Atg5-/-+ DN-rab5**).


A
Atg5+/+
Atg5-/-
Vector
DN-rab5
Vector
DN-rab5
C S R C S R C S R C S R
P-S6K
S6K
P-S6
S6
Atg5-Atg12
GFP-DN-rab5
Actin


B
Relative ACAT Activity
(fold of change)
3
2
1
0
*** **
***
ns
Control
Starvation
Re-Feeding
Atg5+/+
Atg5+/+/DN-Rab5
Atg5 -/-
Atg5 -/-/DN-Rab5

C

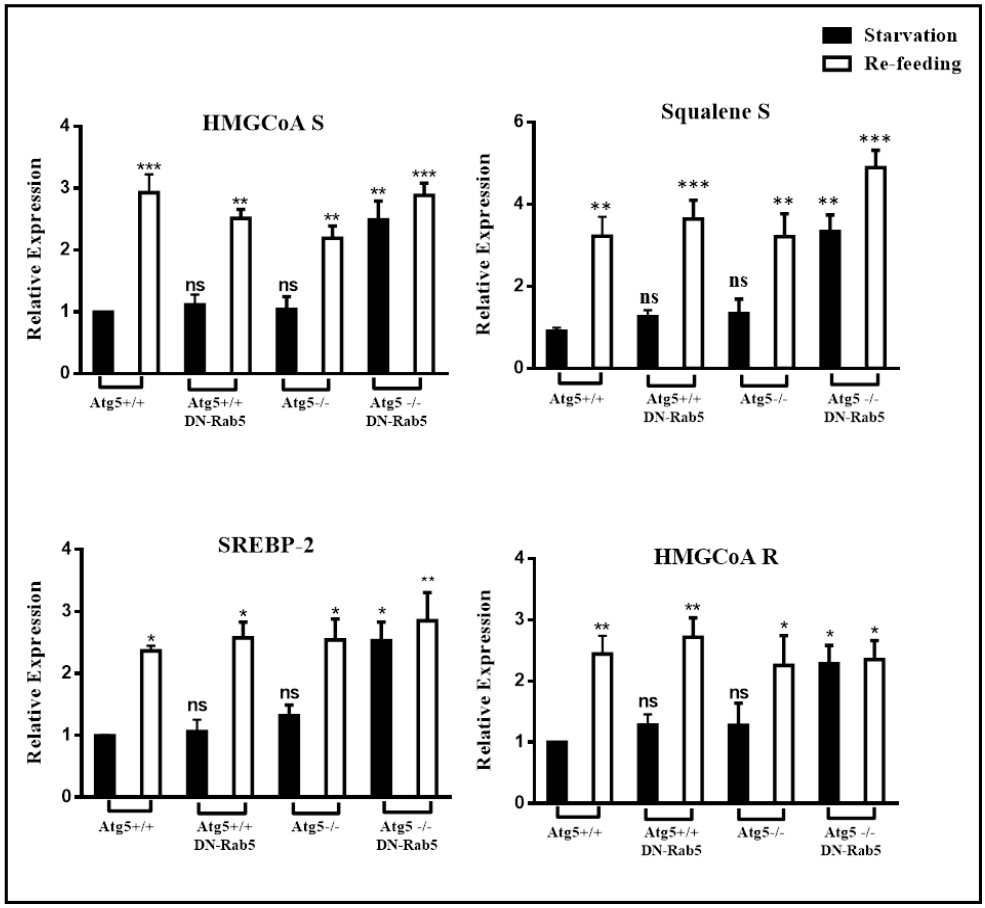


Figure 19: Combination of autophagy and re-routed endosomal trafficking contributes to cholesterol trafficking from the lysosome to the ER to suppress SREBP-2.

(A) Atg5 Tet-off inducible MEFs were cultured in growth medium for four days in the presence or absence of doxycycline (10ng/ml). Some cells were transfected with either a control vector or DN-rab5 DNA on Day 2. Cells were then subjected to starvation or starvation/re-feed as in **Figure 1A**, cells were lysed and protein analyzed by SDS-PAGE followed by western blotting with indicated antibodies.

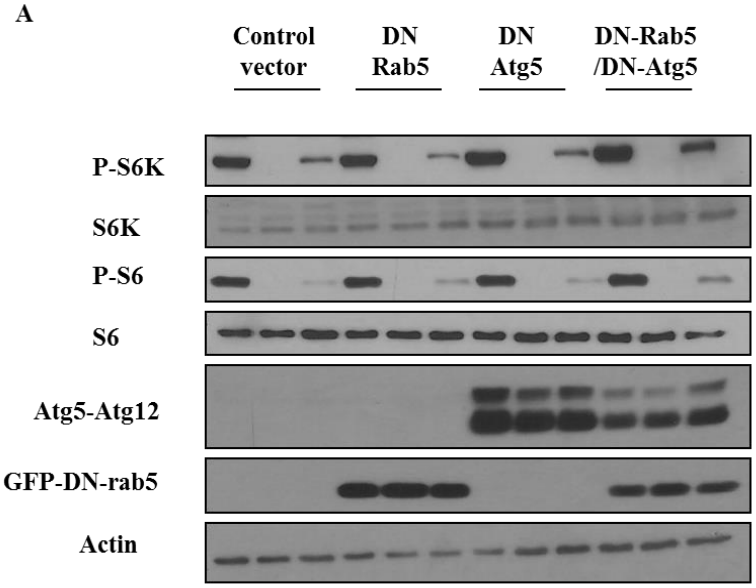
(B) ACAT activity in Atg5 Tet-off inducible MEFs with or without DN-rab5 transfection. Data are the average +/- SEM of three independent experiments.

(C) Relative expression of SREBP-2 target genes in Atg5 Tet-off inducible MEFs with or without DN-rab5 transfection. Result shows fold increase of gene expression in cells after re-feeding relative to cells in starvation. Results are the average +/- SEM of four independent experiments.

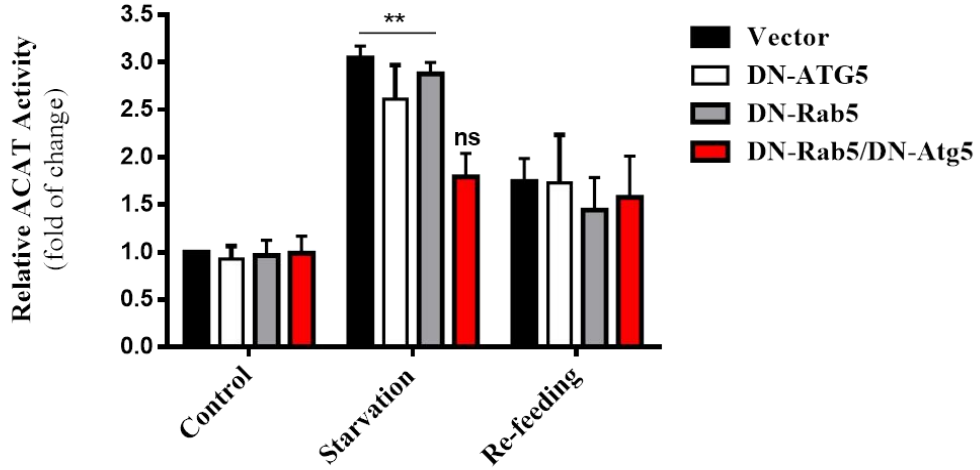
P-value: *P<0.05, **P<0.005, ***P<0.0001, ns=not significant.

Similar results were observed with HEK cells, expressing DN-Atg5 or DN-rab5 or both (see **Figure 20A**). Only dual blockade of both pathways prevented ER cholesterol from increasing (see **Figure 20B, red bars**) and, as a result, SREBP-2 dependent gene expression was high regardless of nutrient conditions (see **Figure 20C)**.

A


Control vector
DN Rab5
DN Atg5
DN-Rab5 /DN-Atg5
P-S6K
S6K
P-S6
S6
Atg5-Atg12
GFP-DN-rab5
Actin

B


Relative ACAT Activity
(fold of change)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
**
ns
Control
Starvation
Re-feeding
Vector
DN-ATG5
DN-Rab5
DN-Rab5/DN-Atg5

C

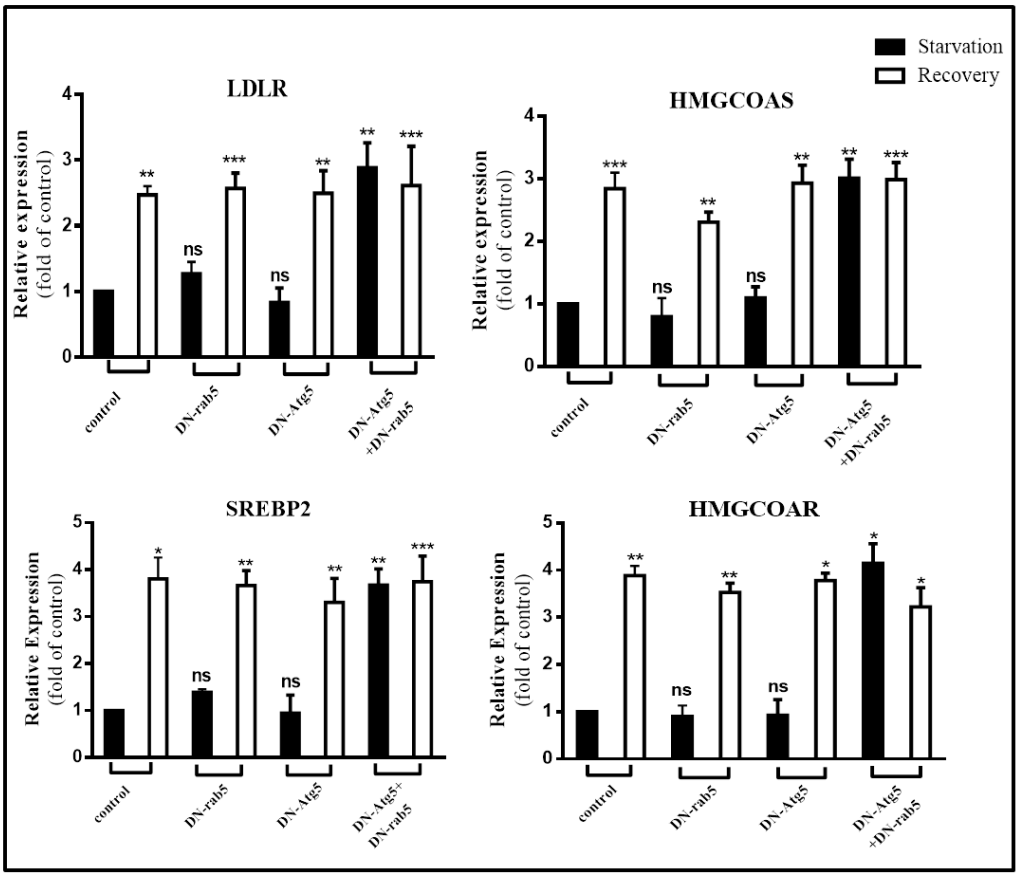


Figure 20: Defective autophagy and endocytic pathways suppresses lysosomal cholesterol trafficking and induces SREBP-2 activity.

(A) Western blot of HEK 293T cells transfected with either a control vector, DN-Atg5, DN-rab5 or a combination of DN-Atg5/DN-rab5. Cells were then subjected to the same treatment as in **Figure 7A,** lysed and processed SDS-PAGE followed by western blot analysis for the indicated antibodies.

(B) ACAT activity of HEK293T cells. Results represent fold increase of CE formation relative to cells in control medium. Data are the average +/- SEM of three independent experiments.

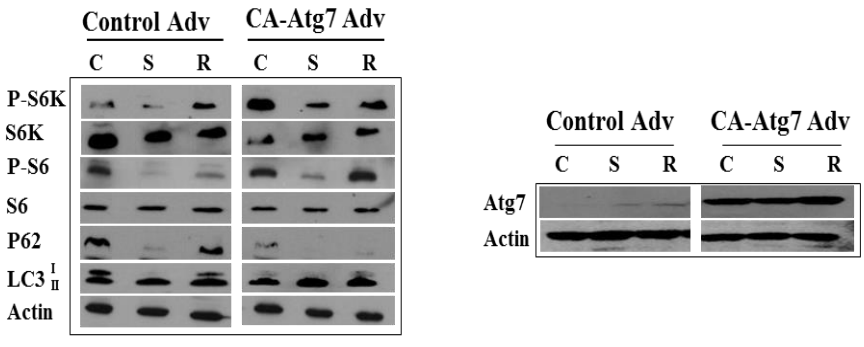
(C) Relative expression of SREBP-2 target genes in transfected HEK cells. Results show fold increase after re-feeding relative to cells in starvation. Data are the average +/- SEM of four independent experiments.

P-value: *P<0.05, **P<0.005, ***P<0.0001, ns=not significant.

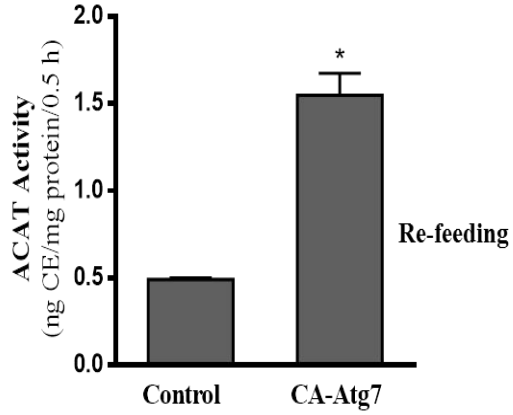
3.3.4 Constitutively active autophagy induces FC trafficking into the ER under amino acid rich condition and suppresses SREBP-2 activation

To understand the consequence when autophagy is overly stimulated with high mTORC1 activity, I overexpressed a constitutive active (CA) Atg7 in MEF cells using CA-Atg7 adenovirus (see **Figure 21A, *b***). This activates autophagy even when mTORC1 activity was high (in normal or re-feeding medium), as reported previously (Yang et al. 2010). Indeed, I observed that little p62 remained in CA-Atg7 expressing cells, even in re-feeding medium (see **Figure 21A, *a***), indicative of over-active autophagy. p62 is a ubiquitin-binding scaffold protein and serves as part of the autophagic machinery that is eventually degraded in the lysosome. Therefore, p62 level decreases when autophagy is active (Pankiv et al. 2010). In addition, I also saw that CA-Atg7 expressing cells accumulated LC3II, an autophagy product, under all nutrient conditions. Indeed, in comparison to cells infected with control virus, CA-Atg7 expressing cells not only had more active autophagy, but also higher ER cholesterol, even when cells were exposed to amino acid rich medium (see **Figure 21B**). This was correlated with suppression of SREBP-2 target gene, HMG-CoAR, expression (see **Figure 21C**).

A


Control Adv
CA-Atg7 Adv
C S R C S R
P-S6K
S6K
P-S6
S6
P62
LC3 I II
Actin
Control Adv
CA-Atg7 Adv
C S R C S R
Atg7
Actin

B


ACAT Activity
(ng CE/mg protein/0.5 h)
2.0
1.5
1.0
0.5
0.0
*
Re-feeding
Control
CA-Atg7

C

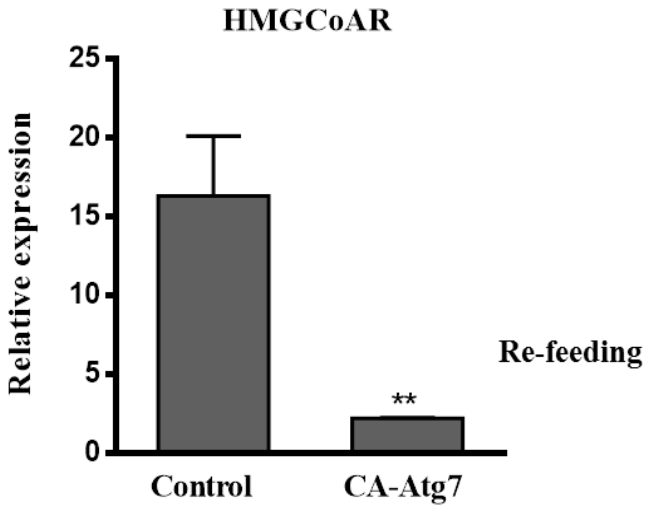


Figure 21: Accelerated autophagy effect on cholesterol trafficking and SREBP2 expression.

(A) Western analysis of wt MEFs transfected with CA-Atg7 or control adenovirus for one day then cultured for three days.

(B) ACAT activity of cells transfected with CA-Atg7 or control adenovirus. Data represent the cells in the re-feeding condition.

(C) HMGCoA-R mRNA expression in MEFs transfected with CA-Atg7 or control adenovirus. Data are the average +/- SEM of three independent experiments.

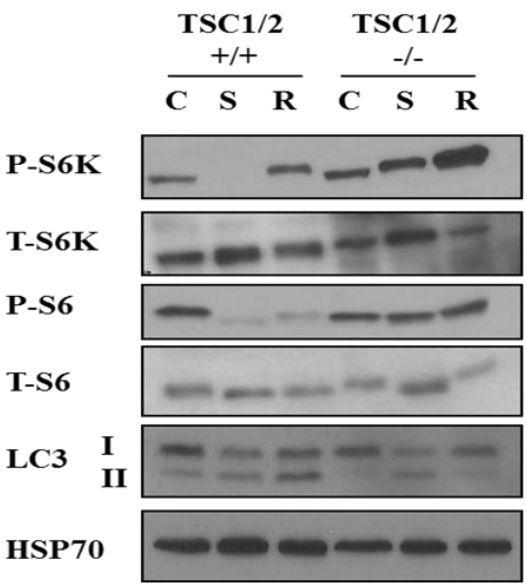
P-value: *P<0.05, **P<0.005, ***P<0.0001, ns=not significant.

3.4 Cholesterol trafficking to the ER is a proximal regulator of SREBP-2 activity, despite high mTORC1 activity, in TSC1/2$^{-/-}$ MEFs.

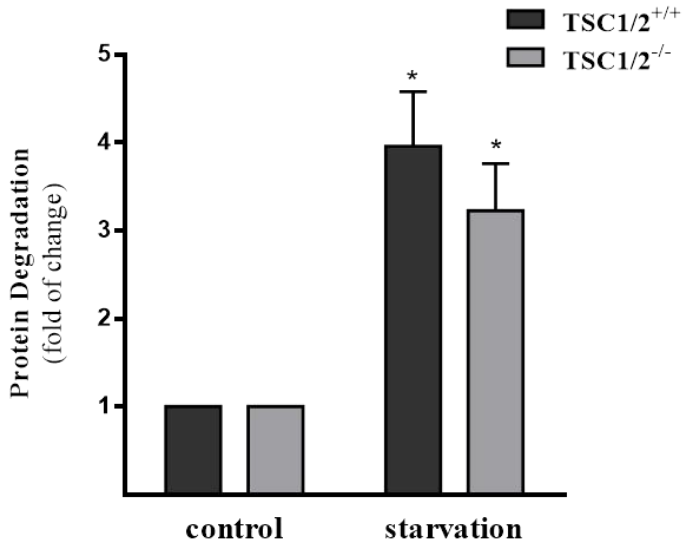
I next attempted to see whether high mTORC1 activity is sufficient for SREBP-2 activation. TSC1/2 are endogenous inhibitors of mTORC1 activation (HUANG and MANNING 2008). Growth factors such as insulin and phosphorylate TSC1/2 release the TSC inhibition of Rheb and activate mTORC1. In cells deficient in TSC1/2, mTORC1 is constitutively activated, regardless of nutrient conditions (K. Du et al. 2010). I observed that mTORC1 activity in TSC1/2$^{-/-}$ MEFs, based on p-S6K and p-S6 levels, remained little changed by nutrient conditions and remained high under starvation (see **Figure 22A**).

Nevertheless, TSC1/2$^{-/-}$ MEFs remained responsive to starvation, indicated by up-regulation of autophagy (LC3I to LC3II processing) (see **Figure 22A**), protein degradation (see **Figure 22B)**, and ACAT activation (see **Figure 22C**), all identical to that of wt MEFs. This agrees with previous reports (K. Du et al. 2010). Remarkably, SREBP-2 target genes were suppressed by starvation in TSC1/2$^{-/-}$ MEFs (see **Figure 22D**), despite persistently elevated mTORC1 activity (see **Fig. 22A)**. This observation suggested that active mTORC1 is not sufficient to activate SREBP-2. Rather, it is cholesterol trafficking to the ER that is a more proximal regulator of SREBP-2. Thus, cholesterol trafficking from the lysosomes to the ER likely functions downstream of mTORC1 to regulate SREBP-2.

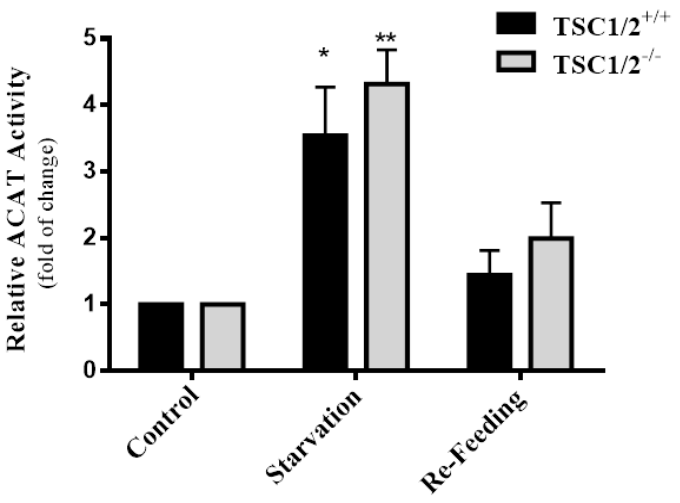
A


TSC1/2
+/+
TSC1/2
-/-
C S R C S R
P-S6K
T-S6K
P-S6
T-S6
LC3 I
II
HSP70

B


TSC1/2+/+
TSC1/2-/-
Protein Degradation
(fold of change)
5
4
3
2
1
*
*
control
starvation

C


TSC1/2+/+
TSC1/2-/-
Relative ACAT Activity
(fold of change)
0
1
2
3
4
5
*
**
Control
Starvation
Re-Feeding

D

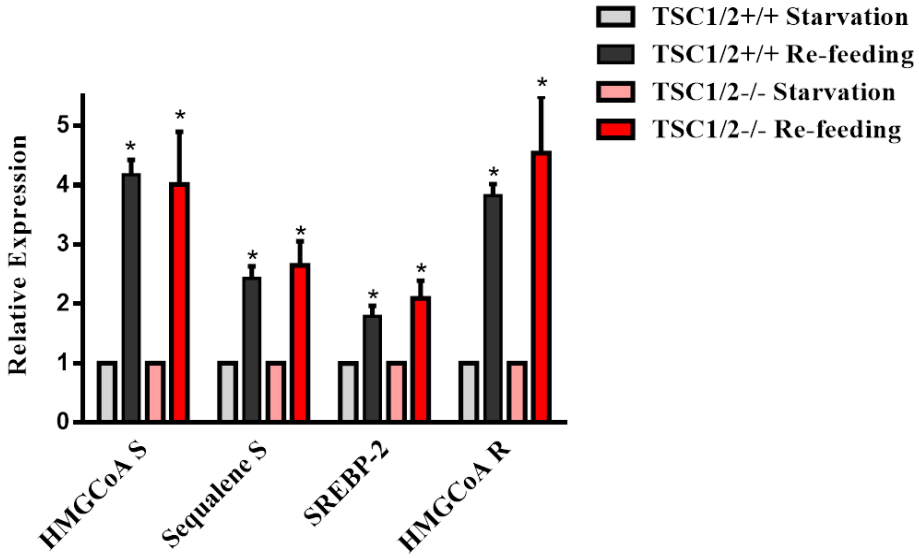

TSC1/2+/+ Starvation
TSC1/2+/+ Re-feeding
TSC1/2-/- Starvation
TSC1/2-/- Re-feeding
Relative Expression
0
1
2
3
4
5
*
HMGCoA S
Sequalene S
SREBP-2
HMGCoA R

Figure 22: Cholesterol trafficking regulates SREBP-2, regardless of mTORC1 activity.

(A) wt and TSC $1/2^{-/-}$ MEFs were incubated in control (C), starvation (S) and starvation/re-feeding (R) medium as in **Figure 1A**. Cells were then lysed and subjected to SDS-PAGE, followed by western blot analysis with indicated antibodies.

(B) Protein degradation in wt and TSC $1/2^{-/-}$ MEFs. Data are the average +/- SEM of three independent experiments.

(C) ACAT activity in wt and TSC$1/2^{-/-}$ MEFs as in (A). Results are the average +/- SEM of three independent experiments.

(D) Relative expression of SREBP-2 target genes in wt and TSC$1/2^{-/-}$ MEFs. mRNA levels were determined by real-time qPCR and normalized to 18S mRNA. Data are presented as the fold increase in gene expression relative to cells in starvation medium and represent the average +/- SEM of four independent experiments.

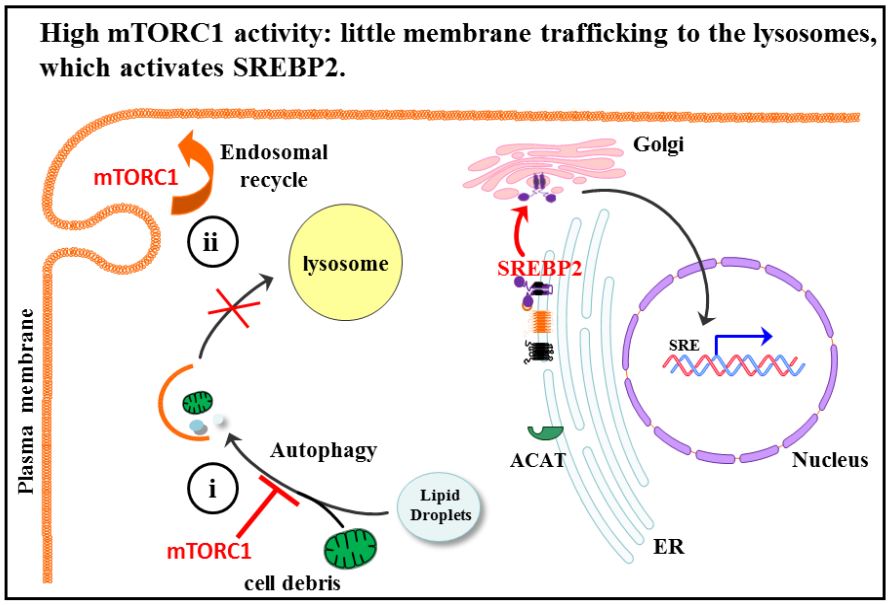
P-value: *P<0.05, **P<0.005, ***P<0.0001.

3.5 Conclusions

Overall, this work demonstrates, for the first time, that mTORC1 regulates membrane trafficking, namely autophagy and endosomal recycling. This in turn regulates cholesterol delivery to the lysosomes. It is lysosome-derived cholesterol that determines cholesterol levels in the ER, which then regulates SREBP-2 activities. Specifically, when mTORC1 activity is high (see **Figure 23A**) (for example, when nutrients are available), autophagy is suppressed and endosomes are actively recycling to return to the plasma membrane. Consequently, little cholesterol appears in the lysosomes and is transported to the ER resulting in SREBP-2 activation.

Conversely, when mTORC1 activity is low **(Fig. 23 B)**, either by starvation or by pharmacological inhibition, autophagy is activated and delivers membrane-rich cargo to the lysosomes. Endosomes with receptors are also targeted to the lysosomes. This creates cholesterol-rich lysosomes, which is then released from lysosomes in a NPC1-dependent manner. This raises cholesterol level in the ER and suppresses SREBP-2 activation.

A



B

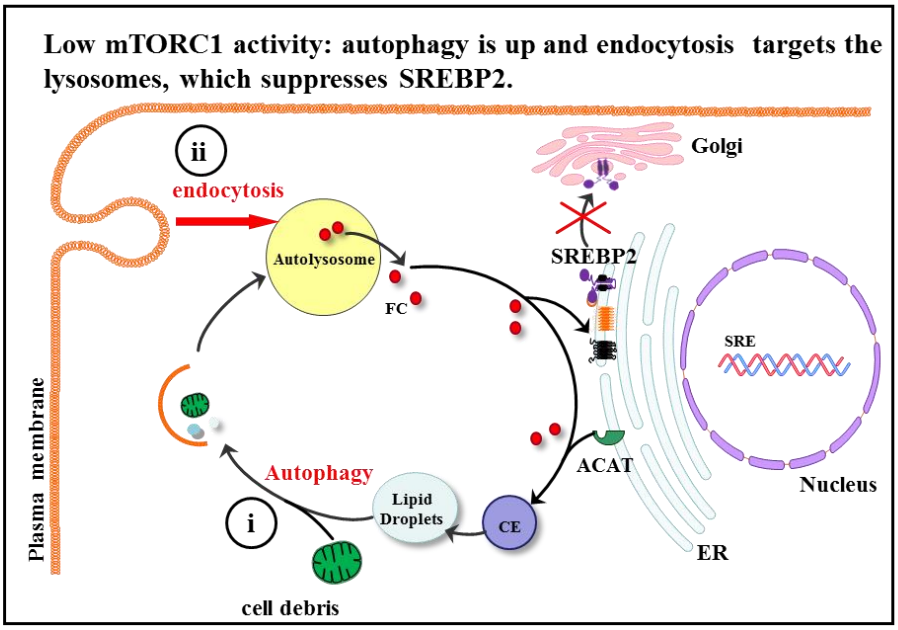


Figure 23:Model of mTORC1/autophagy/endocytosis dependent regulation of SREBP-2 transcriptional activities. During (A) high mTORC1 activity (growth condition) and (B) low mTORC1 activity (starvation condition).

4 DISCUSSION

4.1 mTORC1 Activates SREBP-2

The mTORC1 signaling pathway serves as a central hub that senses and integrates intra- and extra-cellular cues to regulate cellular processes and maintain homeostasis. Deregulation results in human diseases such as cancer and type 2 diabetes (Zoncu, Efeyan, and Sabatini 2011). In particular, mTORC1 is known to control lipogenesis by promoting SREBP activation. When mTORC1 is high, there is increased SREBP proteolytic processing, nuclear accumulation, and transcriptional upregulation of lipogenic genes (Porstmann et al. 2009). However, the precise molecular mechanism by which mTORC1 promotes SREBP activation is largely unknown. In this study, we focused on SREBP-2, a master regulator of cholesterol synthesis and acquisition. We provided a mechanism by which mTORC1 regulates membrane trafficking to the lysosomes to control SREBP-2 activity.

4.2 Autophagy and Re-routed Endosomes, Through mTORC1 Suppression, Promotes Cholesterol Trafficking from the Lysosomes to the ER

All mammalian cells express ACAT in the ER membrane and can store cholesterol as CE, though with variable capacity among different tissues and cell types. This CE pool apparently is constantly hydrolyzed to cholesterol, by both acidic lipases in the lysosomes and neutral lipases in the cytosol. Cholesterol is then re-esterified by ACAT in the ER to CE in the so-called “CE cycle,” a process documented by Brown et al. in the 1980s (Michael S. Brown, Ho, and Goldstein 1980). It was noted at the time that each CE cycle consumes at least one ATP for a seemingly futile process. In the current study, we discovered that mTORC1 apparently regulates at least one arm of such cycles, namely supplying ACAT in the ER with lysosome-released cholesterol. When mTORC1 is low, both autophagosomes

and endosomes fuse with acid lysosomes or enodosmes to form autolysosomes. The lysosomes then digest the membranes and release cholesterol by a NPC-1-mediated mechanism. This newly released cholesterol is transported to the ER membrane, along with other cellular membranes, to suppress SREBP-2. On the other hand, high mTORC1 activity suppresses autophagy and promotes recycled endosomes back to the plasma membrane. This deprives the lysosomes of the cholesterol, thereby lowering ER cholesterol and activating SREBP-2. In this context, the CE cycle seems to serve as a metabolic cue to reflect metabolic state (i.e. mTORC1 activity); perhaps maintaining a dynamic and responsive cellular cholesterol recycling pool. Indeed, this concept is consistent with several recent studies in cells with abundant LD, such as macrophages and hepatocytes. For example, a type of autophagy called lipophagy, suppresses lipogenesis under various metabolic conditions in microphages (Ouimet et al. 2011). There, LD was shown to be sequestered by a lipophagy process for delivery to the lysosome. CE was then hydrolyzed to release cholesterol with the consequence of suppressed lipogenesis. Noticeably, LD is prevalent in lipid-rich cells such as hepatocytes or adipocytes. There, they also seem to use autophagy to suppress lipogenesis (Yang et al. 2010). How autophagy or lipophagy suppresses lipogenesis in the above studies is not clear. Based on the results of this study, we propose that the active CE cycle may participate in mTORC1 signaling and dynamically supply cholesterol to the ER membrane, thereby suppressing SREBP activation.

In MEFs and HEK cells, used in this thesis, mTORC1 activity is regulated by amino acid availability. Autophagy is activated by amino acid starvation or mTORC1 inhibition by Torin-1. Starvation caused the release of cholesterol from the lysosomes, which led to higher ER cholesterol, as indicated by enhanced ACAT activity. Furthermore, it was also

established that autophagy is not the only route to provide cholesterol, in the form of membranes and debris, to the lysosomes during starvation. We showed that $Atg5^{-/-}$ MEFs, defective in canonical autophagy, responded to starvation normally, with both an increase in protein degradation and a rise in ER cholesterol. It is thus apparent that other membrane trafficking processes must also be initiated by starvation in addition to autophagy. It was then discovered that starvation (thus low mTORC1) re-directs the endocytic recycling process to the lysosomes, in addition to initiating autophagy (Dauner et al. 2017). There, starvation, as well as mTORC1 inhibition by Torin-1, delivers cholesterol-rich endosomes, along with their embedded transferrin receptors and LDL receptors, to lysosomes, independent of Atg5. We further show here that this re-routing of endosomes was sufficient to raise ER cholesterol to activate ACAT and suppress SREBP-2.

I then tested the possibility that blocking the endocytic pathway by DN-rab5 would effect on the ER cholesterol. Interestingly, the rise in ER cholesterol during starvation is minimally affected by blocking either pathway alone (autophagy or endocytic recycling). Only when both pathways were blocked simultaneously did the ER cholesterol fail to rise during starvation and SREBP-2 remained activated.

Together, this suggests that the CE cycle may play a role in controlling the ER cholesterol level. In this context, it may not be futile: it provides an interface between the mTORC1/autophagy/endocytic pathway and lipid metabolism, by keeping a continuous CE cycle and maintaining the ER membrane cholesterol level at a near-critical point (five percent) (Radhakrishnan et al. 2008), nutrient/energy signals can be rapidly translated into SREBP activation or suppression.

It should also be noted that CE hydrolysis, assessed by acid lipase inhibitor, lalistat-1, did not seem to play much role in our experiments with HEK and MEF cells. This could be due to the fact that these cells have limited LD and, within a relatively short experimental time frame (four hours), these cells primarily use membrane as the main source of cholesterol. It remains to be seen whether regulatory pathways we observed here for SREBPs are present in LD-rich cells, such as hepatocytes or macrophages.

4.3 Lysosomes are at the center of Cholesterol Trafficking to the ER and mTORC1 Activation

Lysosomes are recognized as a major digestive organelle for dietary cholesterol entering the cell. For example, LDL, the major exogenous source of cholesterol, is taken into cells by LDL receptors in clathrin-coated pits and delivered to the lysosomes for degradation, which releases cholesterol to the cell (Ikonen 2008). Endogenous cholesterol can also be redistributed through the lysosomes, as was shown here, to achieve metabolic regulatory roles. Here, during starvation, cholesterol from cellular membranes is directed to the lysosome for hydrolysis and recycling. This caused an increase in ER membrane cholesterol and suppression of SREBP-2 activation. Consistent with our current knowledge of lysosomes, cholesterol could only perform this regulatory role if its efflux mechanism was intact (i.e. functional NPC 1 and 2). U16666A or NPC1 siRNA abolishes cholesterol export from lysosomes and activates SREBP-1 even when mTORC1 is suppressed.

Significantly, the lysosomes are also identified as the cellular location where mTORC1 is activated. For example, when nutrients are available, Rag GTPases, binds mTORC1 and anchors it to the lysosome to interact with Rheb GTPase. This activates mTORC1 kinase activity (Bar-peled et al. 2012). In this context, it is interesting to note lysosomal cholesterol

seem to directly regulate mTORC1 anchorage to the lysosomes. Cholesterol depletion by MCD dissociates mTORC1 from the lysosomes, independent of nutrient conditions, while repleting cholesterol with either LDL or MCD/cholesterol restored its anchoring. Apparently, in nutrient-rich medium, lysosomes sense cholesterol through SLC38A9, a lysosomal integral membrane protein, and require minimal cholesterol levels to maintain SLC38A9 configuration to anchor mTORC1 (Castellano et al. 2017).

On the other hand, if cholesterol accumulates in the lysosome over a threshold, mTORC1 is also inactivated, although the precise mechanism there is yet to be determined (J. Xu et al. 2010). The lysosomes can directly control mTORC1 anchoring/activation through their membrane cholesterol content and also participate in cholesterol trafficking downstream of mTORC1 signaling to regulate ER activity such as SREBP-2 activation.

4.4 mTORC1 Regulates SREBP-2 Translocation to the Golgi through Control of Autophagy/Re-routed Endocytic Membrane Trafficking to the Lysosomes

SREBPs exist as membrane-bound precursor proteins on the ER membrane. Low cholesterol is sensed by SCAP in the ER membrane consequently disassociates from Insig to escort SREBPs to the Golgi where they undergo proteolytic cleavage to release the active transcription factor to enter the nucleus for lipogenic genes expression (R. Sato 2010). Numerous studies have presented the possibility that mTORC1 may influence SREBP processing (i.e. its transport from the ER to the Golgi) (Shao and Espenshade 2012; Porstmann et al. 2008; S. Li, Brown, and Goldstein 2010; Peterson et al. 2011; Moon et al. 2012; K. Du et al. 2010). It was also reported that rapamycin inhibits mTORC1-mediated

induction of nuclear SREBP accumulation, similar to the effect of cholesterol (Porstmann et al. 2008). Perhaps most importantly, hepatic *SCAP* deletion suppressed lipogenesis and prevented steatosis in the obese mouse model, although mTORC1 is hyper-activated in this model (Moon et al. 2012).

SREBP-1a and -2 are the predominant isoforms in cultured cells that activate fatty acid and cholesterol synthesis, respectively (Jay D Horton, Goldstein, and Brown 2002). SREBP-1c is expressed primarily in the liver where it is regulated by multiple signals related to nutrient and energy status. For example, insulin activates liver SREBP-1c through mTORC1 (S. Li, Brown, and Goldstein 2010). This thesis focused on SREBP-2, which is most abundant in culture cells (Jay D Horton, Goldstein, and Brown 2002). At least in the case of SREBP-2, mTORC1 regulates its processing by lowering ER cholesterol.

Indeed, I observed that mTORC1 inactivation leads to induction of autophagy and re-routing of endocytic activity. Resulting in increased lysosomal cholesterol release. Once sensed by the ER membrane, cholesterol released by the lysosome likely leads to the retention of SREBP-2 in the ER and prevents its proteolytic cleavage. Although most studies on animal models concentrate on SREBP-1c, our conclusion is, consistent with previous reports that autophagy regulates lipid content, particularly cholesterol levels. For example, blocking autophagy caused increased cholesterol level in cultured hepatocytes (R. Singh et al. 2009). Also, obese mice severely down-regulate autophagy in the liver, resulting in lipid accumulation and defective insulin signaling. Restoration of autophagy in these animals enhanced insulin sensitivity and reduced lipid accumulation (Yang et al. 2010). Interestingly, autophagy is also decreased with ageing (Cuervo et al. 2005). The decline autophagy is

thought to contribute to susceptibility to hepatic lipid accumulation and metabolic syndrome, common with older animals and humans (Ford, Giles, and Dietz 2002).

Taken together, our conclusion that mTORC1 regulates SREBP-2 processing and hence cholesterol biosynthesis is consistent with a large body of studies.

4.5 The Role of Lipin-1 in SREBP Activation by mTORC1

Until now, the only mechanistic explanation of how mTORC1 activates SREBPs came from a study with lipin-1. It was reported there that, in mice with constitutive active lipin-1 in the liver, a high fat diet and thus, high mTORC1 activity, failed to upregulate lipogenesis and prevented hepatic steatosis (Peterson et al. 2011). However, lipin-1 is known to be phosphorylated and retained in the cytoplasm by mTORC1. Without sufficient mTORC1 activity, lipin-1 is then dephosphorylated, thereby being activated and entering nucleus. This activated nuclear form of lipin-1 was shown to sequester mature SREBPs to the nuclear envelope, thereby preventing them from binding SREs in the promoters of lipogenic genes.

In addition, lipin-1 promotes degradation of mature nuclear SREBP proteins potentially by an autophagy-related process (Peterson et al. 2011). This further limits the function of mature SREBPs and lipogenesis when mTORC1 activity is low. However, as mentioned earlier, high mTORC1 activity promotes all stages of SREBP activation, beginning from SCAP-mediated SREBP translocation and eventual nuclear localization. Lipin-1 in its native form, on the other hand, exerts its function in the nucleus and on mature SREBPS when mTORC1 is low (Peterson et al. 2011). Thus, lipin-1 may represent an additional layer of regulation to rapidly suppresses SREBP activity once mTORC1 is turned off.

We observed that, when both autophagy and endocytosis are blocked, SERBP-2 is fully activated without active mTORC1. This is a condition under which lipin-1 should be in the nucleus and function as a negative regulator of SREBP. However, we did not observe any inhibition of SREBP-2 activity. This suggests that lipin-1 may not sufficiently suppress the function of mature SREBP-2 in the nucleus, at least in the absence of autophagy. Future studies will be required to see whether lipin-1 is able to enter the nucleus under these experimental conditions where autophagy and endocytosis are blocked and mTORC1 activity is low (starvation).

4.6 Additional Regulatory Mechanisms are Involved in SREBP-1 Activation

This thesis has shown the mechanism by which mTORC1 activates SREBP-2 by limiting ER cholesterol. In theory, similar mechanisms should apply to other SREBPs as they can use the SCAP to sense ER cholesterol (Radhakrishnan et al. 2008). Nevertheless, with the exception of FAS, I failed to detect change in SREBP-1a activity upon starvation or refeeding.

It is not entirely clear why SREBP-1a failed to respond to nutrient conditions, even though ER cholesterol is clearly altered. However, we detected SREBP-1a translocation from the ER to the Golgi by immunofluorescence staining (see **Figure 14B**). It is noteworthy that such detection may or may not entirely be reliable as antibody specificity to specific SREBP isoforms is often ambiguous. Several attempts to identify a mature form of SREBP-1a by western plotting were unsuccessful. Nevertheless, FASN may be regulated in a more complicated manner than that of other sterol-regualted genes. It seemed reasonable to assume that FASN would not solely depend on ER cholesterol

availability. Perhaps there are additional regulatory mechanisms in the nucleus after SREBP-1 proteolytic processing and nuclear entry.

Indeed, poly-unsaturated fatty acids (PUFAS) were implicated in the suppression of SREBP-1 (Ye and DeBose-Boyd 2011). PUFAS inhibit the transcription of SREBP-1 by acting as antagonist to LXR. This prevents LXR from binding to the LXR response element on the promoter region of SREBP-1, thereby inhibiting its mRNA expression (Repa et al. 2000). PUFAS are also believed to accelerate the degradation of SREBP-1 mRNA, resulting in a decreased SREBP-1 protein level (J. Xu et al. 2001).

Interestingly, it was shown that the expressions of genes targeted by SREBP-1 were responsive to nutrient conditions in both HEK and MEF cells in an earlier study (Peterson et al. 2011). There, HEK and MEF cells were subjected to six- and eight-hour starvation respectively. This is significantly longer than our experimental condition (four hours), which might partially explain the discrepancy. In this study, we found that 6–8-hour nutrient starvation was too severe on cell variability. Further studies will be required to address these issues.

4.7 Starvation-induced Cholesterol Trafficking to ER in TSC1/2$^{-/-}$ MEFs

I showed that TSC1/2$^{-/-}$ MEFs, known to have a hyper-activated mTORC1 during starvation, can respond to nutrient conditions normally (for example, starvation-triggered appearance of LC3-II, an autophagy marker, protein degradation and ACAT activation, all of which are identical to those in wt MEFs). Accordingly, SREBP-2 target genes were suppressed during starvation, despite high mTORC1 activities in these cells. This may emphasize our conclusion that cholesterol trafficking from the lysosomes to the ER is a more

proximal regulator of SREBP-2 than mTORC1 activity per se, albeit downstream of mTORC1.

It is not entirely clear why TSC2$^{-/-}$ MEFs can still activate autophagy with high mTORC1 activity. It was reported that that amino acid starvation activates phosphatase 2A (PP2A), which dephosphorylates ULK1 and thus activates autophagy in some cancer cells with high mTORC1 activity (Wong et al. 2015). Alternatively, in starved TSC2$^{-/-}$ MEFs, mTORC1, bound to Rheb-GTP and thus constitutively active, may not be in the correct intracellular location. Rag-GTPases are known to mediate amino acid signaling to mTORC1 as well as inducing changes to mTROC1 localization (Jewell, Russell, and Guan 2013). In the absence of amino acids, Rag GTPases in their inactive form may not direct mTORC1 to lysosomes. Therefore, ULK1 remains dephosphorylated and thus initiates autophagy (Sancak et al. 2007).

We have shown here that autophagy alone is sufficient to raise ER cholesterol and suppress SREBP-2 in DN-rab5 expressing cells. Starved TSC2$^{-/-}$ MEFs also seem to have sufficient autophagy, which would explain sufficient SREBP-2 suppression. It remains to be determined whether endocytic recycling is targeted to the lysosomes in starved TSC2$^{-/-}$ MEFs.

4.8 Autophagy, Protein Degradation and ACAT

Another interesting issue arising from this thesis is how to assay autophagy. Since the discovery of autophagy, several assays have been established to monitor the autophagic process. The classic method is the measurement of long-lived protein degradation, in line with “self-eating” and “rescue oneself,” as the name of autophagy implies. Specifically, this assay measures the appearance of amino acid in the medium during starvation, presumably

from the lysosomal protein degradation. However, the majority of amino acids released from protein degradation in the lysosomes would be used immediately by the cell to survive, the purpose of the autophagy. Thus, the amount of amino acid appearing in the medium is likely small and dependent on many other factors, such as membrane permeability to amino acid. This assay is therefore intrinsically low sensitivity (Mizushima, Yoshimori, and Levine 2010) (Sword, Shintani, and Klionsky 2004). Due to such difficulty, several other assays have been proposed and used with variable interpretations, which generated great confusion in the field. This confusion even prompted a Keystone Symposium consented guideline (Klionsky et al. 2012). Further guidelines will be needed to promote advancement of autophagy.

During my thesis study, I observed that autophagy induced by starvation could be readily detected by a whole cell ACAT assay, a well-established protocol in cholesterol research. I believe that this ACAT assay, in combination with LC3 processing, presents a novel and far superior method to detect autophagy than previous assays. Indeed, throughout my thesis work, the results from the ACAT assay were completely in agreement with the results obtained by long-lived protein degradation and, importantly, with much higher sensitivity. Future in-depth research will be required to quantitatively establish the sensitivity and reliability of ACAT assays in the context of autophagy.

5 CONCLUSION

In summary, the results from this thesis demonstrate for the first time that mTORC1 plays a significant role in regulating membrane trafficking to the lysosomes. mTORC1 is usually activated by nutrient-rich conditions, common in highly proliferating cells and some cancer cells (Saxton and Sabatini 2017). High mTORC1 activity (see **Figure 23A**) has two effects on membrane trafficking: (**i**) suppressing autophagy and (**ii**) maintaining endosomal recycling to the plasma membrane. The net effect is that membrane organelles and cell debris do not reach lysosomes. Thus, a minimal cholesterol is delivered to the lysosomes. This limits cholesterol supply to the ER membrane and activates SREBP-2, including translocation, proteolytic processing, nuclear entry and eventual transcription of target genes. On the other hand, low mTORC1 activity (see **Figure 23B**) (**i**) triggers autophagy that directs cholesterol, as part of cell membrane debris, to lysosomes and (**ii**) re-directs cholesterol-rich endosomes to lysosomes. Cholesterol-rich lysosomes subsequently release cholesterol in a NPC-1-dependent manner. This leads to a rise in ER cholesterol levels and suppression of SREBP-2.

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Publications

1. Dong, Fumin, Zhongcheng Mo, **Walaa Eid**, Kevin C. Courtney, and Xiaohui Zha. "Akt Inhibition Promotes ABCA1-Mediated Cholesterol Efflux to ApoA-I through suppressing mTORC1." *PloS one* 9, no. 11 (2014): e113789.

2. Ferrier, Andrew, Yves De Repentigny, Anisha Lynch-Godrei, Sabrina Gibeault, **Walaa Eid**, Daniel Kuo, Xiaohui Zha, and Rashmi Kothary. "Disruption in the autophagic process underlies the sensory neuropathy in dystonia musculorum mice." *Autophagy* 11, no. 7 (2015): 1025-1036.

3. Dauner, Kristin, **Walaa Eid**, Riya Raghupathy, John F. Presley, and Xiaohui Zha. "mTOR complex 1 activity is required to maintain the canonical endocytic recycling pathway against lysosomal delivery." *Journal of Biological Chemistry* 292, no. 14 (2017): 5737-5747.

4. **Eid, Walaa**, Kristin Dauner, Kevin C. Courtney, AnneMarie Gagnon, Robin J. Parks, Alexander Sorisky, and Xiaohui Zha. "mTORC1 activates SREBP-2 by suppressing cholesterol trafficking to lysosomes in mammalian cells." *Proceedings of the National Academy of Sciences* (2017): 201705304.

Accreditations and licenses

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Awards

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