REGULATION OF AUTOPHAGY BY LIPID SPECIES

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Declaration

I hereby declare that this thesis is my original work and it has been written by me in its entirety.

I have duly acknowledged all the sources of information which have been used in the thesis.

This thesis has also not been submitted for any degree in any university previously.

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16/8/2013

Date

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Summary

Lipotoxicity refers to the cytotoxic effects of excess fat accumulation in cells and it has been implicated as one of the contributing factors to diseases like obesity, diabetes and non-alcoholic fatty liver. Macroautophagy (referred to as autophagy hereafter in this thesis) is an evolutionarily conserved and regulated catabolic process where cellular components (proteins, lipids and organelles) are sequestered in double membrane vesicles called autophagosomes which fuse with lysosomes for degradation by lysosomal enzymes. At present, the lipotoxic effects of free fatty acids (FFAs) have been well studied, while the role of FFAs in the regulation of autophagy is still controversial. In the first part of our study we sought to examine effects of palmitic acid (PA) and oleic acid (OA), two of the most common dietary FFAs on the autophagic process. We found that PA, but not OA, was able to cause an increase in autophagic flux, evidenced by LC3-II accumulation and formation of Green Fluorescent Protein (GFP)-LC3 puncta. Notably, PA-induced autophagy was found to be independent of the Mechanistic Target of Rapamycin Complex 1 (MTORC1) regulation. Next, in search of the mechanism mediating PA-induced autophagy, we found increased levels of diacylglycerol (DAG) species and Protein Kinase C (PKC) activation in PA-treated cells; and inhibition of classical PKC isoforms (PKC-α) was able to effectively suppress PA-induced autophagy. Finally, we showed that inhibition of autophagy sensitized the cells to PA-induced apoptosis, suggesting the pro-survival function of autophagy induced by PA. Taken together, results from this study reveal a novel mechanism underlying free fatty acids-mediated autophagy. Furthermore, the pro-survival function of autophagy suggests that modulation of autophagy as a potential therapeutic strategy in protection of cells against lipotoxicity and lipid-related metabolic diseases

In the second part of our study, we tried to investigate how modulation of endogenous saturated and monounsaturated fatty acids (MUFAs) would affect cellular autophagic activity. Stearoyl-CoA Desaturase 1 (SCD-1) is an endoplasmic reticulum bound enzyme that catalyzes formation of the first double bond at the cis-Δ9 position of saturated fatty acids (SFA) to form monounsaturated fatty acids (MUFA). There is increasing evidence indicating that autophagy plays an important role in regulating lipid metabolism, while little is known whether key enzymes of lipogenesis like SCD-1 can regulate autophagy. In this study, we examined the roles of SCD-1 in autophagy using the Tuberous sclerosis complex 2 ($Tsc2)^{-/-}$ mouse embryonic fibroblasts (MEFs) possessing constitutively active MTORC1 as a cellular model. TSC2 (also known as tuberin) forms a stable complex by interacting with TSC1 (also known as hamartin) and this TSC1-TSC2 complex act as a GTPase-activating protein (GAP) in cells by inhibiting the activity of the GTPase protein Rheb (Ras homolog enriched in brain) which is a direct upstream activator of MTOR. Therefore, cells that have lost the functional TSC1-TSC2 complex are known to possess constitutively activated MTORC1 signaling pathway independent of growth factors regulation. We found that mRNA and protein levels of SCD-1 are significantly elevated in the $Tsc2^{-/-}$ MEFs compared to $Tsc2^{+/+}$ MEFs, resulting in significant increase in levels of various lipid classes. Furthermore, inhibition of SCD-1 activity by either a chemical inhibitor or genetic knockdown resulted in an increase of autophagic flux only in the $Tsc2^{-/-}$ MEFs. Induction of autophagy was independent of MTORC1 regulation as MTORC1 activity was not suppressed by SCD-1 inhibition. Loss of phosphorylation on Akt-S473 was observed upon SCD-1 inhibition and such Akt inactivation was due to disruption of membrane lipid raft formation, without affecting the formation and activity of MTORC2. Increased nuclear translocation of FoxO1 was observed following Akt inactivation, leading to increased

transcription of genes involved in the autophagic process. The *Tsc2*$^{-/-}$ MEFs were more susceptible to apoptosis induced by SCD-1 inhibition and blockage of autophagy sensitized the cell death response. These results thus reveal a novel function of SCD-1 on regulation of autophagy via lipogenesis and the lipid rafts-Akt-FoxO1 pathway. In summary, in this study we have shown conclusively that treatment of cells with exogenous FFAs like PA but not OA induces autophagy via activation of PKC-α signaling pathway. Furthermore, autophagy can also be induced by modulating endogenous FFAs levels through inhibition of SCD-1 in cells with hyperactivated MTORC1 signaling pathway. Data from this study support the notion that changes in the intracellular levels of lipid species play important roles in regulation of autophagy and such inducible autophagy generally serves as a pro-survival mechanism against the lipotoxicity. Therefore, results from our study suggest that manipulation of autophagy can be a potential therapeutic strategy against different types of diseases. Induction of autophagy upon lipotoxic stresses can be utilized to promote cell survival and limit the cytotoxic effects of excess intracellular lipid accumulation while on the other hand; inhibition of autophagy can also be utilized in tandem with SCD-1 inhibition to target cells with hyperactivated MTORC1 signaling pathway in diseases like cancer.

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List of Abbreviations

ACC	Acetyl-CoA Carboxylase
ACL	ATP Citrate Lyase
AMPK	AMP-Activated Protein Kinase
BSA	Bovine Serum Albumin
CAY	CAY-10566
CE	Cholesteryl Ester
CHO	Water Soluble Cholesterol
CMA	Chaperone Mediated Autophagy
CQ	Chloroquine
CTxB	Cholera Toxin Subunit B
DAG	Diacylglycerol
DEPTOR	DEP Domain containing MTOR Interacting Protein
DGAT1	Diacylglycerol Acyltransferase 1
DMEM	Dulbecco's Modified Eagle's Medium
DOX	Doxycycline
DRF	Detergent Resistant Fraction
DSF	Detergent Soluble Fraction
EBSS	Earles' Balanced Salt Solution
ER	Endoplasmic Reticulum
ERK	Extracellular Signal-Regulated Kinase
EV	Empty Vector
FASN	Fatty Acid Synthase

FBS	Fetal Bovine Serum
FB1	Fumonisin B1
FFA	Free Fatty Acid
FM	Full Medium
GAP	GTPase-Activating Protein
GAPDH	Glyceraldehyde-3- Phosphate Dehydrogenase
GFP	Green Fluorescence Protein
HLH-LZ	Helix-Loop-Helix Leucine Zipper
HFD	High Fat Diet
HPLC	High Performance Liquid Chromatography
IP	Immunoprecipitation
IP_3	myo-Inositol 1,4,5-Triphosphate
IRS-1	Insulin Receptor Substrate-1
JNK	c-Jun N-Terminal Kinase
KO	Knock-out
LC3	Microtubule-Associated Protein 1 Light Chain
LD	Lipid Droplet
MAM	Mitochondria-Associated ER Membrane
MBCD	Methyl-β-Cyclodextrin
MEF	Mouse Embryonic Fibroblasts
mLST8	Mammalian Lethal with Sec-13 Protein 8
MRM	Multiple Reaction Monitoring
MTOR	Mechanistic Target of Rapamycin

MTORC1	Mechanistic Target of Rapamycin Complex 1
MTORC2	Mechanistic Target of Rapamycin Complex 2
MUFA	Monounsaturated Fatty Acid
NDRG	N-myc Downstream-Regulated Gene
NPC	Niemann-Pick Type C
OA	Oleic Acid
PA	Palmitic Acid
PARP	Poly(ADP-ribose) Polymerase
PBS	Phosphate Buffer Saline
PC	Phosphatidylcholine
PDK1	Phosphoinositide-Dependent Kinase-1
PE	Phosphatidylethanolamine;
PG	Phosphatidylglycerol
PhA	Phosphatidic Acid
PI	Phosphatidylinositol
PI3K	Phosphatidylinositol-3-Kinase
PIP2	Phosphatidylinositol (4,5)-Biphosphate
PIP3	Phosphatidylinositol (3,4,5)-Triphosphate
PKC	Protein Kinase C
PLC	Phospholipase C
PLD1	Phospholipase D1
Protor1/2	Protein Observed with Rictor 1/2
PS	Phosphatidylserine

PtdIns3P	Phosphatidylinositol-3-Phosphate
PUFA	Polyunsaturated Fatty Acid
PVDF	Polyvinylidene Difluoride
RAPTOR	Regulatory-Associated Protein of MTOR
Rheb	Ras Homolog Enriched in Brain
RICTOR	Rapamycin-Insensitive Companion of MTOR
ROS	Reactive Oxygen Species
SCD-1	Stearoyl-CoA Desaturase-1
SFA	Saturated Fatty Acid
Sin-1	Stress Activated Map Kinase Interacting Protein 1
siRNA	short interfering RNA
SK1	Sphingosine Kinase 1
SREBP-1	Sterol Regulatory Element-Binding Protein 1
S1P	Sphingosine-1-Phosphate
TAG	Triacylglycerol
TCA	Tricarboxylic Acid
TET	Tetracycline
TFEB	Transcription Factor EB
TPA	12-*O*-tetradecanoylphorbol-13-acetate
TSC	Tuberous Sclerosis Complex
UPR	Unfolded Protein Response
UVRAG	Ultraviolet Irradiation Resistance-Associate Gene
WT	Wild-Type

List of Publications

1. **Tan, S.H.**, Shui, G., Zhou, J., Shi, Y., Huang, J., Xia, D., Wenk, M.R., and Shen, H.M (2013). Critical role of SCD-1 in autophagy regulation via lipogenesis and lipid rafts-coupled Akt-FoxO1 signalling pathway. (Manuscript Under Review)

2. **Tan, S.H.**, Shui, G., Zhou, J., Li, J.J., Bay, B.H., Wenk, M.R., and Shen, H.M. (2012). Induction of autophagy by palmitic acid via protein kinase C-mediated signaling pathway independent of mTOR (mammalian target of rapamycin). J Biol Chem *287*, 14364-14376.

3. Zhou, J., **Tan, S.H.**, Codogno, P., and Shen, H.M. (2013). Dual suppressive effect of mTORC1 on autophagy: tame the dragon by shackling both the head and the tail. Autophagy. *9*, 803-5.

4. Zhou, J., **Tan, S.H.**, Nicolas, V., Bauvy, C., Yang, N.D., Zhang, J., Xue, Y., Codogno, P., and Shen, H.M. (2013). Activation of lysosomal function in the course of autophagy via mTORC1 suppression and autophagosome-lysosome fusion. Cell Res. 23, 508-23.

5. Zhou, J., Hu, S.E., **Tan, S.H.**, Cao, R., Chen, Y., Xia, D., Zhu, X., Yang, X.F., Ong, C.N., and Shen, H.M. (2012). Andrographolide sensitizes cisplatin-induced apoptosis via suppression of autophagosome-lysosome fusion in human cancer cells. Autophagy *8*, 338-349.

1. Introduction

1.1 Autophagy

1.1.1 Introduction

There are three main types of autophagy: macroautophagy, microautophagy, and chaperon-mediated autophagy (CMA). Macroautophagy (referred to as autophagy hereafter in this thesis) is an evolutionarily conserved and regulated catabolic process where cellular components (proteins, lipids and organelles) are sequestered in double membrane vesicles called autophagosomes which fuse with lysosomes for degradation by lysosomal enzymes (He and Klionsky, 2009; Mehrpour et al., 2010; Mizushima, 2007; Mizushima et al., 2008; Ravikumar et al., 2010b). The degraded macromolecules are then recycled back to the cytoplasm for maintaining cellular homeostasis.

1.1.2 Stages of the autophagic process

Our knowledge of the molecular machinery of autophagy has been greatly facilitated by studies done on the budding yeast *Saccharomyces cerevisiae* in which more than 30 *ATG* (autophagy-related genes) have been identified and many orthologs have been discovered in mammals (He and Klionsky, 2009; Nakatogawa et al., 2009). This has allowed the dissection of the whole autophagy process into several sequential steps that are regulated by the hierarchical function of the ATG proteins. Figure 1 below shows the summary of the different stages of autophagic process as described below (Rubinsztein et al., 2012a). These steps include:

(i) induction or initiation: which depends on a protein complex consisting of ULK1 (ATG1 homologue), ATG13 and FIP2000 proteins, leading to the

formation of the phagophore and is negatively regulated by Mechanistic Target of Rapamycin Complex 1 (MTORC1);

(ii) nucleation: which is mediated by the Beclin 1 and hVps34/class III phosphoinositide 3-kinase (PI3K) complex that produces phosphatidylinositol-3-phosphate (PtdIns3P) and recruits various essential proteins to the autophagosomes;

(iii) elongation, which is required for the completion of autophagosomes by making use of two ubiquitin-like conjugation systems (Atg12-Atg5 system and the LC3/Atg8 system) that results in the covalent binding of phosphatidylethanolamine (PE) to the carboxyl-terminal glycine of LC3/Atg8 protein which is then localized to the autophagosomal membrane;

(iv) maturation and degradation, which involves fusion of the completed autophagosomes with endosomes-lysosomes to form autolysosomes whereby the cargoes sequestered can be degraded by the lysosomal hydrolases.

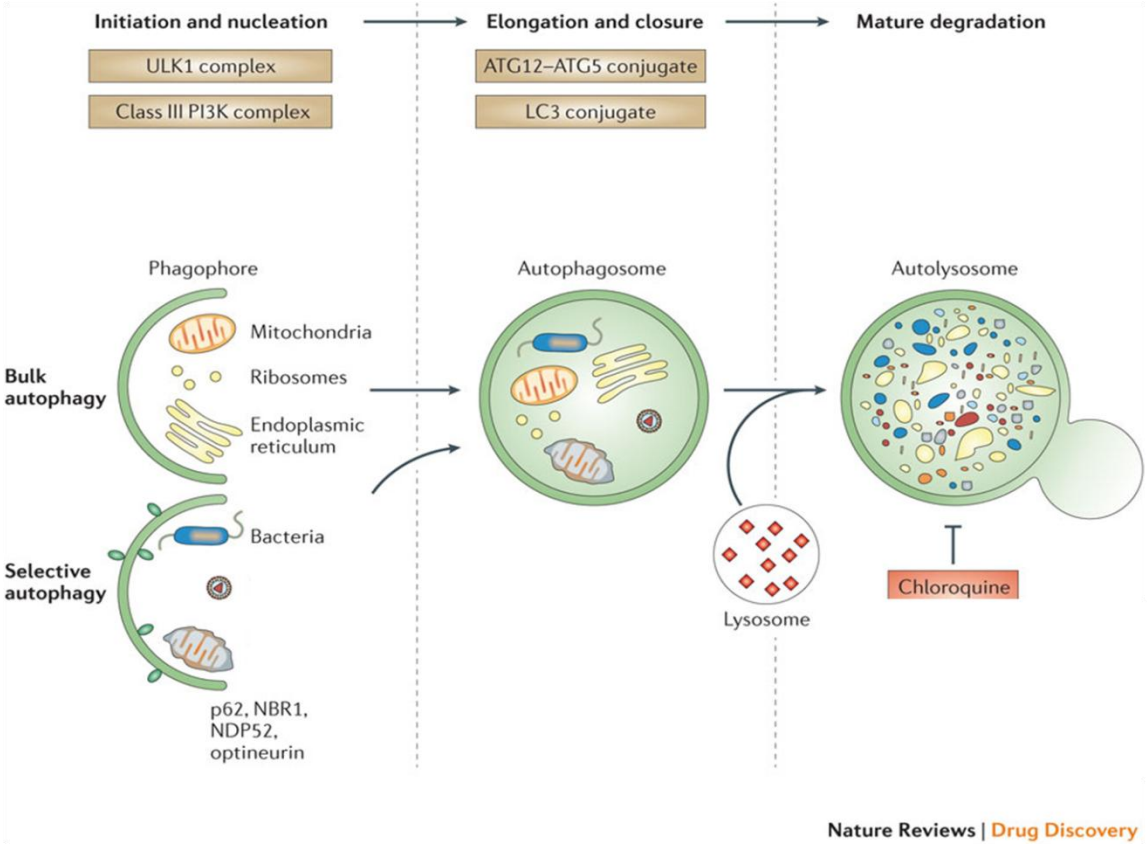


Figure 1. Summary of the different stages of the autophagy process in mammalian cells (Rubinsztein et al., 2012a).

1.1.3 Regulatory pathways of autophagy

The class I PI3K-Akt-MTORC1 signaling network is one of the key negative regulators of autophagy in mammalian cells. When MTORC1 is activated in the presence of nutrients and amino acids, it is able to directly phosphorylate ULK1 and Atg13 to inhibit the ULK1-Atg13-FIP200 complex, thus preventing the initiation step of autophagosome formation (Hosokawa et al., 2009; Jung et al., 2009; Mizushima, 2010). Under nutrient starvation or treatment with chemical inhibitors of MTOR, MTORC1 activity is inhibited,

thus releasing the ULK1-Atg13-FIP200 complex from its inhibitory phosphorylation and leading to initiation of autophagy in the cells (Jung et al., 2010).

As an upstream inhibitor of MTORC1 activity, AMP-activated protein kinase (AMPK) has been shown to be able to induce autophagy during periods of metabolic stresses (Ravikumar et al., 2010b). Apart from its ability to induce autophagy via the inhibition of MTORC1 activity (Gwinn et al., 2008; Inoki et al., 2006; Laplante and Sabatini, 2012), recent studies have identified other molecular mechanisms by which AMPK can directly affect the induction of autophagy. Studies have shown convincingly that under conditions of glucose starvation, AMPK is able to directly phosphorylate ULK1 on multiple sites leading to its activation and ultimately autophagy induction (Egan et al., 2011; Kim et al., 2011). Furthermore, AMPK is able to phosphorylate Beclin-1 that is present in specific Vps34 complexes during energy stresses to promote autophagy (Kim et al., 2013).

Interestingly, there have been other protein kinases that have been described to activate autophagy apart from MTORC1 and AMPK described above. One of the protein kinase shown to be critical for autophagy induction during nutrient starvation is the c-Jun NH_2-terminal kinase (JNK). JNK signaling pathway plays a critical role in regulation of cell fate and is essential for cellular response to stress stimuli (Weston and Davis, 2007). The antiapoptotic protein Bcl-2 binds to Beclin-1 during nutrient sufficiency and thus inhibits the autophagy induction function of Beclin-1 (Pattingre et al., 2005). Under starvation conditions, JNK1 phosphorylates Bcl-2 and prevents its interaction with Beclin-1, thus freeing up Beclin-1 to participate in autophagy induction (Wei et al., 2008a; Wei et al., 2008b). Another protein kinase that has been implicated in autophagy induction is the Extracellular Signal-Regulated Kinase (ERK) signalling cascade. It has been shown that

the activity of ERK can be activated during amino acid starvation leading to the induction of autophagy which is independent or MTORC1 regulation (Pattingre et al., 2003). It was established that ERK activation stimulates autophagy by phosphorylation of the Gα-interacting protein (GAIP) which leads to hydrolysis of GTP and induction of autophagy (Ogier-Denis et al., 2000). More recently, a study has reported that sustained ERK activation can lead to the disassembly of both MTORC1 and MTORC2 while enhancing Beclin-1 activity and resulting in autophagy induction (Wang et al., 2009). A summary of the common signaling pathways regulating autophagy is provided in Figure 2 below (Yang and Klionsky, 2010a).

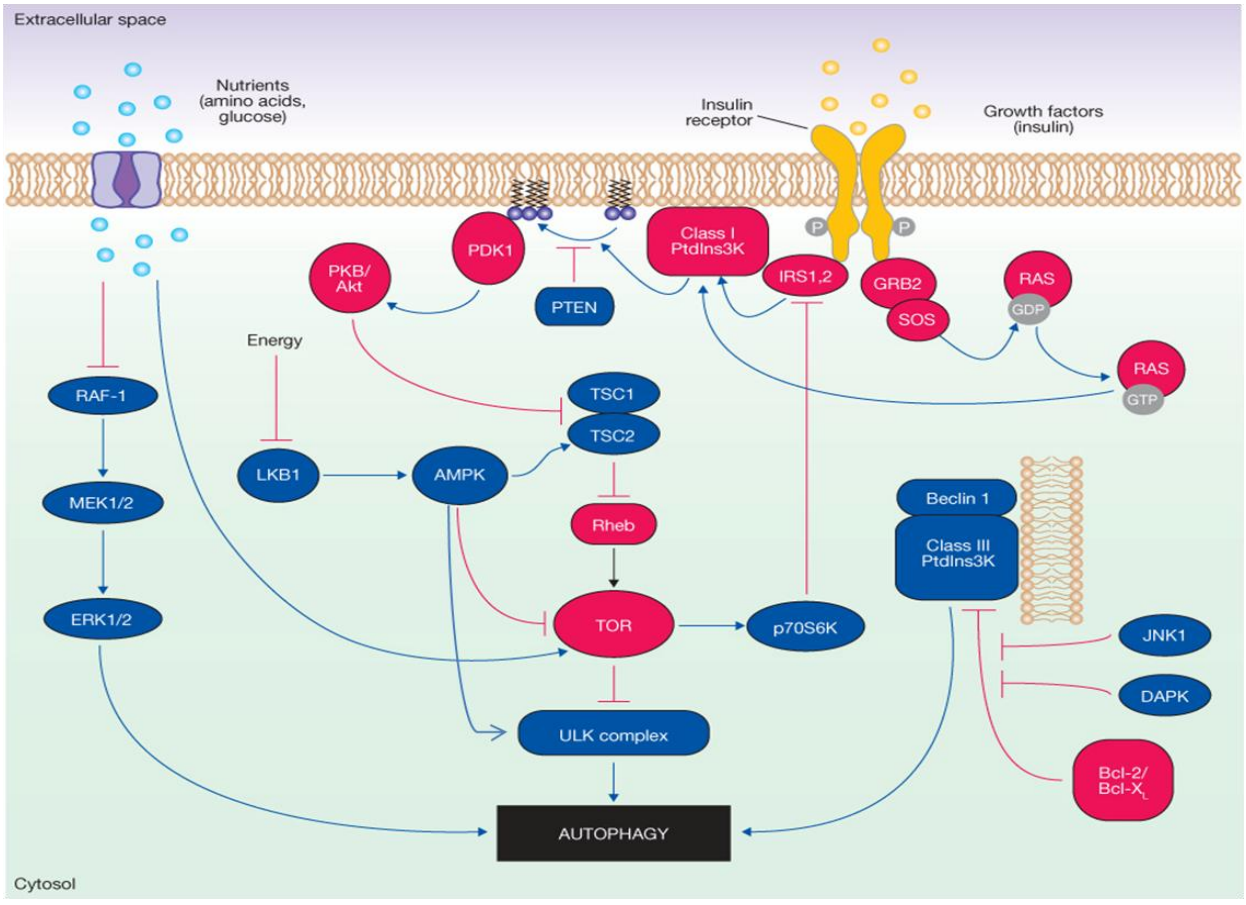


Figure 2. Common signaling pathways involved in the regulation of autophagy (Yang and Klionsky, 2010a).

Recent studies on the regulation of autophagy have also uncovered the existence of MTORC1-independent regulation of mammalian autophagy. The first such evidence was uncovered when it was shown that decreasing the intracellular levels of myo-inositol 1,4,5-triphosphate (IP_3) via pharmacological drugs like lithium which inhibits inositol monophosphatase can lead to autophagy induction that is independent of MTORC1 signaling pathway (Sarkar et al., 2005). It was later shown in a large scale chemical screen that L-type Ca^{2+} channel inhibitors, K^+ ATP channel opener, and Gi signaling activators all have the ability to induce autophagy independent of MTORC1 regulation (Sarkar and Rubinsztein, 2008). These drugs reveal a novel regulatory pathway for autophagy in which cAMP and intracellular levels of Ca^{2+} play a crucial role in autophagy induction independent of MTORC1 (Mehrpour et al., 2010; Ravikumar et al., 2010b). A more detailed study showed that IP_3 receptors (IP_3R) formed a complex with Beclin-1 that was regulated by Bcl-2 (Vicencio et al., 2009). Experiments using genetic knockdown or chemical inhibitors of IP_3R were able to lead to autophagy induction via disruption of the IP_3R-Beclin-1 complex that was again independent of MTORC1 regulation (Vicencio et al., 2009).

Another group of protein kinases that has been implicated in autophagy induction is the Protein Kinase C (PKC) family (Chen et al., 2008; Sakaki and Kaufman, 2008; Sakaki et al., 2008). The PKC family consists of serine/threonine protein kinases that are categorized into the following 3 classes: classical (containing α, β and γ), novel (containing δ, η, ε and θ) and atypical (containing ζ, λ and μ). The PKC family was first identified as protein kinases that were activated by membrane phospholipids and intracellular calcium ions (Ca^{2+}) (Newton, 2001; Takai et al., 1979). Subsequently, the

PKC family was shown to be intracellular receptors for the tumor promoting phorbol esters, making it one of the first kinases shown to be involved in carcinogenesis (Castagna et al., 1982; Kikkawa et al., 1983). Further in depth studies then showed that the hydrolysis of phosphatidylinositol (4,5)-biphosphate (PIP2) by phospholipases like phospholipase C (PLC) to form the second messengers, inositol-1,4,5-triphosphate (InsP3) and diacylglycerol (DAG) are required to trigger the release of Ca^{2+} and PKC activation (Wymann and Schneiter, 2008). Cellular stresses that affect endoplasmic reticulum (ER) homeostasis would disrupt protein folding and activate the unfolded protein response (UPR) and autophagy (He and Klionsky, 2009). Amongst the PKC family, PKC-θ has been shown to be required for the induction of autophagy under ER stress conditions in a Ca^{2+} dependent but MTORC1 independent manner (Sakaki and Kaufman, 2008; Sakaki et al., 2008). It was also reported that another member of the PKC family, PKC-δ is required for induction of autophagy under acute hypoxic conditions (Chen et al., 2008). A recent study has implicated that activation of PKC-δ in the induction of antibacterial autophagy (Shahnazari et al., 2010), thus strengthening the importance of PKC family for autophagy induction under specific stress stimulus in cells.

1.1.4 Biological functions of autophagy

Autophagy has been shown to be an important cellular catabolic process for maintaining cellular homeostasis. Basal levels of autophagy are essential for the removal of unfolded proteins and damaged organelles in the cytosol of the cells to prevent damages to the cells. Autophagy can also be induced during times of nutrient deprivation and cellular stresses where it plays a key role as a pro-survival mechanism via recycling of nutrients such as amino acids and fatty acids (FAs). The most well-established regulatory pathway of

autophagy is the PI3K-Akt-MTORC1 pathway (Mizushima, 2007). The MTORC1 signaling pathway is the convergent point of signaling pathways originating from growth factors like insulin and nutrients such as amino acids, and it has been identified as a key negative regulator directly upstream of the ULK1/Atg1 complex (Jung et al., 2009; Mizushima, 2010). During nutrient deprivation, autophagy is induced to degrade cellular components and to recycle the nutrients for cell survival. For example, it has been shown that activation of autophagy is essential for the survival of newborn mice that were deprived of food and nutrients upon birth (Efeyan et al., 2013; Kuma et al., 2004). Recent studies have reported the role of autophagy in regulating the breakdown of stored lipids in the cells during starvation (Settembre et al., 2013; Singh et al., 2009a) and the details will be discussed in the following sections

Once thought to be a non-selective degradation process, many studies have now shown that autophagy can indeed selectively degrade defective and damaged cellular organelles to maintain cellular homeostasis. For example, mitophagy is a selective form of autophagic process that targets damaged mitochondria for degradation in the cells to prevent intracellular accumulation of these damaged mitochondria which leads to increase of reactive oxygen species (ROS) and occurrence of DNA damages (Kim et al., 2007; Kissova et al., 2007; Zhang et al., 2007). Other types of selective autophagy that have been identified include pexophagy whereby excessive or damaged paroxysms are removed from the cells (Deosaran et al., 2013; Sakai et al., 2006), and reticulophagy which is the selective degradation of ER in cells (Bernales et al., 2006). Autophagy is known to be important for degradation of protein aggregates which are resistant to the ubiquitin-proteasome degradation system (Kirkin et al., 2009). The p62 protein is a

selective substrate of autophagy that can interact with LC3 protein and is sequestered into the autophagosome for degradation during autophagy (Shvets et al., 2011; Weidberg et al., 2011). The impairment of autophagy by the deletion of Atg7 in mice can lead to formation of poly-ubiquitinated p62 protein aggregates or inclusion bodies which can contribute to the pathogenesis of various diseases (Komatsu et al., 2007; Komatsu et al., 2005; Zatloukal et al., 2002), thus suggesting the importance of autophagy in clearance of cytotoxic protein aggregates.

Apart from being implicated as a major cell survival process, autophagy has also been suggested to cause cell death, although this has been the subject of much debate and an area of intense research. It has been suggested that autophagic process contributes to cell death by a process that has been termed Type II cell death or autophagic cell death (Galluzzi et al., 2012), which is characterized by dying cells with increased number of autophagosomes. The widely accepted model for autophagic cell death has come from the lower eukaryotes like the *Dictyostelium discoideum* which is defective in apoptosis, based on the observations that the loss of ATG1 was able to reduce cell death (Kosta et al., 2004). Autophagic cell death has been studied extensively in *Drosophila melanogaster* where it was shown that overexpression of loss of function mutants of *Atg2* and *Atg18* or knockdown of *Atg1* and *Atg18* rescued cell death and delayed clearance of the midgut section (Denton et al., 2010; Denton et al., 2009). On the other hand, the occurrence of autophagic cell death in mammalian systems is more controversial. Indeed, there seems to be overwhelming evidence showing that disruption of autophagy process does not disrupt cell death in many mouse models. For example, apart from the *BECLIN 1* knockout mice which displayed increased embryonic lethality due to enhanced

apoptosis (Yue et al., 2003), no disruption of cell death was observed in the various *ATG* knockout mouse models that have been generated including *ATG5* (Kuma et al., 2004; Qu et al., 2007) and *ATG7* (Komatsu et al., 2005). Most supporting evidence for the pro-cell death effect of autophagy has come mainly from *in vitro* cell culture systems where there is the absence of the apoptotic machinery, such as the Bax-/-Bak-/- double knockout MEFs. Bax and Bak proteins belong to the pro-apoptotic Bcl-2 family and these double knockout MEFs are known to be resistant to apoptotic cell death. When stimulated with cytotoxic reagents like etoposide and staurosporine, the $Bax^{-/-}Bak^{-/-}$ double knockout MEFs died via autophagic cell death and the cell death could be rescued by knockdown of *Atg5* or *Beclin 1* expression (Shimizu et al., 2004; Shimizu et al., 2010). However, a recent report has suggested that autophagy rarely, if ever, serves as an executioner of cell death in cells and that most evidence suggest that autophagy is cytoprotective instead (Shen et al., 2011). In this study, the authors screened more than 1400 chemical compounds possessing anti-tumour capabilities in a panel of cancer cells and found that 59 compounds were able to cause an increase in autophagic flux. Most importantly, none of these compounds identified were able to induce autophagic cell death since inhibition of autophagy via the knockdown of Atg5 or Atg7 did not rescue the cancer cells from cytotoxic effects of the chemical compounds (Shen et al., 2011). Instead, the authors showed convincingly that autophagy inhibition caused an increased rate of cell death in the cancer cells treated with these autophagy inducing chemical compounds, thus suggesting that autophagy is a cytoprotective mechanism (Shen et al., 2011).

1.1.5 Implications of autophagy in human diseases

Recent studies have also brought into focus the role of autophagy in the development of various human diseases like cancer, neurodegenerative diseases and metabolic disorders (Meijer and Codogno, 2009; Mizushima et al., 2008). Mouse models with Atg gene knockout has been developed and these mouse models have shown that the loss of autophagy leads to the development of neurodegenerative conditions like Huntington's disease, Parkinson's disease and Alzheimer's disease (Funderburk et al., 2010; Hara et al., 2006; Komatsu et al., 2006; Sarkar and Rubinsztein, 2008; Yue et al., 2009). For example, it is now known that activation of the autophagic process can help to enhance the clearance of the huntingtin protein aggregates in Huntington's disease (Jeong et al., 2009; Williams et al., 2008). Mouse models with Atg5 or Atg7 knocked-out specifically in neurons have been shown to have excessive accumulation of ubiquitin containing inclusion bodies and excessive neuronal cell death (Hara et al., 2006; Komatsu et al., 2006), suggesting conclusively that removal of cytotoxic protein aggregates from the neurons requires functional autophagy (Hara et al., 2006; Komatsu et al., 2006).

Furthermore, autophagy has been implicated in tumorigenesis and cancer development as well (Apel et al., 2009; Chen and Debnath, 2010; Levine and Kroemer, 2008; Mathew et al., 2007a). Earlier studies had reported an increase in the incidence of tumorigenesis in mice upon the allelic loss of Beclin-1, while this phenotype could be reversed by over-expression of Beclin-1 (Liang et al., 1999; Yue et al., 2003). Furthermore, other components that make up the Beclin-1/Vps34 complex essential for the autophagic process like ultraviolet irradiation resistance-associated gene (UVRAG), Ambra1 and Bif-1 have been shown to have tumour suppressive functions as loss of these proteins and

autophagy activity led to tumour development (Fimia et al., 2007; Takahashi et al., 2007). However, the mechanisms behind the tumour suppressive function of autophagy are still largely unknown. One of the commonly suggested mechanism is that autophagy in cells is able to prevent the accumulation of excessive ROS in the cells and thus limit DNA damage and genome instability that could lead to tumour initiating genetic mutations. Several studies reported that autophagy was critical for the removal of damaged mitochondria and limiting the production of ROS, subsequently led to the suppression of tumorigenesis (Karantza-Wadsworth et al., 2007; Mathew et al., 2007b).

In addition, autophagy has also been shown to be important for the elimination of invading pathogens such as bacteria, parasites and viruses (Levine and Deretic, 2007; Shahnazari et al., 2010). Autophagy has been shown to promote the innate and adaptive immunity responses of infected cells. For example, it has been reported that autophagy is important in promoting MHC class II presentation of cytosolic antigens to boost the adaptive immunity response as the pharmacological and genetic inhibition of autophagy has been shown to decrease the efficiency of MHC class II presentation (Nimmerjahn et al., 2003; Paludan et al., 2005). Autophagy has also been implicated in many other human diseases including muscle myopathies, ageing, liver and cardiovascular diseases which have been extensively reviewed elsewhere (Mizushima and Komatsu, 2011; Mizushima et al., 2008; Ravikumar et al., 2010b) and will not be discussed further here.

1.2 Lipids and autophagy

1.2.1 Introduction to lipids

Lipids make up a major class of macromolecules that are absolutely essential for cell survival and growth. This class of macromolecules is required for a wide variety of

cellular structures and biological functions (van Meer et al., 2008; Wymann and Schneiter, 2008). The different types of lipids that have been identified so far are shown in Figure 3 (Roberts et al., 2008). The most well-established cellular function of lipids is their role in the formation of the phospholipid bilayer of cell membrane or cellular organelles that acts as the barrier to limit permeability of different molecules into cells (Dowhan, 1997; van Meer et al., 2008). This compartmentalization of intracellular organelles is important for specific cellular processes to take place. Another well-established function of lipids is their ability to act as signaling molecules during signal transduction in cells (Dowhan, 1997; Lemmon, 2008; van Meer et al., 2008; Wymann and Schneiter, 2008). For example, the rapid production of PIP3 from PIP2 that is modulated by Class I PI3K during activation of receptor tyrosine kinases is essential for signal transduction for the PI3K-Akt-MTOR signaling pathway. As mentioned earlier, PLC mediates the production of InsP3 and DAG through the hydrolysis of PIP2 at the plasma membrane, leading to Ca^{2+} release from intracellular stores and activation of PKC (Wymann and Schneiter, 2008).

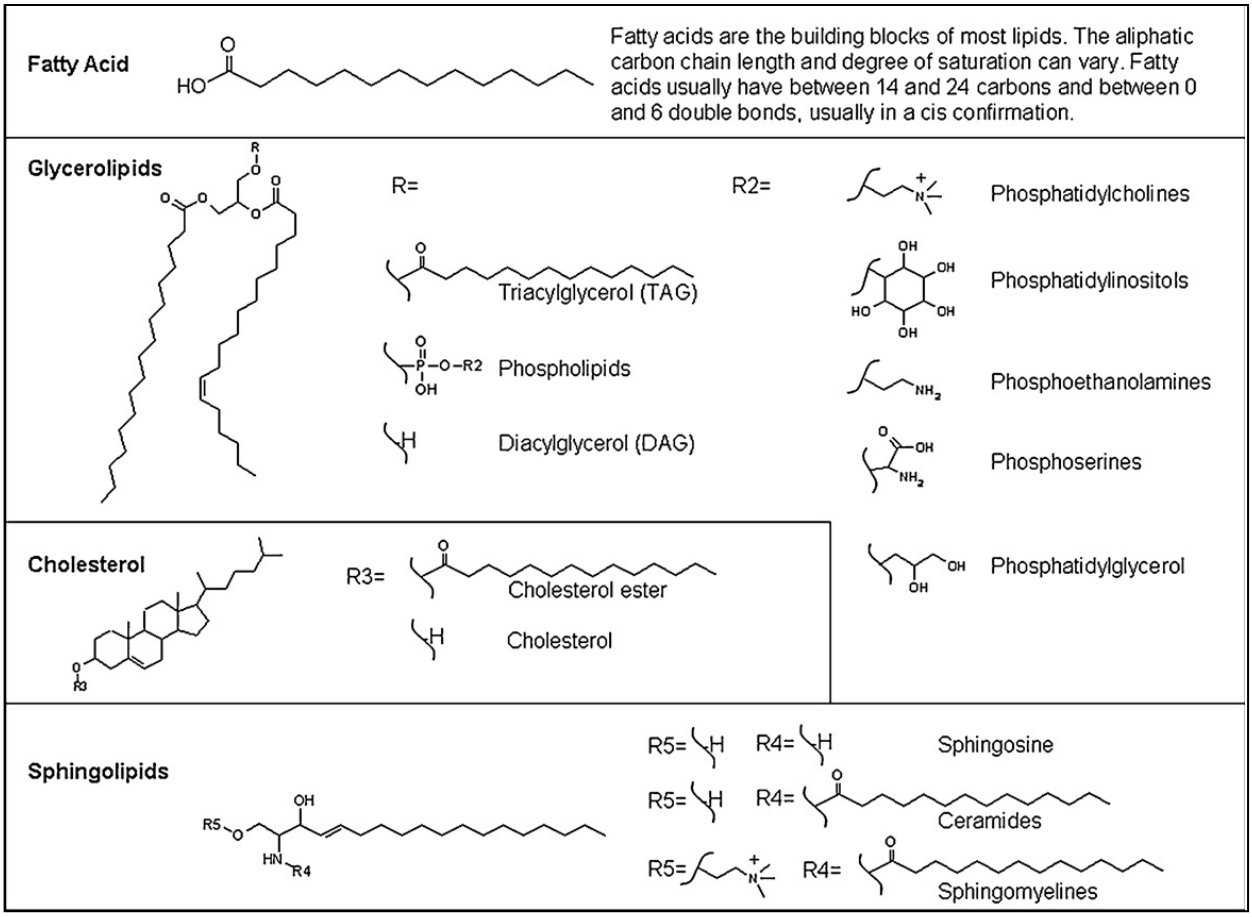


Figure 3: Summary of the different groups of lipid species in cells (Roberts et al., 2008).

Another important function of lipids is to act as storage of excess FAs in cells in the form of lipid droplets (LD) in the cytoplasm of the cells (Martin and Parton, 2006; Thiele and Spandl, 2008). The core of LD consists of neutral lipids like triacylglycerol (TAG) and cholesterol ester (CE) which are surrounded by a monolayer of phospholipid membrane and LD associated proteins (Coleman and Mashek, 2011; Ducharme and Bickel, 2008; Martin and Parton, 2006). This lipid store can act as a reserve of energy store for the cells to access during energy deprivation and the stored lipids can be tapped for synthesis of other important lipid molecules (Ducharme and Bickel, 2008).

1.2.2 Regulatory roles of lipids in autophagy

Lipids are intimately linked to the various steps of the autophagic process and the presence of various lipid species are essential for autophagy to be carried out in the cells. For example, the availability of lipids can influence the formation of the autophagosomal membrane which is absolutely required for the sequestration of autophagic targets in the cytosol to finally form the completed autophagosome structure. A few examples of lipid species which are important for autophagy to occur includes PtdIns3P which is the product of the Class III PI3K, Vps34. PE on the other hand is required for conjugation to LC3 protein. Apart from its involvement in the autophagic process, many studies have shown that lipids species like sphingolipids can also have a regulatory effect on the autophagic process by directly affecting signalling pathways upstream of autophagy. The detailed regulatory roles of various lipids on autophagy are discussed in further details in the following sections.

1.2.2.1 Lipid source of autophagosomal membrane

The main focus of research on the role of lipids in autophagy has largely been on their involvement in the process of the nucleation and formation of the double membrane bound autophagosome. Many studies have suggested that lipids from several sources contribute to autophagosome formation (Hamasaki et al., 2013b; Mari et al., 2011; Rubinsztein et al., 2012b) but the exact origin of the autophagosomal membrane remains unknown. One early model proposed that autophagosomal membrane could be formed by *de novo* lipid synthesis on site, based on the fact that the autophagosomal membrane lacked transmembrane proteins and other cytoplasmic organelle protein marker (Stromhaug et al., 1998; Tooze and Yoshimori, 2010). However subsequent studies have

suggested that the autophagosomal membrane most probably arise from pre-existing organelles in the cells (Tooze and Yoshimori, 2010). Many independent studies have since presented evidences suggesting the ER, mitochondria, plasma membrane or Golgi apparatus as possible sites of origin of the autophagosome membrane (Hailey et al., 2010; Hayashi-Nishino et al., 2009; Ravikumar et al., 2010a; Yla-Anttila et al., 2009; Young et al., 2006). However, recent studies have provided evidence to suggest a new model whereby both the ER and mitochondria contribute to autophagosome membrane synthesis (Hamasaki et al., 2013a; Hamasaki et al., 2013b). In that study, the authors showed that under starvation conditions, the pre-autophagosome/autophagosome marker Atg14L localizes to the ER-mitochondria contact site known as mitochondria-associated ER membrane (MAM) and disruption of MAM leads to defective autophagosome formation (Hamasaki et al., 2013a; Hamasaki et al., 2013b). The results from these studies will certainly help to explain previous results whereby either the ER or mitochondria was identified as the source of autophagosome membrane. However, more work will need to be conducted to investigate if autophagosome formation independent of the MAM can be observed in cells.

1.2.2.2 Role of PtdIns3P in autophagosome nucleation

It is now known that formation of specific lipid species like PtdIns3P is required for the nucleation step of autophagosome formation (Nakatogawa et al., 2009; Yang and Klionsky, 2010b). The Class III PI3K, Vps34 is well conserved from yeast to mammalian and is required for the formation of a complex that consists of other protein components including Atg14L, Beclin 1 and Vps15 (Itakura et al., 2008; Longatti and Tooze, 2009; Zhong et al., 2009). In mammalian cells, this particular complex has been reported to be

localized to a structure at the ER termed omegasome during starvation-induced autophagy (Axe et al., 2008). This complex is essential for autophagy to occur and the only known substrate of Vps34 is the building block of inositol lipid, phosphatidylinositol (PtdIns), which is phosphorylated to form PtdIns3P (Vanhaesebroeck et al., 2001). PtdIns3P is one of the major lipid constituent of autophagosome in yeast (Obara et al., 2008) and has been shown to be able to bind to and recruit proteins containing the FYVE or PH domains (Vanhaesebroeck et al., 2001; Yoshimori and Noda, 2008) to the autophagosomal membrane to participate in the subsequent membrane curvature, fusion and degradation of the autophagosome. Interestingly it has been established that the activation of Vps34 is actually dependent on the presence of amino acids and starvation actually causes a decrease in the lipid kinase activity of the cells leading to a decrease in the total intracellular levels of PtdIns3P (Byfield et al., 2005; Gulati et al., 2008). However, a recent study has identified that Beclin-1 protein that is present in a particular Vps34 complex consisting of Atg14L, Beclin-1 and Vps34 is specifically phosphorylated by ULK1 upon amino acid starvation leading to the activation of the lipid kinase activity of Vps34 and increased PtdIns3P production at autophagosomal membrane (Russell et al., 2013). Results from this study confirm that only Vps34 present in specific complexes that are recruited to the phagophore via Atg14L upon autophagy induction can be activated to produce PtdIns3P. Apart from Vps34, Jumpy (MTMR14) and MTMR3 are 2 PtdIns3P phosphatases that have been shown to inhibit autophagy activity by regulating PtdIns3P turnover as well. The increase in activity of these lipid phosphatases dephosphorylate and decrease the

overall PtdIns3P levels in the cells leading to a decrease in autophagic activity mainly in nutrient rich conditions (Taguchi-Atarashi et al., 2010; Vergne et al., 2009).

1.2.2.3 Role of PE in autophagosome expansion

Another type of lipid involved in autophagosome formation and elongation as mentioned earlier is PE. PE is conjugated to Atg8/LC3 protein during autophagy by an ubiquitin-like system involving Atg7 and Atg3 which are E1 and E2 enzymes, respectively, in the ubiquitylation reaction to form LC3-PE (Ichimura et al., 2000). This lipidated form of LC3 is found on both sides of the isolation membrane and the autophagosome membrane. LC3-PE present within the autophagosomal lumen will eventually be degraded after fusing with the lysosomes while those at the outer autophagosome membrane surface can be recycled for further use (Kirisako et al., 2000; Nakatogawa et al., 2009). This lipidated form of LC3 is essential for the expansion of the autophagosomal membrane after nucleation and loss of LC3 expression has been shown to lead to accumulation of unclosed isolation membrane in cells (Noda et al., 2009; Xie et al., 2008). Furthermore, results from *in vitro* experiments conducted with purified protein components and liposomes containing PE have suggested that this lipidated form of LC3-PE mediates the membrane tethering and hemifusion of the autophagosome, a process presumably required for the fusion with lysosomes (Nakatogawa et al., 2007).

1.2.2.4 Role of sphingolipids in autophagy

Apart from investigating the effects of autophagy on lipid metabolism, many studies have tried to look at how different types of lipid species are able to regulate autophagic activity. Perhaps the most well studied lipid species in this aspect are the sphingolipids.

Sphingolipids are a class of lipids that are major components of lipid membrane and certain species can be produced as second messengers during signal transduction in cells. They have been shown to affect biological processes like cell growth, cell death, tumorigenesis, stress responses and autophagy (Holland and Summers, 2008; Kihara et al., 2007; Mehrpour et al., 2010; Ravikumar et al., 2010b; Zheng et al., 2006). The main species of sphingolipids that have been shown to regulate autophagic activity are sphingosine-1-phosphate (S1P) and ceramide. While ceramide in the cells can be obtained via *de novo* synthesis, S1P in the cells can only be synthesized via two sequential steps: first formation of sphingosine from ceramide via a deacylation process, and then sphingosine is phosphorylated to give rise to S1P (Spiegel and Milstien, 2003). S1P has been shown to promote cell growth and survival while in contrast, ceramide is a known inducer of cell death. Therefore, tight regulation of the ratio of these two lipid metabolites has been referred to as the 'sphingolipid rheostat' in cells and is crucial in determining the cell fate (Cuvillier et al., 1996; Hannun and Obeid, 2008). Overexpression of sphingosine kinase-1 (SK1), which is responsible for producing S1P leads to induction of autophagy via inhibition of MTORC1 signaling without inhibiting the upstream PI3K-Akt signaling pathway (Lavieu et al., 2006). More importantly, SK1 activity was also shown to be elevated during nutrient starvation and knocking down SK1 abrogated starvation-induced autophagy and resulted in increased cell death (Lavieu et al., 2006). Furthermore, knockdown of sphingosine phosphate phosphohydrolase-1 which is responsible for dephosphorylating S1P resulted in autophagy induction due to increased intracellular S1P levels (Lepine et al., 2011). Autophagy induction in this context was independent of MTORC1 regulation but instead dependent on ER stress signalling, based

on the observations that autophagy induction could be inhibited by silencing of proteins involved in propagating the unfolded protein response (UPR) (Lepine et al., 2011). On the other hand, addition of exogenous short chain C2-ceramide was able to cause increased synthesis and accumulation of long-chain ceramide in cancer cells, leading to autophagy induction due to inhibition of Akt activation and subsequent MTORC1 activity (Scarlatti et al., 2004). Inhibition of Akt activity was later suggested to be due to the down regulation of amino acid transporters from the cell surface upon ceramide treatment (Guenther et al., 2008). There was a concomitant increase of Beclin-1 levels in these cells treated with C2-ceramide (Guenther et al., 2008) and similar observations were observed in cells treated with tamoxifen which causes increased production of ceramide in cells (Scarlatti et al., 2004). It was also suggested that ceramide accumulation could lead to JNK activation which phosphorylates Bcl-2 and relieves its inhibitory effect on autophagy by causing the phorphorylated Bcl-2 to dissociate from Beclin-1 (Pattingre et al., 2009). A recent study has implicated mitochondrial localized long chain C18-ceramide produced by ceramide synthase 1 (CerS1) to be involved in mediating autophagic cell death (Sentelle et al., 2012). The authors showed that mitochondrial localized C18-ceramide, through direct binding to lipidated form of LC3 could lead to mitophagy and ultimately cell death due to disruption of mitochondria and energy homeostasis in the cells (Sentelle et al., 2012). Thus more studies are needed to understand how cells regulate the internal ‘sphingolipid rheostat’ in response to different types of stresses and the role of autophagy in response to changing ceramide and S1P levels in the cells.

1.2.2.5 Role of cholesterol in autophagy

Autophagy has been shown to control intracellular cholesterol levels via regulation of cholesterol efflux in macrophages (Ouimet et al., 2011). Interestingly, cholesterol has also been shown in different studies to play contrasting roles in the regulation of the autophagic process. For example, depletion of cholesterol with different chemical inhibitors like methyl-β-cyclodextrin (MBCD) or depletion of lipoprotein from culture medium was first shown to be able to induce autophagy in multiple cell lines (Cheng et al., 2006). Furthermore, the authors showed that depletion of cholesterol led to the inhibition of MTOR activity and activation of autophagy. Interestingly, disruption of lipid raft microdomains by cholesterol depletion using MBCD has already been shown to induce chaperone mediated autophagy (CMA) (Kaushik et al., 2006), while increasing cholesterol content in lysosomes will lead to inhibition of CMA (Kaushik et al., 2006; Rodriguez-Navarro et al., 2012). These studies suggest that autophagy induced by cholesterol depletion could be directly associated with the depletion of lipid raft microdomains in cells. On the other hand, there are studies suggesting that depletion of cholesterol levels will lead to the inhibition of autophagic activity. It was shown in an *in vitro* assay model that autophagic process can be inhibited by specifically depleting cholesterol from isolated autophagosomes and lysosomes, probably via suppression of the formation of autolysosomes (Koga et al., 2010). The authors further presented evidence showing that lysosomes and autophagosomes isolated from mice that were exposed to High Fat Diet (HFD) had decreased levels of cholesterol on their respective membranes, thus supporting their earlier findings that autophagy was inhibited in mice exposed to chronic HFD (Koga et al., 2010; Singh et al., 2009a). On the other hand, disruption of

cholesterol trafficking has been implicated in autophagy induction in the central nervous system of mice suffering from a neurodegenerative disease, Niemann-Pick Type C (NPC) (Liao et al., 2007; Pacheco et al., 2007; Pacheco and Lieberman, 2007). NPC is a type of lipid storage disorder characterized by defects in sphingolipids and cholesterol trafficking due to mutations in NPC1 and NPC2, leading to accumulation of cholesterol in the neurons (Liao et al., 2007; Pacheco et al., 2007; Pacheco and Lieberman, 2007). The studies showed that NPC1 deficiency led to accumulation of intracellular cholesterol, which through an unknown mechanism resulted in increased expression of Beclin-1 in these NPC1 deficient cells. The increase in Beclin-1 levels in the cells was shown to be responsible for the increase in basal autophagic activity in the cells which was independent of MTORC1 inhibition (Pacheco et al., 2007; Pacheco and Lieberman, 2007).

1.2.2.6 Role of DAG in autophagy

Apart from cholesterol, another neutral lipid that has been described to have an effect on the regulation of autophagic activity is DAG. DAG species are important second messengers in cells because of their ability to activate protein kinases such as the PKC family as mentioned earlier (Newton, 2001). DAG has recently been shown to be involved in the induction of autophagy caused by various types of cellular stresses. It was demonstrated that DAG production is essential for effective antibacterial autophagy to be carried out during infection of the cells by *Salmonella typhimurium* and that DAG was found to be co-localized with the bacterial containing phagosomes at the very early stages of autophagy induction (Shahnazari et al., 2010). The authors went on to propose that the source of DAG came from the sequential breakdown of phosphatidylcholine (PC) into phosphatidic acid (PhA) by phospholipase D1 (PLD1) followed by dephosphorylation of

PhA to DAG by phosphatidic acid phosphatase (PAP) (Shahnazari et al., 2010). Furthermore, they further showed that DAG was able to activate PKCδ, an isoform belonging to the novel PKC family. PKCδ activation resulted in activation of JNK which in turn phosphorylates Bcl-2 protein to cause it to dissociate from its inhibitory interaction with Beclin-1, resulting in the activation of autophagy (Shahnazari et al., 2010; Wei et al., 2008a). It has been well established that the activation of classical PKC family is not only Ca^{2+}-dependent, but also requires both DAG and phosphotidylserine (PS); while activation of the novel PKC family is Ca^{2+}-independent but dependent on DAG and PS and finally, activation of the atypical family of PKC is independent of either Ca^{2+} or DAG (Newton, 2001). As mentioned earlier, novel PKC members like PKC-θ (Sakaki and Kaufman, 2008; Sakaki et al., 2008) and PKC-δ (Chen et al., 2008) are required for autophagy induction caused by stress factors such as hypoxia and ER stress. On the other hand, there is report showing the importance of PLD1 activity in autophagy induction (Dall'Armi et al., 2010). The authors showed that during nutrient starvation, PLD1 was found to localize to outer membrane of autophagosome-like structure and an increase in its catalytic activity was observed resulting in a subsequent increase in the PhA levels in the cell (Dall'Armi et al., 2010). PhA has also been shown to be a direct binding partner of MTOR and is essential for the stability and activity of MTORC1 and MTORC2 (Fang et al., 2001; Foster, 2013; Toschi et al., 2009). In particular, MTORC1 is sensitive to changes in PhA levels in cells and PLD1 is localized on lysosomal membrane in response to amino acids and could increase the production of PhA to activate MTORC1 (Yoon et al., 2011). Therefore, more work will have to be done to investigate whether the increase in PhA levels will ultimately lead to an increase in DAG levels as well as to determine

the downstream targets of PKC to elucidate how the PLD1-PhA-DAG-PKC signaling pathway in the control of autophagy. It will also be important to investigate whether other sources of PhA and DAG can contribute to autophagy induction.

1.2.2.7 Role of Free Fatty Acids and lipotoxicity in Autophagy

Free fatty acids (FFAs) are the basic building blocks of lipids and have been implicated in the cause of metabolic diseases such as diabetes. FAs are aliphatic monocarboxylic acids and most natural FAs have chain lengths between C_4 and C_{22} (Fahy et al., 2005). There are generally 2 major classes of FAs, namely the saturated and unsaturated fatty acids. Saturated FAs (SFAs) contain no double bonds in their aliphatic carbon chains while unsaturated FAs contain single or multiple carbon-carbon double bonds forming monounsaturated (MUFA) or polyunsaturated (PUFA) FAs, respectively. FFAs in the cytosol of cells can be converted to fatty acyl-CoA by fatty acyl-CoA synthetase (FACS) and then be utilized as precursors for synthesis of other lipid species. Furthermore, the fatty acyl-CoA chains are an important energy source for cells as they can be transported into the mitochondria and then broken up via a multi-step process called β-oxidation to produce acetyl-CoA that is ultimately used to generate ATP (Li et al., 2010; Lopaschuk et al., 2010).

When there is an excess supply of FFAs either from the breakdown of fats in the diet or from increased *de novo* lipogenesis, the excess FFAs will be converted to TAG and stored as LD in the cytoplasm of the cells (Martin and Parton, 2006; Thiele and Spandl, 2008). Storage of FFAs as LDs is important because LDs not only act as a store of energy source but also sequesters the toxic FFAs in an inert form within cells. Normally, adipocytes are the main cell type in the body that can store large amounts of lipids, while

non-adipocytes like the hepatocytes, cardiac myocytes, pancreatic β cells and skeletal muscles can also store lipids, albeit to a much smaller extent (Brookheart et al., 2009; Schaffer, 2003). High levels of lipid accumulate intracellularly due to an imbalance between the levels of FFA import/synthesis and utilization. Excess fatty acids are normally esterified and stored as LD that can be utilized when broken down by cellular lipases. In cases where there are elevated FFAs in the body, lipid storage within the non-adipocytes might exceed the limit and the excess FFAs present in the cells thus causes lipotoxicity. Lipotoxicity refers to excessive accumulation of lipid in non-adipose tissues/cells which ultimately leads to loss of cellular functions and cell death (Brookheart et al., 2009; Unger, 2002). This phenomenon has been shown in many studies where the excessive levels of serum FFA leads to, for example, dysfunction and ultimately the death of pancreatic β cells in diabetes and obesity (Cunha et al., 2008; Shimabukuro et al., 1998b), development of insulin resistance in skeletal muscle (Kim et al., 2000) and death of mouse myocardiocytes that could lead to heart failure (Chiu et al., 2001). As a result, lipotoxicity has been hypothesized to be one of the underlying causes of diseases associated with excess lipid accumulation in the body, such as cardiac diseases, obesity and diabetes (Brookheart et al., 2009; Chavez and Summers, 2010; Eckel et al., 2005). Furthermore, the lipid induced programmed cell death caused by lipotoxicity in the cells has been termed lipoapoptosis as cells dying from lipotoxicity actually undergo via the apoptotic machinery (Shimabukuro et al., 1998a; Unger, 2003; Unger and Orci, 2002). Interestingly, a recent study has shown that cells under stress from lipotoxicity actually die via mitochondria-dependent necrosis (Rockenfeller et al., 2010).

One of the major areas of active research regarding lipotoxicity is focused on elucidating the group of lipid metabolites responsible for the toxicity. It is known that the long chain saturated fatty acids are generally more toxic to cells than the long chain unsaturated fatty acids and the secondary lipid metabolites that are synthesized from the saturated FFAs are important for inducing cell death (Cunha et al., 2008; Listenberger et al., 2003; Listenberger et al., 2001; Maedler et al., 2001; Malhi et al., 2006; Schaffer, 2003). Palmitic acid (16C:0, PA) and oleic acid (18C:1, OA) are the two most common dietary saturated and monounsaturated FA found in the body, respectively, and thus have been the most widely studied FA species for lipotoxicity. Dysfunction and ultimately cell death of pancreatic islets brought about by lipotoxicity in different mouse models of obesity are known to lead to the development of type 2 diabetes (T2D) (Lupi et al., 2002; Unger and Zhou, 2001). Culturing of human or rat pancreatic islets in medium containing PA resulted in reduced cell proliferation and increase in cell death, while treatment with unsaturated FA like palmitoleic acid (C16:1) or OA did not have any major detrimental effects on cell growth (Maedler et al., 2003; Maedler et al., 2001). PA is an essential precursor for the generation of ceramide in cells. Therefore, the authors went on to show that treatment with a ceramide analog, C_2-ceramide had similar effects on cell death seen in PA treatment, suggesting that metabolism of PA into ceramide species could be the reason for PA-mediated lipotoxicity (Maedler et al., 2003; Maedler et al., 2001). Another study has also reported that apoptosis was induced in the Chinese hamster ovary (CHO) cells treated with PA but not OA (Listenberger et al., 2001). Moreover, inhibition of *de novo* ceramide synthesis either via chemical or genetic means did not rescue PA-induced cell death in CHO cells. Instead, PA-mediated cell death could be blocked by the addition

of ROS scavengers, suggesting that cell death was mediated by the generation of ROS upon PA treatment (Listenberger et al., 2001).

Interestingly, lipotoxicity studies in different tissues like pancreatic islets and rat myocytes have shown that co-treatment with both PA and OA was able to significantly prevent loss of cell viability that was seen with PA treatment alone (de Vries et al., 1997; Maedler et al., 2003). This observation was confirmed in another study using CHO cells in which the authors went on further to show that OA was taken up and converted to TAG to be stored as the non-toxic LDs whereas PA was preferentially converted to the cytotoxic ceramide (Listenberger et al., 2003). Furthermore, treatment of cells concurrently with PA and OA resulted in the channelling of PA species into TAG formation, thus preventing PA-induced cell death (Listenberger et al., 2003). To investigate the importance of TAG storage upon lipotoxic stress, the authors made use of Diacylglycerol Acyltransferase 1 (DGAT1) knock-out mouse embryonic fibroblasts (MEFs) which could not produce TAG as this enzyme is required for the final step of TAG synthesis from DAG. Indeed, OA treatment in the *Dgat1*$^{-/-}$ MEFs did not lead to accumulation of TAG but induction of cell death to a similar extent seen in PA treatment alone (Listenberger et al., 2003). These studies have suggested that the differences in lipotoxic effects of saturated and unsaturated FAs are most probably due to the different lipid metabolites which the individual lipid species are channelled into.

Based on the known effects of FFAs in inducing various cellular stresses such as oxidative stress and ER stress which are also known to be involved in regulation of autophagy (Cunha et al., 2008; Listenberger et al., 2001; Malhi et al., 2006; Schaffer, 2003), many studies have thus been carried out to investigate whether FFAs can regulate

the autophagic pathway in a positive or negative way. However, currently there is no agreement as to whether FFAs are able to induce or inhibit autophagic activity in the various tissues and systems. A few studies using pancreatic β cells have shown that FFAs treatment is indeed able to induce autophagy (Choi et al., 2009; Ebato et al., 2008; Komiya et al., 2010; Martino et al., 2012). PA and OA were both shown to be able to induce an increase in autophagic flux in pancreatic β cell line and autophagy was identified to play a role in the maintenance of normal islet morphology and function like insulin secretion, especially in mice given HFD (Ebato et al., 2008). Conversely, loss of autophagic activity in the islets led to development of insulin resistant state and subsequently loss of pancreatic cells function in the mice, thus highlighting the importance of autophagy in response to lipotoxic stresses (Ebato et al., 2008). These results were also supported by other groups showing that PA could activate autophagy in pancreatic β cells and activation of autophagy played an important role for the cell survival upon lipotoxic stresses (Choi et al., 2009; Martino et al., 2012). Furthermore, the double stranded RNA-dependent protein kinase (PRK)-JNK signaling pathway was subsequently shown to be required for the activation of autophagy in PA-treated pancreatic β cells (Komiya et al., 2010). This result was confirmed recently in a study showing that PA and other saturated FAs was able to inhibit STAT3 which leads to activation of PKR-eIF2α signaling pathway and thus activation of autophagy in numerous of cancer cell lines tested (Shen et al., 2012). However, although JNK signaling pathway was also activated downstream of PKR, the authors did not pursue whether JNK activation did have a role in activation of autophagy in their model system (Shen et al., 2012).

On the contrary, FFAs treatments have been shown to inhibit autophagic process as well. For example, exposure of pancreatic β cells to PA or OA for up to 22 hours resulted in inhibition of autophagy due to the inhibition of the autophagosome-lysosome fusion and inhibition of the hydrolase activity (Las et al., 2011). Specifically, they found that PA or OA treatment could result in mitochondria damage, leading to decreased ATP production and ultimately affecting lysosomal acidification due to decreased function of the V-ATPase pump (Las et al., 2011). However, results from another study in cultured and primary hepatocytes concluded that OA was able to induce autophagy while PA had an inhibitory effect on autophagy (Mei et al., 2011). Activation of autophagy by OA was found to be independent of MTOR regulation and attributed to the generation of ROS (Mei et al., 2011). Furthermore, the authors showed that PA could induce apoptosis in hepatocytes and at the same time inhibited autophagy by activating caspase-mediated cleavage of Beclin-1 (Mei et al., 2011). Interestingly, it was shown in an earlier study that OA treatment had no effect on the formation of autophagosomes in hepatocytes but inhibited lysosomal degradation of autophagic substrates (Singh et al., 2009a).

1.2.3 Autophagy regulates lipid metabolism

As discussed earlier, LDs containing TAG can be broken down by cells into FFAs for cellular functions including energy production in a process known as lipolysis (Finn and Dice, 2006; Zechner et al., 2005). Lipolysis was previously thought to be dependent only on lipases that were present in the cytosol of the cells (Finn and Dice, 2006; Zechner et al., 2005). However, recent studies have demonstrated that autophagy can regulate lipid metabolism in cells by participating in the breakdown of intracellular lipid droplet stores into FFAs that can be fed into the mitochondria for β-oxidation to maintain cellular

energy homeostasis (Liu and Czaja, 2013; Singh et al., 2009a). The breakdown of LDs by autophagy (termed lipophagy) was suggested to be upregulated during nutrient deprivation or when the cells were exposed to extra-cellular lipid stimulus like fatty acids or cholesterol (Singh et al., 2009a). Furthermore, *in vivo* studies with a mouse model deficient in autophagy in the liver also displayed increases in the number and size of LDs in the hepatocytes together with increased levels of TAG and cholesterol contents (Singh et al., 2009a). More importantly, the study reported that chronic exposure of the mice to high fat diet (HFD) containing high levels of fatty acids could ultimately lead to inhibition of autophagy and lipophagy (Singh et al., 2009a). The importance of lipophagy as an event upstream of fatty acid oxidation in mice during starvation has been demonstrated in a recent report (Settembre et al., 2013). The transcription factor EB (TFEB) was previously identified as a master regulator of lysosomal biogenesis and autophagy (Sardiello et al., 2009; Settembre et al., 2011). The same group reported that TFEB is induced during starvation and activates lipid catabolism by inducing both lipophagy and expression of peroxisome proliferator-activated receptor coactivator 1α (*Ppargc1α*) and peroxisome proliferator-activated receptor α (*Pparα*) (Settembre et al., 2013). More importantly, the authors also demonstrated that breakdown of LDs by lipophagy is essential for subsequent processes like fatty acid β-oxidation in the mitochondria. Furthermore, HFD-induced obesity in the mice was prevented by the overexpression of TFEB which culminates in elevated levels of lipid catabolism (Settembre et al., 2013).

There has been other studies that suggest that functional autophagy in hepatocytes can help to dampen ER stress and maintain normal insulin sensitivity in mouse models of

obesity (Yang et al., 2010). The loss of autophagy subsequently led to elevated ER stress and disruption of systemic glucose tolerance (Yang et al., 2010). These results strongly suggest that lipophagy is essential for regulation of lipid storage in the cells and disruption of autophagy contributes to the development of pathological conditions such as liver steatohepatitis. Apart from the liver, it has been reported that upon exposure of macrophages to oxidized lipoproteins (LDL), autophagy is upregulated to degrade CEs within the LDs into free cholesterol for efflux and transport to the liver. (Ouimet et al., 2011). Subsequently, the loss of autophagic activity in the macrophages led to accumulation of cholesterol within the cells (Ouimet et al., 2011). Separate studies from other groups have instead concluded that autophagy is actually essential for the normal development of adipocytes during adipogenesis (Singh et al., 2009b; Zhang et al., 2009). By utilizing mice with *atg7* knocked out in the adipocytes, it was demonstrated that the loss of autophagy led to lean mice with reduction in accumulation of adipose tissue. Specifically, in the autophagy deficient mouse there was a major reduction in the formation of white adipose tissue with a concomitant increase in brown adipose tissue (Singh et al., 2009b; Zhang et al., 2009). Furthermore, these mice possessed enhanced levels of insulin sensitivity and were also resistant to HFD-induced obesity (Singh et al., 2009b; Zhang et al., 2009). Thus results from these studies highlight that the role of autophagy might be tissue and cell-type specific.

1.4 MTORC1 and *De Novo* Lipogenesis

1.4.1 MTORC1 Signaling pathway

The MTOR signaling network is responsible for integrating various nutritional signals to regulate cell growth and proliferation by activating or inhibiting a whole range of

anabolic and catabolic processes that helps to maintain cellular metabolic homeostasis. The MTOR protein is a serine/threonine kinase that makes up the catalytic subunit of 2 distinct complexes in mammalian cells named MTOR complex 1 (MTORC1) and MTOR complex 2 (MTORC2). These two distinct protein complexes share similar protein components in containing the catalytic subunit MTOR, mammalian lethal with sec-13 protein 8 (mLST8), DEP domain containing MTOR interacting protein (DEPTOR) and the Tit1/Tel2 complex (Laplante and Sabatini, 2012). Protein components uniquely found in MTORC1 include the regulatory-associated protein of MTOR (Raptor) and proline-rich Akt substrate 40kDA (PRAS40), while rapamycin-insensitive companion of MTOR (Rictor) and mammalian stress activated map kinase interacting protein 1 (Sin-1) and protein observed with Rictor 1 and 2 (protor1/2) are specifically found in MTORC2 (Bhaskar and Hay, 2007). Both complexes not only differ in their protein components, but also differ in their regulatory mechanisms and biological functions. Many studies have now shown that MTORC1 is the key sensor and convergent point of nutrients, amino acids, growth factors, energy and stress in the cells (Laplante and Sabatini, 2012; Zoncu et al., 2011b). In the presence or absence of these signals, MTORC1 regulates major cellular processes like protein and lipid synthesis, mitochondrial biogenesis and autophagy (Jewell and Guan, 2013; Laplante and Sabatini, 2012; Yuan et al., 2013). MTORC1 is known to be activated by upstream signals originating from growth factors and nutrients including amino acids and glucose. When growth factors such as insulin binds to its insulin receptor substrate-1 (IRS-1), it triggers the recruitment and activation of the PI3K which subsequently phosphorylates and converts phosphatidylinositol (4,5)-biphosphate (PIP2) to phosphatidylinositol (3,4,5)-triphosphate (PIP3) (Andjelkovic et al.,

1997; Bhaskar and Hay, 2007; Jewell and Guan, 2013). Akt is then recruited to the plasma membrane by PIP3 and activated subsequently through phosphorylation by phosphoinositide-dependent kinase-1 (PDK1) at T308 (Alessi et al., 1997) and by MTORC2 at S473 (Sarbassov et al., 2005). The fully activated Akt protein then phosphorylates and inhibits the Tuberous sclerosis complex 2 (TSC2). The TSC1-TSC2 complex acts as a GTPase-activating protein (GAP) in cells (Hay and Sonenberg, 2004). A recent study has further identified TBC1D7 as a new and essential component of the TSC1-TSC2 complex by helping to enhance the interaction between TSC1 and TSC2 and its GAP activity (Dibble et al., 2012). The physiological role of the TSC1-TSC2 complex in cells was first elucidated in studies using *Drosophilla* where loss of functional TSC1 and TSC2 proteins resulted in uncontrolled increase in cell size (Gao and Pan, 2001; Potter et al., 2001). The TSC1-TSC2 complex inhibits the activity of the GTPase protein Rheb (Ras homolog enriched in brain) by driving the hydrolysis of bound GTP in the Rheb protein through its GAP activity and converting Rheb to its GDP bound state which is inactive (Hay and Sonenberg, 2004; Jewell et al., 2013). Further studies then firmly established Akt as the upstream kinase that directly phosphorylates and inhibits TSC2's GAP activity (Inoki et al., 2002; Manning et al., 2002). Rheb is a direct upstream activator of MTOR and activates MTOR's kinase activity via a still unknown mechanism and thus inhibition of TSC1-TSC2 complex by Akt will ultimately lead to the activation of MTORC1 activity in the presence of growth factors (Inoki et al., 2003; Yuan et al., 2013). Therefore, cells with a defective TSC1-TSC2 complex have highly elevated levels of GTP-bound RHEB and thus a constitutively activated MTORC1 signaling pathway independent of growth factors regulation (Bhaskar and Hay, 2007; Tee et al., 2002). This

phenomenon is particularly manifested in diseases like TSC. TSC is an autosomal dominant genetic disorder that is characterized by the growth of benign tumors in multiple organs due to loss-of-function mutations in either *TSC1* or *TSC2* gene, resulting in the loss of functional TSC1-TSC2 protein complex (Kwiatkowski and Manning, 2005). On the other hand, MTORC1 activity is known to be tightly regulated by its ability to sense the presence of amino acids as MTORC1 activity cannot be fully activated in the absence of amino acids despite the presence of growth factors and other nutrients (Sancak et al., 2008). The exact mechanisms through which intracellular amino acids are sensed by MTORC1 have being gradually elucidated by a series of studies. The Rag GTPases family in particular have been implicated as the main component of amino acid sensing by MTORC1 (Kim et al., 2008; Sancak et al., 2008). Amino acids are able to activate heterodimers of Rag proteins by causing GTP-loaded RagA or RagB to bind to GDP-loaded RagC or RagD. These activated heterodimers then recruit MTORC1 to the lysosomal surface (Sancak et al., 2010; Sancak et al., 2008). The Ragulator complex was identified as a guanine nucleotide exchange factor for RagA/B that activates the Rag GTPases (Sancak et al., 2010) and further studies have shown that this complex is regulated by the lysosomal vacuolar H^+- adenosine triphosphatase (v-ATPase) that is sensitive to the intralysosomal amino acids levels (Zoncu et al., 2011a). Altogether, these studies have suggested strongly that MTORC1 is activated by signals from both the plasma membrane and intracellular compartments such as lysosomes.

MTORC1 is also sensitive to the levels of glucose and ATP in the cells through AMPK, which is a serine/threonine kinase that acts as a sensor of the intracellular AMP:ATP ratio (Hardie, 2007). AMPK is activated upon metabolic stresses such as glucose starvation

that leads to increased levels of AMP and ADP together with decreased levels of ATP in cells. Activated AMPK can directly phosphorylate TSC2 and activate its GAP activity, leading to inhibition of MTORC1 activity (Inoki et al., 2006). Figure 4 below shows a detailed summary of the MTORC1 signaling pathways and the various signals that converges on MTORC1, as discussed above (Zoncu et al., 2011b).

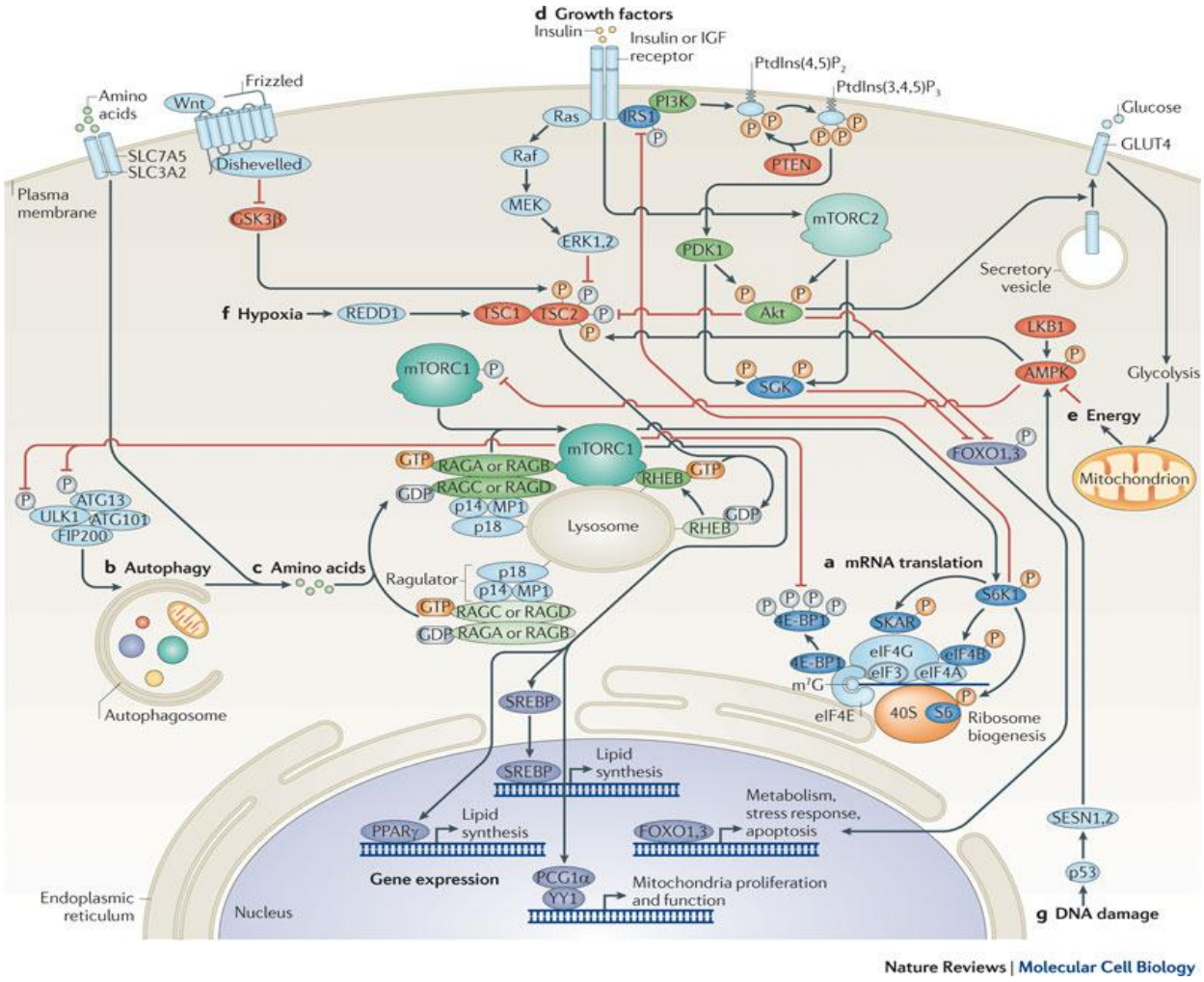


Figure 4: The MTORC1 signaling pathway and the various signals that converges on MTORC1 (Zoncu et al., 2011b).

1.4.2 MTORC1 activates *De Novo* Lipogenesis

The PI3K-Akt-MTOR signaling pathway, specifically MTORC1 has been identified as the upstream activator of sterol regulatory element-binding proteins (SREBPs) to stimulate lipogenesis in cells (Caron et al., 2010; Duvel et al., 2010; Laplante and Sabatini, 2009; Peterson et al., 2011; Porstmann et al., 2005; Porstmann et al., 2008). SREBPs are a class of basic helix-loop-helix leucine zipper (bHLH-LZ) transcription factors that are known to be the master regulator of almost all the genes encoding enzymes involved in the fatty acid and cholesterol biosynthesis (Horton et al., 2002; Shao

and Espenshade, 2012). There are two SREBP genes encoded in mammalian cells, SREBP-1 and SREBP-2 that give rise to 3 SREBP isoforms (SREBP-1a, SREBP-1c and SREBP-2) (Horton et al., 2002; Shao and Espenshade, 2012). SREBP-1a is known to activate biosynthesis of fatty acid and cholesterol, SREBP-1c activates fatty acid biosynthesis and lastly SREBP-2 regulates cholesterol biosynthesis and uptake. SREBP activity is tightly regulated through a series of complex signaling events to maintain lipid homeostasis in the cells. SREBPs are normally synthesized as their inactive forms on the surface of the ER and bound by a protein named SREBP cleavage-activating protein (SCAP). When the intracellular levels of lipids are depleted, SREBP will move from the ER to the Golgi apparatus where is it is cleaved two times in succession to produce the active form of the protein and finally translocates to the nucleus and turn on expression of its downstream target genes (Horton et al., 2002; Jeon and Osborne, 2012; Shao and Espenshade, 2012). Studies using cells expressing constitutively active Akt protein or possessing constitutively active MTORC1 have shown that MTORC1 activity is crucial to activate the lipogenic phenotype in these cells via the activating the nuclear translocation of SREBP-1 and thus inducing its transcriptional activity (Duvel et al., 2010; Peterson et al., 2011; Porstmann et al., 2005; Porstmann et al., 2008). The activation of SREBP-1 subsequently leads to the increased expression of lipogenic enzymes like ATP Citrate Lyase (ACL), Acetyl-CoA Carboxylase (ACC), Fatty Acid Synthase (FASN) and Stearoyl-CoA desaturase 1 (SCD-1) (Duvel et al., 2010; Peterson et al., 2011; Porstmann et al., 2005; Porstmann et al., 2008). The exact mechanism of how MTORC1 mediates SREBP-1 activation is currently under intense investigation. One particular candidate that has been proposed to mediate SREBP-1 activation from MTORC1 signaling is Lipin-1.

Lipin-1 is a phosphatidic acid phosphatase that has been identified to serve as a transcriptional coactivator as well (Peterson et al., 2011). Under nutrient rich conditions, Lipin-1 is phosphorylated by MTORC1 on multiple sites and excluded from the nucleus. SREBP-1 is then activated and translocates to the nucleus to activate transcription of lipogenic genes. The authors then showed that dephosphorylation of Lipin-1 upon MTORC1 inhibition leads to accumulation of Lipin-1 in the nucleus. Nuclear localized Lipin-1 inhibits the nuclear translocation of SREBP-1 and thus blocks the expression of lipogenic enzymes. The exact mechanism behind how Lipin-1 is able to impair SREBP-1 translocation is still unknown although Lamin A which is a component of the nuclear matrix has been proposed to play an important role. (Peterson et al., 2011; Ricoult and Manning, 2013). Further studies will need to be done to elucidate the mechanism through which phosphorylation of Lipin-1 can regulate SREBP-1 localization and activity.

1.4.3 Altered lipid metabolism in cancer

Actively proliferating cells usually have to satisfy the increased demand for energy and for the synthesis of biomolecules to ensure the doubling of protein, lipids and nucleic acids for each round of cell division. It is therefore understandable that cancer cells are often found to contain genetic mutations that lead to alterations in their cellular metabolic pathways to enable them to keep up with their dependence on the constant supply of nutrients for cell proliferation (Hanahan and Weinberg, 2011; Schulze and Harris, 2012; Vander Heiden et al., 2009; Ward and Thompson, 2012). Deregulated cellular metabolism in cancer cells has emerged as one of the important hallmarks of cancer and also as an effective cancer therapeutic target. Alteration of cellular metabolism in cancer cells was first reported by Otto Warburg in the 1920s when he showed that these cells

had elevated rates of glucose uptake coupled with lactate formation to produce ATP the presence of oxygen via a process termed 'aerobic glycolysis' (Warburg, 1956; Warburg et al., 1927). This observation is known as the Warburg effect and is one of the most fundamental changes attributed to cancer cells. Although the early studies on cancer cell metabolism focused on the reprogramming of glucose metabolism in cancer cells, it is now clear that cellular metabolism of amino acids and fatty acids are altered in these cells and targeting these metabolic pathways represents possible therapeutic targets for cancer therapy (Cheong et al., 2012; Jones and Schulze, 2012).

1.4.4 *De Novo* Lipogenesis in cancer

One particular area that of cancer metabolism that has gained recognition over the last few years is lipid metabolism in cancer cells. One of the most commonly perturbed pathways in human cancers is the PI3K-Akt-MTOR signaling pathway and recent studies have placed it as an important upstream regulator of lipogenic phenotype in cancer cells via its ability to positively regulate the SREBP. (Ricoult and Manning, 2013; Santos and Schulze, 2012; Yecies and Manning, 2011). As summarized in Figure 5, mutations in a variety of oncogenes and tumour suppressors that lead to constitutive activation of MTORC1 will lead to elevated activity of SREBP-1 and expression of lipogenic enzymes that drive *de novo* lipogenesis (Laplante and Sabatini, 2009). One of the metabolic changes that is well characterized in cancer cells is the increase in *de novo* fatty acid synthesis to satisfy the increased demand for lipids that are required for membrane biogenesis during cell growth and proliferation (Vander Heiden et al., 2009). Although most cells can obtain their lipids in the form of FFAs from the bloodstream, cancer cells obtain most of their required lipids through *de novo* fatty acid synthesis.

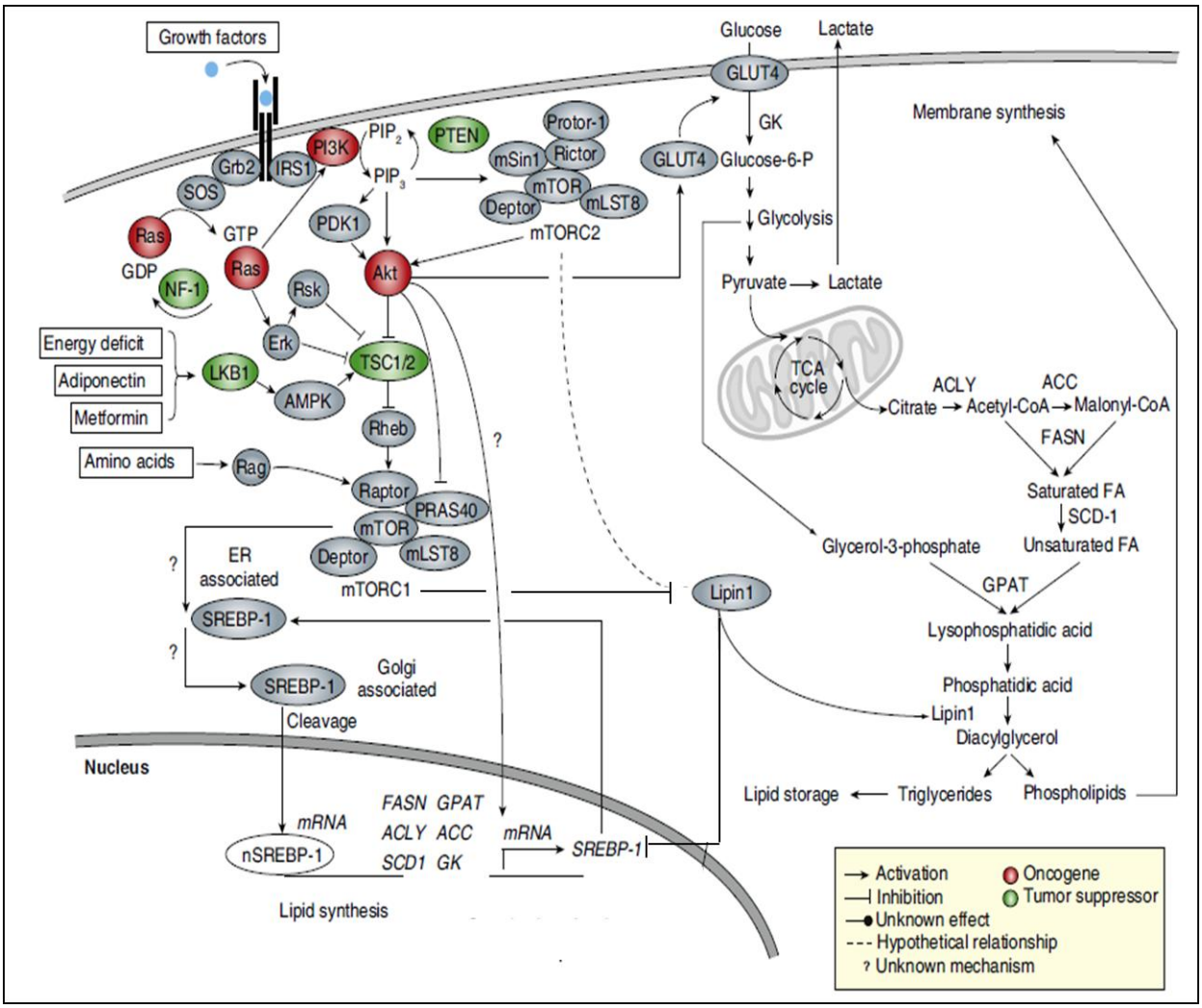


Figure 5: Constitutively activated MTORC1 leads to elevated levels of *de novo* lipogenesis through activation of SREBP-1 (Laplante and Sabatini, 2009).

The increased rate of *de novo* fatty acid synthesis in cancer cells was first recognized 50 years ago when elevated levels of fatty acid synthesis were observed in neoplastic tissue slices (Medes et al., 1953; Santos and Schulze, 2012). It is known that most of the membrane phospholipids in proliferating cancer cells are obtained from esterified fatty acids that are synthesized *de novo* (Pizer et al., 1996; Swinnen et al., 2003). However, the importance of *de novo* fatty acid synthesis in cancer cells has only recently re-emerged when Kuhadja and Co-workers identified that the oncogenic antigen-519 (OA-519)

which is associated with poor prognosis in breast cancer patients is actually the enzyme FASN (Kuhajda et al., 1994). Majority of the subsequent studies on the lipogenic phenotype in cancer cells has been focused on elucidating the mechanisms controlling the increased rate of fatty acid synthesis in cancer cells, as well as on targeting FASN for cancer therapy (Mashima et al., 2009; Menendez and Lupu, 2007). It is now known that apart from increased expression of FASN, other enzymes such as ACL, ACC and long chain elongase ELOVL7 which are involved in *de novo* fatty acid synthesis are up-regulated as well (Chajes et al., 2006; Deberardinis et al., 2008; Hatzivassiliou et al., 2005; Tamura et al., 2009). In cancer cells, excess pyruvate which is the end product of glycolytic cycle can be directed to *de novo* fatty acid synthesis. Pyruvate is converted to citrate in the mitochondria via the tricarboxylic acid (TCA) cycle and the citrate is then transported out to the cytosol and converted to acetyl-CoA by ACL. The rate limiting step of fatty acid synthesis requires conversion of acetyl-CoA to malonyl-CoA via ACC, a process that requires the consumption of an ATP molecule. FASN then performs the successive condensation reactions of acetyl-CoA and malonyl-CoA to produce the final product, the 16 carbon saturated PA. Elongation of PA can then take place through the elongases to form longer chain FAs and desaturation of the FAs can also take place that involves desaturases to produce unsaturated FAs (Deberardinis et al., 2008; Menendez and Lupu, 2007; Santos and Schulze, 2012).

1.5 Role of SCD-1 in cancer metabolism

1.5.1 Introduction to SCD-1

While FASN has been the main lipogenic enzyme being studied primarily as a potential therapeutic target in cancer (Mashima et al., 2009; Menendez and Lupu, 2007), the potential of targeting SCD-1 in cancer therapy has recently gained substantial attention. SCD-1 is an ER bound enzyme that catalyzes the formation of the first double bond at the cis-Δ9 position of SFAs to form MUFAs, the essential building blocks for a whole range of lipids, including neutral lipids and glycerolphospholipids (Hodson and Fielding, 2012; Ntambi and Miyazaki, 2003). SCD-1 converts palmitoyl-CoA (16C:0) and stearoyl-CoA (18C:0) to palmitoleoyl-CoA (16C:1) and oleoyl-CoA (18C:1), respectively. Therefore, the primary role of SCD-1 is to regulate the ratio of the saturated and unsaturated fatty acyl species of phospholipids that make up the cellular membrane to ensure the optimal membrane fluidity in the cells (Hodson and Fielding, 2013; Ntambi and Miyazaki, 2003). In mouse, there are four different isoforms of SCD (1-4), of which SCD-1 is ubiquitously expressed in most tissues and has been the best studied isoform. In humans, two SCD (1 and 5) isoforms have been found with 85% homology to the mouse SCD-1 (Sampath and Ntambi, 2006). Expression of SCD-1 is highly regulated in cells and sensitive to a wide range of nutrients, including carbohydrates (Miyazaki et al., 2004), fatty acids and cholesterol (Ntambi, 1999). Apart from nutrient regulation, SCD-1 expression in the cells can be positively regulated by numerous hormones and growth factors such as insulin (Waters and Ntambi, 1994), transforming growth factor-β (Samuel et al., 2002), fibroblast growth factor receptor 3 (Du et al., 2012) and retinoic acid (Samuel et al., 2001) as well. The role of SCD-1 in the development of metabolic diseases such as obesity and

diabetes has been well documented. It was observed that there was no accumulation of TAG and cholesterol esters in the livers of SCD-1 knock-out mice and TAG levels could not be restored despite supplementation with a lipogenic diet (Miyazaki et al., 2001). These findings thus suggest that *de novo* synthesized MUFAs by SCD-1 are the main substrates for production of TAG and cholesterol esters in the liver (Man et al., 2006). Furthermore, other studies have reported that SCD-1 played a role in energy homeostasis in the body as SCD-1 knock-out mice or mice injected with SCD1-specific antisense oligonucleotide inhibitors displayed reduced amount of adipose tissues, increased insulin sensitivity and were resistant to obesity (Hodson and Fielding, 2012; Jiang et al., 2005; Ntambi et al., 2002; Sampath and Ntambi, 2006).

1.5.2 SCD-1 as therapeutic target in cancer

Recent studies into malignant cell transformation and subsequent proliferation have suggested a critical role for SCD-1 in the development of cancer. As discussed above cancer cells have increased rate of glycolysis and *de novo* fatty acid synthesis. Apart from increased expression of SCD-1 due to increased SREBP-1 activity in cells with hyperactivated MTORC1 (Duvel et al., 2010; Peterson et al., 2011; Porstmann et al., 2005; Porstmann et al., 2008), there is increased levels of MUFAs in cancer cells, implicating MUFAs as an important end product of altered glucose metabolism (Igal, 2010; Scaglia et al., 2005). Initial studies found elevated levels of SCD-1 expression in the livers of mice that are susceptible to hepatocarcinogenesis, indicating an important role of SCD-1 in tumor formation (Falvella et al., 2002). It was then proposed that the SCD-1 activity was required for the anchorage-independent cell growth and survival, a major characteristic of cancer cell (Scaglia et al., 2005; Scaglia and Igal, 2005, 2008).

Furthermore, SCD-1 was identified through a siRNA screen to be a potential target for inducing cytotoxicity in cancer cells (Morgan-Lappe et al., 2007). Indeed, SCD-1 was found to be over expressed in many types of cancer cells and suppression of SCD-1 using either chemical inhibitors or genetic knockdown of SCD-1 was able to inhibit cell proliferation and ultimately induce cell death both *in vitro* and in mice xenograft models (Fritz et al., 2010; Hess et al., 2010; Mason et al., 2012; Minville-Walz et al., 2010; Scaglia et al., 2009). Interestingly, in comparison to inhibition of other lipogenic enzymes such as FASN and ACC, inhibition of SCD-1 was found to be most potent in killing of colon cancer cells in vitro and in vivo with the xenograft model (Mason et al., 2012). These results not only imply that SCD-1 is essential for cancer cell proliferation and survival, but also suggest that SCD-1 is important for lipogenesis in cancer cells and production of SFAs by FASN alone is not sufficient to sustain cancer cell growth. More importantly, inhibition of SCD-1 activity only affects the proliferation and survival of cancer cells but not normal, non-transformed cells indicate that specificity of this potential cancer therapeutic strategy (Hess et al., 2010; Minville-Walz et al., 2010; Scaglia et al., 2009).

The exact mechanisms through which SCD-1 regulates cancer cell proliferation and how SCD-1 inhibition can lead to cell death are not well known. As discussed earlier, SFAs like PA which are the end products of *de novo* fatty acid synthesis by FASN are known to cause lipotoxicity in cells. Since cancer cells have elevated rates of fatty acid synthesis, it is imperative for them to convert excess SFAs to the non-lipotoxic MUFAs via SCD-1. The fact that overexpression of SCD-1 in cells treated with supra-physiological amounts of PA can rescue the cells from lipotoxicity is proof of the cytoprotective effect of SCD-1

in response to excess SFAs (Listenberger et al., 2003; Matsui et al., 2012). Inhibition of SCD-1 activity has also been suggested to lead to inhibition of signaling pathways required for cell growth and proliferation. For example, loss of SCD-1 activity was shown to directly lead to inhibition of PI3K-Akt-MTOR signaling pathway in both lung and prostate cancer models (Fritz et al., 2010; Scaglia and Igal, 2008). Akt is important for the activation of MTORC1 that regulates major cellular processes such as protein and lipid synthesis, mitochondrial biogenesis and energy homeostasis (Jewell and Guan, 2013; Laplante and Sabatini, 2012; Yuan et al., 2013). Furthermore, Akt inactivation will lead to dephosphorylation and activation of proteins including the FoxO family and glycogen synthase-3β (GSK-3β), all of which have been implicated in promoting cell cycle inhibition and induction of cell death (Burgering and Kops, 2002; Jope and Johnson, 2004). The mechanism through which SCD-1 can activate PI3K-Akt-MTOR signaling pathway remains unknown. A very recent study has suggested that SCD-1 activity is required to maintain the MUFA/SFA ratio of the fatty acyl chains on the phospholipids of the plasma membrane in human lung cancer cells (Nashed et al., 2012). The authors showed that inhibition of SCD-1 activity will lead to alterations in the membrane fluidity and thus affect the lateral mobility of the lipid microdomain or lipid rafts, ultimately leading to defective EGFR signaling and inhibition of downstream Akt/Erk signaling (Nashed et al., 2012). These observations thus suggest that inactivation of Akt signaling caused by SCD-1 inhibition could be due to disruption of signaling cascade at the tyrosine kinase receptors at the plasma membrane. Proper functioning of tyrosine kinase receptors is known to be dependent on lipid rafts on the plasma membrane (Pike, 2009; Simons and Toomre, 2000). Therefore, the increased activity of SCD-1 in cancer cells

might be essential to maintain functional lipid raft structures on the plasma membrane via the production of MUFAs. More studies will need to be conducted to investigate whether lipid rafts are indeed affected by SCD-1 inhibition in cancer cells and whether this inactivation of growth signaling pathways is the main cause of cell death in cancer cells when SCD-1 is inhibited.

1.6 Aims of the Study

Thus far the role of autophagy in regulating lipid metabolism has been reported (Settembre et al., 2013; Singh et al., 2009a), while the regulatory functions of specific lipid species on the autophagic process remain largely controversial. On the other hand, the role of FFAs in lipotoxicity has been well studied (Schaffer, 2003; Unger, 2002), while the involvement of autophagy in such lipotoxicity has not been fully elucidated. Therefore, in the first part of this study our main objectives are to focus specifically on different types of FFAs in regulation of autophagic activity, with the following specific aims:

1. To investigate whether different types of FFAs, namely SFA and MUFA can regulate autophagy during lipotoxic stresses.
2. To determine the secondary lipid metabolites and the relevant molecular mechanisms directly involved in the regulation of autophagy upon FFAs-induced lipotoxicity.
3. To examine the physiological roles of autophagy during FFAs induced lipotoxicity.

On the other hand, cells deficient in the functional TSC1-TSC2 complex possess a constitutively activated MTORC1 signaling pathway that is independent of growth

factors regulation (Li et al., 2004; Tee et al., 2002). The activation of MTORC1 signaling pathway results in increased *de novo* lipogenesis in the cells via increased expression of lipogenic enzymes like FASN and SCD-1 (Duvel et al., 2010; Peterson et al., 2011; Porstmann et al., 2005; Porstmann et al., 2008). However, it is not known whether alteration of intracellular fatty acid levels in cells can affect the autophagic process. Therefore, in the second part of this study, we decided to investigate how alterations of intracellular SFAs and MUFAs will impact on the autophagic activity in the cells, with the following specific aims:

1. To investigate whether alterations of intracellular SFA and MUFA levels via the inhibition of SCD-1 can regulate autophagy levels.
2. To elucidate the signalling mechanisms involved in autophagy regulation upon inhibition of SCD-1 activity.
3. To determine the physiological roles of autophagy during SCD-1 inhibition in cells deficient of TSC2 protein.

2. Material and Methods

2.1 Antibodies and Reagents

BAPTA-AM, chloroquine diphosphate (CQ), crystal violet, Doxycycline, Earles' balanced salt solution (EBSS), methyl-β-cyclodextrin (MBCD), propidium iodide, 12-*O*-tetradecanoylphorbol-13-acetate (TPA), water-soluble cholesterol, rapamycin, myriocin, fumonisin B1, anti-HA, anti-LC3 and anti-tubulin antibodies were purchased from Sigma-Aldrich. Palmitic acid (PA), Oleic acid (OA) and essentially fatty acid free bovine serum albumin (BSA) were purchased from Sigma-Aldrich and both fatty acids were conjugated to the fatty acid free albumin as described previously (Listenberger and Brown, 2007). Anti-Atg5-Atg12 antibodies were purchased from Nanotools. Anti-PKC-α and anti-caveolin-1 antibody was purchased from BD Transduction Laboratory. Anti-Atg7 antibody was obtained from ProSci while anti-phospho tyrosine antibody Clone 4G10 and anti-phospho-PKC-α antibodies were obtained from Millipore. The PKC chemical inhibitors GF109203X, Gö6976 and Rottlerin were purchased from Calbiochem. Anti-Sin 1 antibody was purchased from Bethyl Laboratories. The SCD-1 chemical inhibitor CAY-10566 (CAY) was purchased from Cayman Chemicals. DQ^{TM} Red BSA and Cholera Toxin Subunit B (CTxB) was purchased from Invitrogen. Anti-SCD-1 antibody was purchased from Santa Cruz. The FoxO1 chemical inhibitor AS1842856 (AS) was purchased from Merk. All other antibodies were obtained from Cell Signaling.

2.2 Cells and Cell Culture

Mouse embryonic fibroblasts (MEFs) and HepG2 cells were maintained in Dulbecco's modified Eagle's medium (DMEM, from Sigma) containing 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen) (defined as full medium in this

study) in a 5% CO2 atmosphere at 37 °C. The Wild-type (WT) and *Atg5* knock-out (KO) mouse embryonic fibroblasts (MEFs) and the Tet-off Atg5 MEFs were provided by Dr. N. Mizushima (Tokyo Medical and Dental University) (Hosokawa et al., 2007). In most of the experiments, cells were treated with 0.25mM of PA or OA in high glucose, serum free DMEM for 4 hours, unless otherwise stated. The $Tsc2^{+/+}$ $p53^{-/-}$ and $Tsc2^{-/-}$ $p53^{-/-}$ MEFs were a generous gift from Dr. D. J. Kwiatkowski (referred to as $Tsc2^{+/+}$ and $Tsc2^{-/-}$ MEFs hereafter) and maintained in Dulbecco's modified Eagle's medium (DMEM, from Sigma) containing 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin (Invitrogen) in a 5% CO2 atmosphere at 37°C as well. The $Tsc2^{-/-}$ MEFs reconstituted with either empty vector (+EV) or TSC2 protein (+TSC2) were a generous gift from Dr. BD Manning (Huang et al., 2008) and maintained in the same conditions as above.

2.3 Immunoblotting and Immunoprecipitation

Immunoblotting analysis was performed following the established procedures. The cells were lysed in Laemmli SDS buffer containing: 62.5 mM Tris at pH 6.8, 25% glycerol, 2% SDS, phosphatase inhibitor mixture (Pierce), and the protease inhibitor mixture (Roche Applied Science). Equal amount of protein for each sample was resolved on SDS-PAGE gel and transferred onto polyvinylidene difluoride (PVDF) membrane (Bio-Rad). After it was blocked with 5% non-fat milk, the membrane was then probed with the designated primary and secondary antibodies and subsequently developed with the enhanced chemiluminescence method (Pierce) and visualized by a Kodak Image Station 4000R (Kodak). The immunoprecipitation (IP) assay was performed as described previously with slight modifications (Ikenoue et al., 2009). Briefly the cells were lysed for 30 minutes in the IP buffer (40 mM HEPES, pH 7.4, 120 mM NaCl, 2 mM EDTA, 0.3%

CHAPS, 10 mM pyrophosphate, 10 mM glycerophosphate, 50 mM NaF, phosphatase and protease inhibitor mixture). Cell lysates containing the same amount of protein were incubated with 1 µg of antibody and mixed overnight at 4°C with gentle rocking. After incubation overnight, 30 µl of Sepharose protein A/G-agarose beads were added to the cell lysates and mixed for 1 hour at 4°C with gentle rocking. After incubation, the beads were washed extensively with the IP buffer for five times, and the immunoprecipitated proteins were eluted by boiling for 5 minutes in sample buffer (Bio-Rad) before being resolved on SDS-PAGE gel and transferred onto PVDF membrane (Bio-Rad) for immunoblotting. All densitometry and quantification for the LC3-II western blots were calculated using the Kodak 1D 4.5.1 software by normalizing to the levels of LC3-II in the control treatments.

2.4 Sample Processing and Imaging for Transmission Electron Microscopy

MEFs were seeded on 4-chambered coverglass (NUNC Lab-tek Chambered Coverglass System) at a density of 2 X 10^4 cells/ml. After the respective treatments for 4 hours, cells were fixed with 2.5% glutaraldehyde and washed 3 times with 1 x phosphate buffer saline (PBS). Subsequently, post-fixation with 1% osmium tetroxide was performed followed by dehydration with ascending series of alcohol before being embedded in araldite for 24 hours. Ultrathin sections of 99 nm thickness were cut with a glass knife on the Reichert Ultracut E ultramicrotome, mounted onto copper grids and doubly stained with uranyl acetate and lead citrate. Images were acquired using the JEOL JEM1010 transmission electron microscope at voltage level of 100.0 KeV.

2.5 Confocal Microscopy

Cells were seeded to a coverglass slide chamber (NUNC Lab-tek Chambered Coverglass System) and after the designated treatments, the cells were examined under a confocal microscope (Olympus Fluoview 1000). GFP-LC3 puncta in the cells were quantified by counting the number in cells as described previously (Wu et al., 2010). Briefly, the GFP-LC3 puncta were manually counted under the confocal microscope. 30 cells were randomly selected from each treatment to calculate the average number of GFP-LC3 puncta per cell. The data shown is from one representative experiment of three independent repeats. For the DQ^{TM} Red BSA assay, DQ^{TM} Red BSA (10 μg/ml) was added for the last hour of treatment and cells were then observed under the confocal microscope. For staining of lipid rafts, the cells were incubated with Cholera Toxin Subunit B (CTxB, 10 μg/ml) at 4°C for 15 minutes. The cells were then washed with PBS for 2 times and incubated for another 30 minutes at 37°C and observed immediately under the confocal microscope.

2.6 Analysis of lipids using High Performance Liquid Chromatography/Mass Spectrometry

An Agilent high performance liquid chromatography (HPLC) 1200 system coupled with an Applied Biosystem Triple Quadrupole/Ion Trap mass spectrometer (3200 Qtrap) was used to quantify individual phospholipids. An Agilent high performance liquid chromatography (HPLC) 1100 system coupled with an Applied Biosystem Triple Quadrupole/Ion Trap mass spectrometer (4000 Qtrap) was used for quantification of individual phospholipids. Samples were introduced into the mass spectrometer by loop injections with chloroform:methanol (1:1) as a mobile phase for both positive ESI mode

and negative ESI mode, respectively. Two comprehensive sets of multiple reaction monitoring (MRM) transitions were set up for quantitative analysis of various phospholipids (Shui et al., 2011b). Levels of individual lipid were quantified using spiked internal standards that include PC-14:0/14:0, PE-14:0/14:0, PS-14:0/14:0, PG-14:0/14:0 (Avanti Polar Lipids, USA), and dioctanoyl PI (Echelon Biosciences, Inc., USA). Neutral lipids were analyzed using an Agilent 1200 HPLC system coupled with an Applied Biosystem Triple Quadrupole/Ion Trap (3200Qtrap) mass spectrometers. Glycerol lipids were analyzed using a sensitive HPLC/ESI/SIM method modified from a previous method (Shui et al., 2010). HPLC conditions: Luna 3-μm silica column (i.d. 150 × 2.0 mm); mobile phase A (chloroform:methanol:ammonium hydroxide, 89.5:10:0.5), B (chloroform:methanol:ammonium hydroxide:water, 55:39:0.5:5.5); flow rate 300 μl/min; 5% B for 3 min, then linearly switched to 30% B in 24 min and maintained for 5 min, and then linearly changed to 70% B in 5 min and maintained for 7 min. Then, the composition of the mobile phase was returned to the original ratio over 5 min and maintained for 6 min before the next sample was analyzed. Multiple reaction monitoring (MRM) transitions for individual glycerolphospholipid species and sphingolipids were set up at different elution stages for LC-MS analysis (Shui et al., 2010; Shui et al., 2011b). Levels of individual lipid levels were quantified using spiked internal standards. Neutral lipids were analyzed using a sensitive HPLC/ESI/MRM method modified from a previous method (Shui et al., 2010). TAGs were calculated as relative contents to the spiked d5-TAG 48:0 internal standard (CDN isotops), while DAGs were quantified using 4ME 16:0 Diether DG (Avanti) as an internal standard. Triacylglycerol (TAGs) were calculated as relative contents to the spiked d5-TAG 48:0 internal standard (CDN

isotops), while diacylglycerol (DAGs) were quantified using 4ME 16:0 Diether DG (Avanti) as an internal standard. Cholesterol and cholesteryl esters (CE) were quantified using HPLC/APCI/MS/MS as previously described with corresponding d6-cholesterol and d6-C18 cholesterol ester (CDN isotopes) as internal standards (Shui et al., 2011a).

2.7 Transient Small Interfering RNA (siRNA) Transfection

The nonspecific siRNA oligonucleotides and siRNA oligonucleotides targeting mouse and human Atg7 and PKC-α, mouse DGAT1, SCD-1 and FoxO1 (ON-TARGETplus SMARTpoolTM) were obtained from Dharmacon (Layfayette, CO). The siRNAs was transfected into the respective cells as indicated using the DharmaFECT 4 transfection reagent according to the manufacturer's protocol.

2.8 Detection of Cell Death

Flow cytometry was used to determine the cell viability using the live cell propidium iodide exclusion test, as previously described (Wu et al., 2008a). In brief, cells were trypsinized at the end of the experiments. Cells were washed once with PBS and resuspended in PBS containing 1 mg of propidium iodide/ml. The levels of propidium iodide incorporation were quantified by flow cytometry using a FACSCalibur flow cytometer. Cell size was evaluated by forward-angle light scattering. Propidium iodide-negative cells with normal size were considered to be live cells. Morphological changes of treated cells under phase-contrast microscope were also observed to detect cell death after treatment.

2.9 Colony Formation Assay

The cells were first treated as designated and then re-seeded into six-well plates at a cell density of 5000 cells/well. After 2 weeks, the surviving clones were stained with 0.5% crystal violet solution for 1 hour and photographed using a digital camera.

2.10 Plasmids and Transfection

The GFP-tagged LC3 plasmid was kindly provided by Dr. T. Yoshimori (Osaka University, Japan). The HA-Akt and Myr-HA-Akt plasmids were kindly provided by Dr. J. S. Gutkind (National Institutes of Health, USA). Transient transfection was performed using the Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocol. Briefly, the cells were transfected with the plasmids for 24 hours and re-seeded overnight before being subjected to the designated treatments.

2.11 Separation of Detergent-Soluble and Detergent-Resistant Fraction

Separation was conducted as described previously with modifications (Wu et al., 2008b). Cells were collected after their designated treatments and lysed in TNE buffer containing 150 mM NaCl, 0.5% Triton X-100, 3mM EDTA, 20 mM Tris HCl (pH 7.5) with phosphatase and protease inhibitor mixture. The cells were then homogenized by passage through a 23-gauge needle for 10 times and incubated for 1 hour on ice. The lysates were then centrifuged at 14,000 g for 30 minutes at 4°C and the supernatants were collected as the detergent-soluble fraction. The remaining pellets were resuspended and briefly sonicated in the same lysis buffer supplemented with 0.5% SDS and 2 mmol/L DTT and the supernatant were collected as the detergent-resistant fractions.

2.12 Preparation of Nuclear and Cytosolic Extracts

After designated treatments, the nuclear and cytosolic extracts were prepared using the NE-PER® nuclear and cytoplasmic extraction reagents (Pierce), according to the manufacturer's protocol.

2.13 Dual-Luciferase Reporter Assay

The WT FoxO1-Luc and mutant FoxO1 (mut 1 +2)-luc vectors were described in a previous study (Al-Mubarak et al., 2009). Transient transfection of FoxO1-Luc and the FoxO1(mut 1+2)-luc vectors were done in *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's protocols. The *Renilla* luciferase vector was cotransfected together as a transfection control. The cells were then treated with CAY for the time indicated 24 hours after transfection. The luciferase activity was then measured using a Dual-Luciferase Reporter Assay System (Promega, E1960) based on the protocol provided by the manufacturer. Briefly, the treated cells were lysed with the addition of cell lysis reagent and the individual cell lysates were collected. The firefly luciferase activity was determined using the luminometer (Promega) after addition of the luciferase assay substrate to a fixed amount of cell lysate. The *Renilla* luciferase activity was subsequently measured after the addition of the Stop&Glo substrate.

2.14 Reverse Transcription and Quantitative Real-Time PCR

The total RNA was extracted with the RNeasy kit (Qiagen). A reverse transcription reaction was then performed using 1 µg of total RNA with High Capacity cDNA Reverse Transcription kit (Applied Biosystems). The mRNA expression levels of target genes

were then determined by real-time PCR using SsoFast™ EvaGreen® Supermix (Bio-Rad) and CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. The primers used in the study are listed as below:

ATG4: Forward primer: ATT GCT GTG GGG TTT TTC TG

Reverse primer: AAC CCC AGG ATT TTC AGA GG

ATG12: Forward primer: GGC CTC GGA ACA GTT GTT TA

Reverse primer: CAG CAC CGA AAT GTC TCT GA

BNIP3: Forward primer: TTC CAC TAG CAC CTT CTG ATG A

Reverse primer: GAA CAC CGC ATT TAC CAG AAC AA

*BNIP3*L: Forward primer: TTG GGG CAT TTT ACT AAC CCT G

Reverse primer: TGC AGG TGA CTG GTG GTA CTA A

BECLIN1: Forward primer: GGC CAA TAA GAT GGG TCT GA

Reverse primer: CAC TGC CTC CAG TGT CTT CA

LC3B: Forward primer: CGT CCT GGA CAA GAC CAA GT

Reverse primer: ATT GCT GTC CCG AAT GTC TC

VPS34: Forward primer: TGT CAG ATG AGG AGG CTG TG

Reverse primer: CCA GGC ACG ACG TAA CTT CT

FASN: Forward primer: CAG CAG AGT CTA CAG CTA CCT

Reverse primer: ACC ACC AGA GAC CGT TAT GC

SCD-1: Forward primer: ACG CCG ACC CTC ACA ATT C

Reverse primer: CAG TTT TCC GCC CTT CTC TTT

GAPDH: Forward primer: TCA CCA TCT TCC AGG AGG GA

Reverse primer: GCA TTG CTG ACA ATC TTG AGT GAG

2.15 Statistics

The numerical results are all expressed as mean ± standard deviation (S.D.) from at least 3 independent experiments. The significance level was set at $*p<0.05$ and $**p<0.01$ using student's t-test.

3. Results

3.1 Induction of autophagy by palmitic acid via protein kinase C-mediated signaling pathway independent of MTOR

3.1.1 PA, but not OA, induces autophagy

As mentioned above many studies have concluded that SFAs, such as PA are more cytotoxic compared to unsaturated fatty acids, such as OA (Cunha et al., 2008; Listenberger et al., 2003; Listenberger et al., 2001; Maedler et al., 2001; Malhi et al., 2006; Schaffer, 2003). However, it is not known if autophagy plays a role promoting or preventing the cytotoxic effect of SFAs. In order to rule out the effects of FFAs that are commonly found in the serum that is added to culture medium, all the subsequent treatments with either OA or PA were conducted in serum free DMEM. Furthermore, cells that were cultured in DMEM without serum were also used as comparison against PA or OA treated cells to rule out the fact that any increase in autophagy levels could be due to the absence of serum. We first treated cells with PA in a time course to look at whether the autophagic levels could be affected in the treated cells. As shown in Figure 6A, treatment of MEFs with PA resulted in a significant increase in the levels of LC3-II for up to 24 hours, in comparison to the control cells treated with BSA as control. In order to measure the actual changes in the autophagic flux of the PA treated cells, we used chloroquine (CQ) to block lysosomal function and the late degradation stage of autophagy (Mizushima et al., 2010). With the addition of CQ, we were able to observe a further increase of LC3-II level in PA-treated cells at the various time points (Fig. 6B), thus clearly suggesting that PA is able to induce autophagy flux in MEFs. To further confirm the observation that PA treatment does indeed induce autophagy in the MEFs,

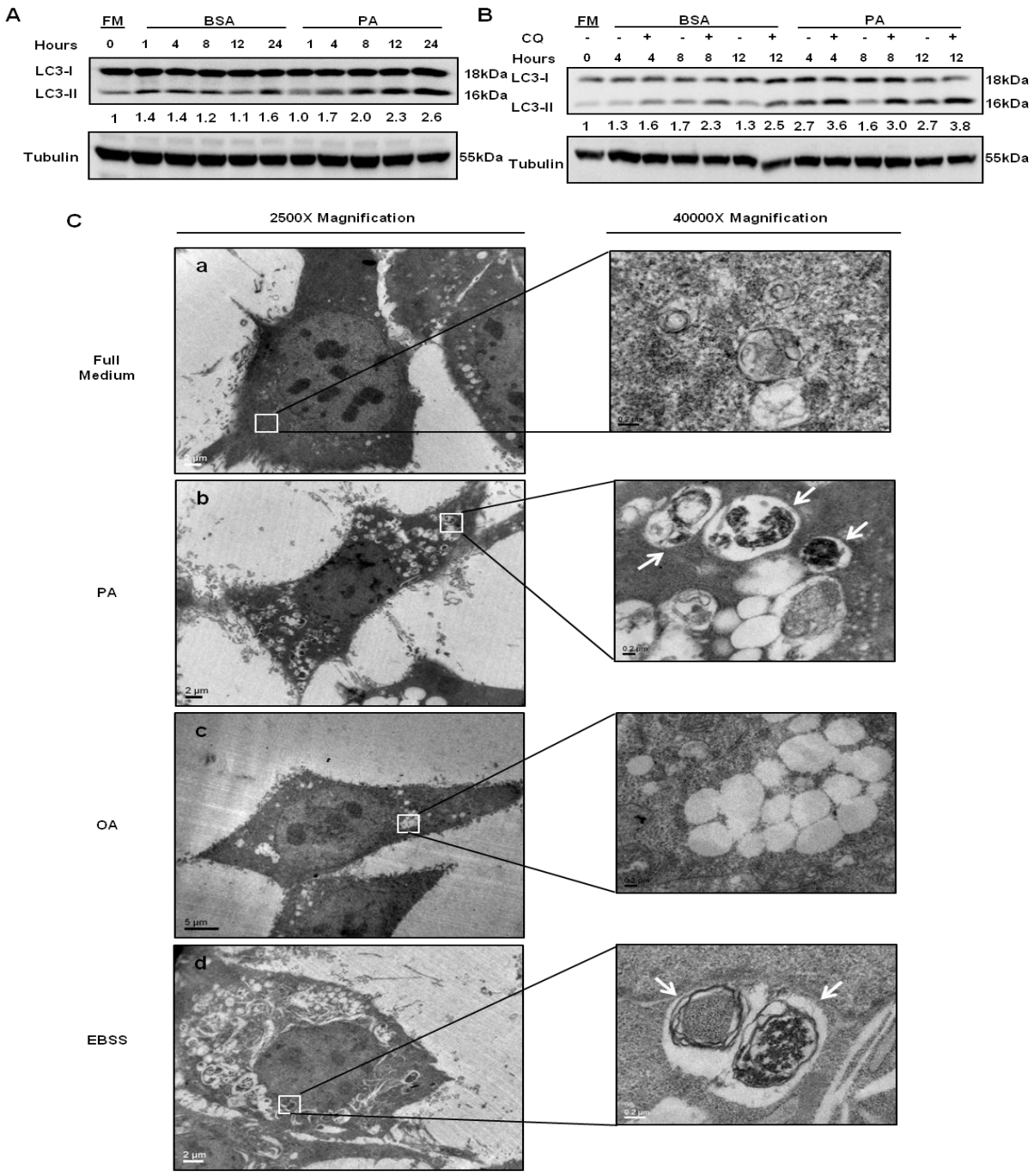


Figure 6. PA treatment induces autophagy. (A) MEFs were treated with PA (0.25 mM) conjugated to fatty acid free BSA for the different time points as indicated. Cells treated with BSA acted as control. FM: full medium (DMEM) with 10 % FBS. After treatments, cell lysates were collected and subject to western blot. (B) MEFs were treated with PA (0.25mM) for the indicated time points, with or without the addition of CQ (10 µM). C. MEFs were treated with BSA (panel a), PA (0.25 mM, panel b), OA (0.25 mM, panel c) or EBSS (panel d) for 4 hours before being processed and cellular images were then taken using an electron microscope at 2500X and 40000X magnification.

transmission electron microscopy studies were performed on MEFs under various treatments. As shown in Figure 6C, autophagosome like vacuoles were hardly seen in BSA-treated control cells (panel a). In contrast, we observed an increase in the formation of autophagosome like structures containing cytosolic contents in the PA-treated MEFs after 4 hours (panel b, white arrows). Similar autophagosome like vacuoles containing cytosolic contents were observed as well in MEFs treated with EBSS as a positive control (Fig. 6C, panel d, white arrows). However, no autophagosome like vacuoles containing cytosolic contents were observed in MEFs treated with OA for 4 hours (Fig. 6C, panel d), suggesting that OA treatment was not able to induce autophagy.

ATG5 protein is essential for the completion of the autophagosome and loss of ATG5 leads to disruption of autophagosome formation and inhibits the autophagic process (Nakatogawa et al., 2009; Yang and Klionsky, 2010b). However, a recent study has shown that autophagy can be induced despite the loss of ATG5 protein in cells (Nishida et al., 2009). Therefore we next examined whether PA-induced autophagy is dependent on the presence of ATG5 and the canonical autophagy machinery. As shown in Figure 7A, no LC3-I to LC3-II conversion was observed in the *Atg5* knock-out (KO) MEFs we used compared to the WT cells and we observed a dose dependent increase in the LC3-II levels upon treatment with increasing doses of PA in the WT MEFs. Similar observation was seen using the WT and *Atg5* KO MEFs when they were starved in the EBSS medium as well. These results suggest that PA-induced autophagy is dependent on the canonical autophagy induction machinery. To further validate the results, we utilized the MEFs with the inducible *Atg5* deletion system that stably express GFP-LC3 (Hosokawa et al., 2007) to further evaluate PA-induced autophagy. There was a significant increase in

GFP-LC3 puncta in PA-treated cells expressing the ATG5 protein where no doxycycline (DOX) was added (Fig. 7B). Consistently, we observed that addition of CQ together with PA treatment further enhanced both the size and the number of such GFP-LC3 puncta in the cells, suggesting an increase in autophagic flux. Similar to the results in Figure 6A, deletion of *Atg5* by addition of DOX abolished the effect of PA on GFP-LC3 puncta formation further confirming that autophagy induction by PA treatment is dependent on the canonical autophagy machinery. Finally, we compared the effects of PA treatment with that of the MUFA, OA on autophagic activity in the treated MEFs. Treatment of the MEFs with OA at similar concentration to that of PA (0.25 mM) for 4 hours did not cause any significant increase in the levels of LC3-II conversion, even with the presence of CQ (Fig. 7C). On the other hand, treatment of the cells with PA and other known autophagy inducers (EBSS and rapamycin) were able to cause an evident increase in the autophagic flux (Fig. 7C).

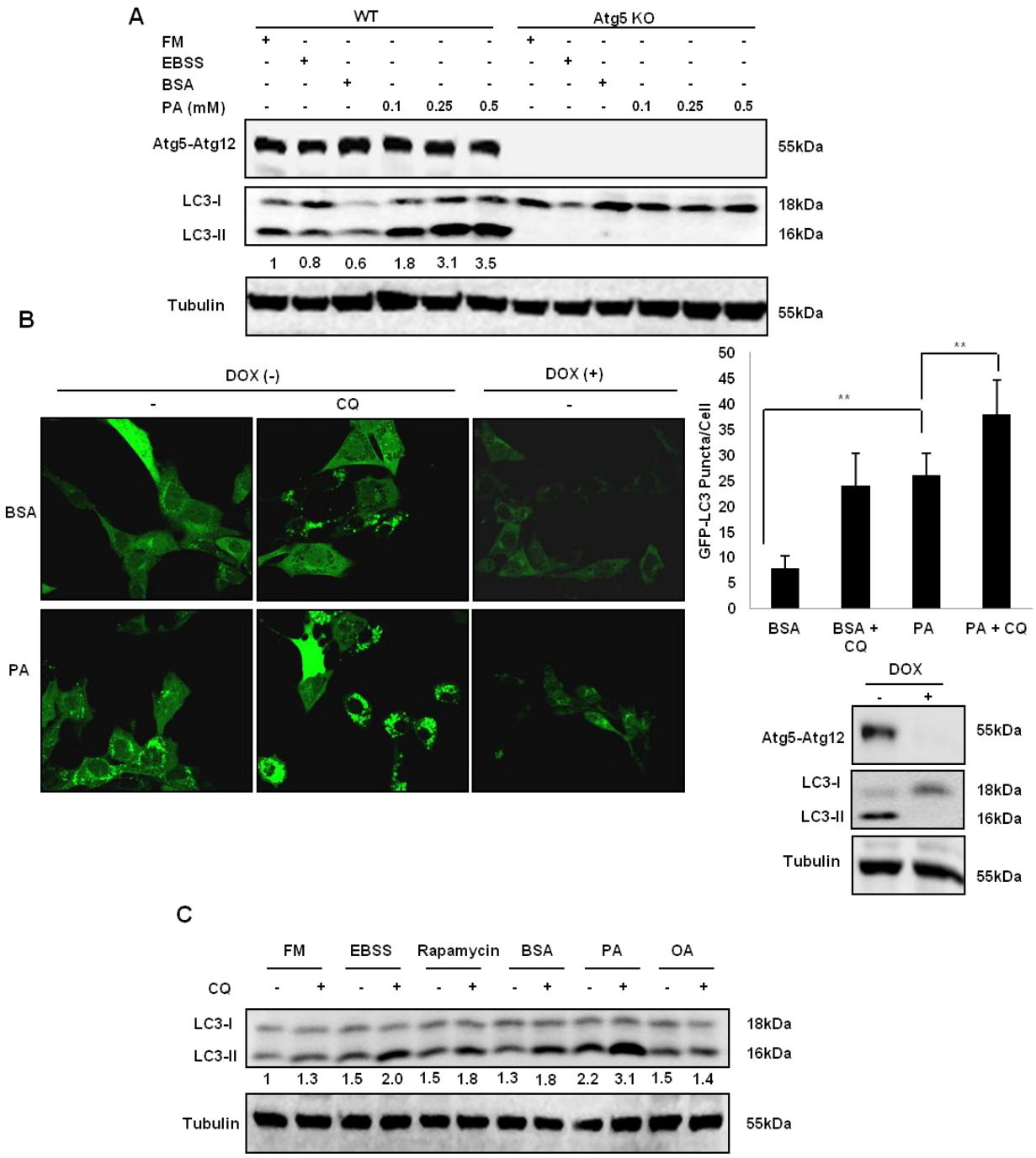


Figure 7. PA induced autophagy is dependent on the canonical autophagy machinery. (A) WT and *Atg5* KO MEFs were treated various concentrations of PA for 4 hours. **(**B) Atg5 Tet-off MEFs stably expressing GFP-LC3 were treated with PA (0.25 mM) for 4 hours and GFP-LC3 puncta formation was observed using confocal microscopy. Atg5 protein expression was inhibited in the cells cultured with DOX for 4 days. The number of GFP-LC3 puncta/cell were then counted (**p<0.01, student's t-test). (C) MEFs were treated with either BSA, PA (0.25 mM) or OA (0.25 mM) for 4 hours with or without the addition of CQ.

3.1.2 PA-induced autophagy is independent of the MTORC1 signaling pathway

Since the MTORC1 signaling pathway is well established as the key negative regulator of the autophagic process (Jung et al., 2009; Mizushima, 2010), we next investigated the role of the MTORC1 signaling pathway in PA-induced autophagy. The p70 S6 kinase (p70S6K) protein is a direct substrate of MTORC1 and its phosphorylation status can be used as an indicator of the activity of the MTORC1 pathway (Laplante and Sabatini, 2012; Zoncu et al., 2011b). When MEFs were treated with either BSA or PA in FBS-free medium, there was a marked reduction of the p-p70S6K level compared to the cells in normal full medium (FM) conditions (Fig. 8A). However, we observed no further decrease in the levels of p-p70S6K levels in the PA treated cells compared to the BSA treated cells, especially at the earlier time points (Fig. 8A). Interestingly, PA increased the p-p70S6K level at later time points (12 and 24 hours). The same trends were also observed for the direct substrate of p70S6K, the S6 ribosomal protein (Fig. 8A), indicating the possibility that PA may activate MTORC1 with prolonged treatment.

To further confirm the above observations, the MEFs were then treated with PA in FM which contains 10% fetal bovine serum (FBS). Other known inducers of autophagy like EBSS treatment and rapamycin are known to induce autophagy via inhibition of the MTORC1 signaling pathway. As shown in Figure 8B, treatment of MEFs with EBSS or rapamycin resulted in complete loss of phosphorylation on the p70S6K and S6 ribosomal proteins implying inhibition of MTORC1 concurrently with an increase in autophagic activity (Fig. 8B). Furthermore, treatment with PA for 4 hours led to an increased conversion of LC3-I to LC3-II levels and addition of the lysosomal inhibitor CQ further enhanced the increase of LC3-II levels, indicating an increase in autophagic flux in the

PA-treated cells. On the other hand, there was no further increase in autophagic flux observed in the cells treated with BSA. Consistently, we observed that PA treatment failed to reduce the phosphorylation levels of p70S6K and S6 ribosomal protein despite the induction of autophagy observed in the MEFs (Fig. 8B), suggesting that MTORC1 activity was not inhibited upon PA treatment. Thus the results here suggest that PA-induced autophagy is independent of the canonical MTORC1 signaling pathway regulation.

In order to investigate whether PA-induced autophagy can be observed in other cell types, we then treated the liver cell line HepG2 with various autophagy inducers. Indeed, treatment of HepG2 cell with PA significantly enhanced the levels of autophagic flux observed compared to the BSA treated cells (Fig. 9A). Furthermore, OA treatment did not did not result in any further increase in the autophagic flux when compared to the BSA-treated control cells (Fig. 9A), confirming the earlier observations that only PA treatment resulted in autophagy induction. Consistently, when the HepG2 cells were treated with PA in FM containing 10% FBS for 4 hours, an increase in autophagic flux was observed with no inhibition of the MTORC1 signaling pathway (Fig. 9B). Such results enforced the observation that induction of autophagy by PA is independent of the MTORC1 signaling pathway.

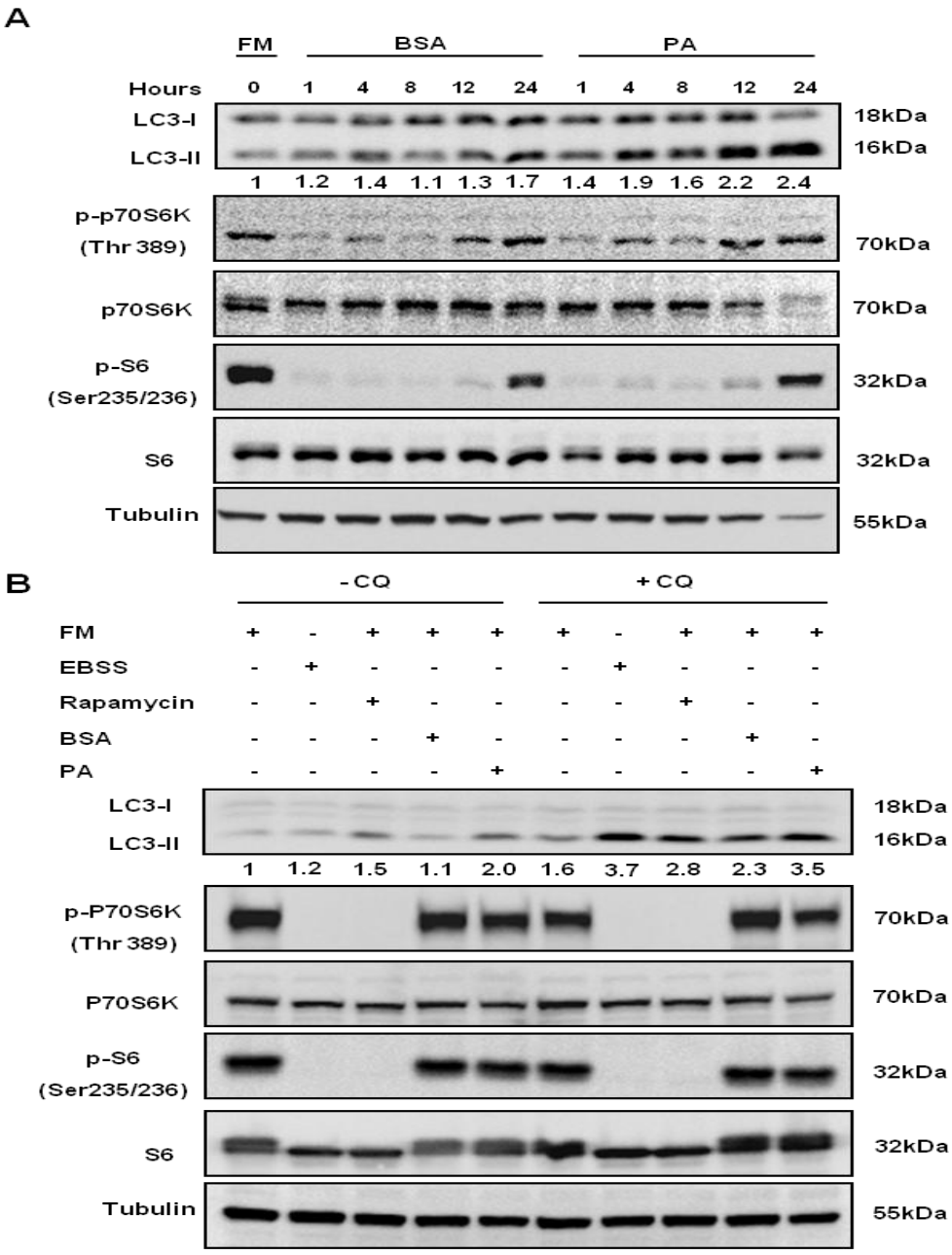


Figure 8. PA-induced autophagy is independent of the MTORC1 regulation. (A) MEFs were treated with BSA or PA (0.25 mM) for various time points as indicated. Cell lysates were collected and subject to western blot. (B) MEFs were treated with BSA control or PA (0.25 mM) for 4 hours in FM. Cells were treated with EBSS and the MTOR inhibitor, rapamycin (20 nM) for 4 hours as positive controls. CQ (10 μM) was added to the treated cells for measuring autophagic flux.

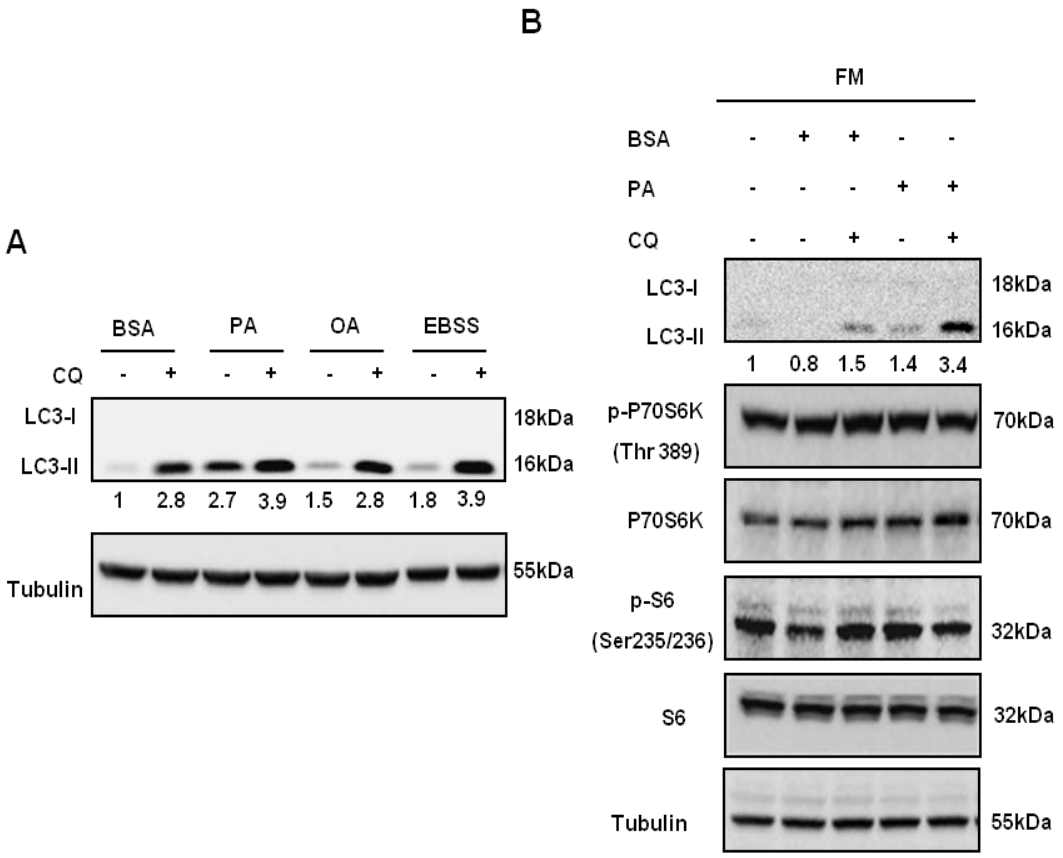


Figure 9. PA also induces autophagy in HepG2 cells which is independent of MTORC1 regulation. (A) HepG2 cells were treated with BSA, PA (0.25 mM) or OA (0.25 mM) for 4 hours with or without the addition of CQ (10 μM). Treatment with EBSS was used as a positive control for autophagy induction in the HepG2 cells. (B) HepG2 cells were treated with BSA control or PA (0.25 mM) for 4 hours in FM. CQ (10 μM) was added to the treated cells to determine the level of autophagic flux in the cells.

3.1.3 Accumulation of intracellular ceramide is not related to PA-induced autophagy

The accumulation of intracellular ceramides have been linked to the progression of diseases like obesity and other metabolic diseases as described earlier (Holland and Summers, 2008; Kihara et al., 2007; Zheng et al., 2006). The first and rate-limiting step of *de novo* ceramide synthesis involves the condensation of serine with palmitoyl-CoA to form 3-ketodihydrosphingosine which is then converted via subsequent reactions into ceramides (Kihara et al., 2007). The treatment of the cells with exogenous PA could possibly cause an increase in ceramide production in the cells since PA can act as a source of palmitoyl-CoA. More importantly, a few studies have reported that treatment with short chain ceramides can induce autophagy in a variety of cancer cell lines due to promotion of long chain ceramide synthesis (Guenther et al., 2008; Pattingre et al., 2009; Scarlatti et al., 2004). Therefore we next investigated whether ceramides played any role in the induction of autophagy caused by PA treatment. Here we first measured the levels of intracellular ceramides in cells treated with either PA or OA. Treatment with PA for 4 hours resulted in an increase of ceramides by about 1.4 fold compared to the cells treated with BSA control although it was not statistically significant; while very marginal increase was found in the OA-treated cells (Fig. 10A). To further test the effects of intracellular ceramides on PA-induced autophagy, cells were treated with the chemical inhibitor of serine palmitoyltransferase, myriocin or the inhibitor of sphingosine N-acyltransferase fumonisin B1 (FB1), as both of these enzymes are essential for the *de novo* synthesis of ceramide in cells (Pattingre et al., 2009; Scarlatti et al., 2004). Addition of myriocin or FB1 to PA-treated cells did not inhibit the increase in LC3-II conversion

observed when compared to the cells treated with PA only (Fig. 10B). Furthermore, the increase in overall autophagic flux was not affected by the inhibition of ceramide synthesis as observed by the cells treated together with CQ (Fig. 10B). To further validate these results, we then inhibited ceramide synthesis using FB1 in the MEFs stably expressing GFP-LC3. Consistently, inhibition of *de novo* ceramide synthesis with FB1 failed to reduce the formation of GFP-LC3 puncta formed in the PA-treated cells, with or without the presence of CQ (Fig. 10C). Altogether, results from this part of the study strongly suggest that endogenous ceramide levels are not related to PA-induced autophagy.

3.1.4 PA, but not OA, induces accumulation of intracellular diacylglycerol

FFAs are the immediate precursors that can be used for *de novo* synthesis of neutral lipids like TAG and DAG (Coleman and Mashek, 2011). Both DAG and TAG are glycerolipids, with either 2 or 3 fatty acid chains linked to the glycerol molecule respectively. When taken up by cells, excess FFAs can be converted into their respective acyl-CoA derivatives (Coleman and Mashek, 2011). These acyl-CoA derivatives can then be incorporated and stored in the cells in the form of neutral lipids like DAG and TAG (Li et al., 2010; Listenberger et al., 2003). Thus we hypothesized that the intracellular levels of DAG and TAG will increase substantially during PA or OA treatment. Therefore we proceeded to measure the levels of intracellular DAG and TAG after treatment with various concentrations of PA or OA. MEFs were treated with either BSA as control or 0.125 mM and 0.25 mM of either PA (Fig. 11A) or OA (Fig. 11B) for 1, 2 and 4 hours. We then measured the relative levels of DAG and TAG accumulation in the treated cells.

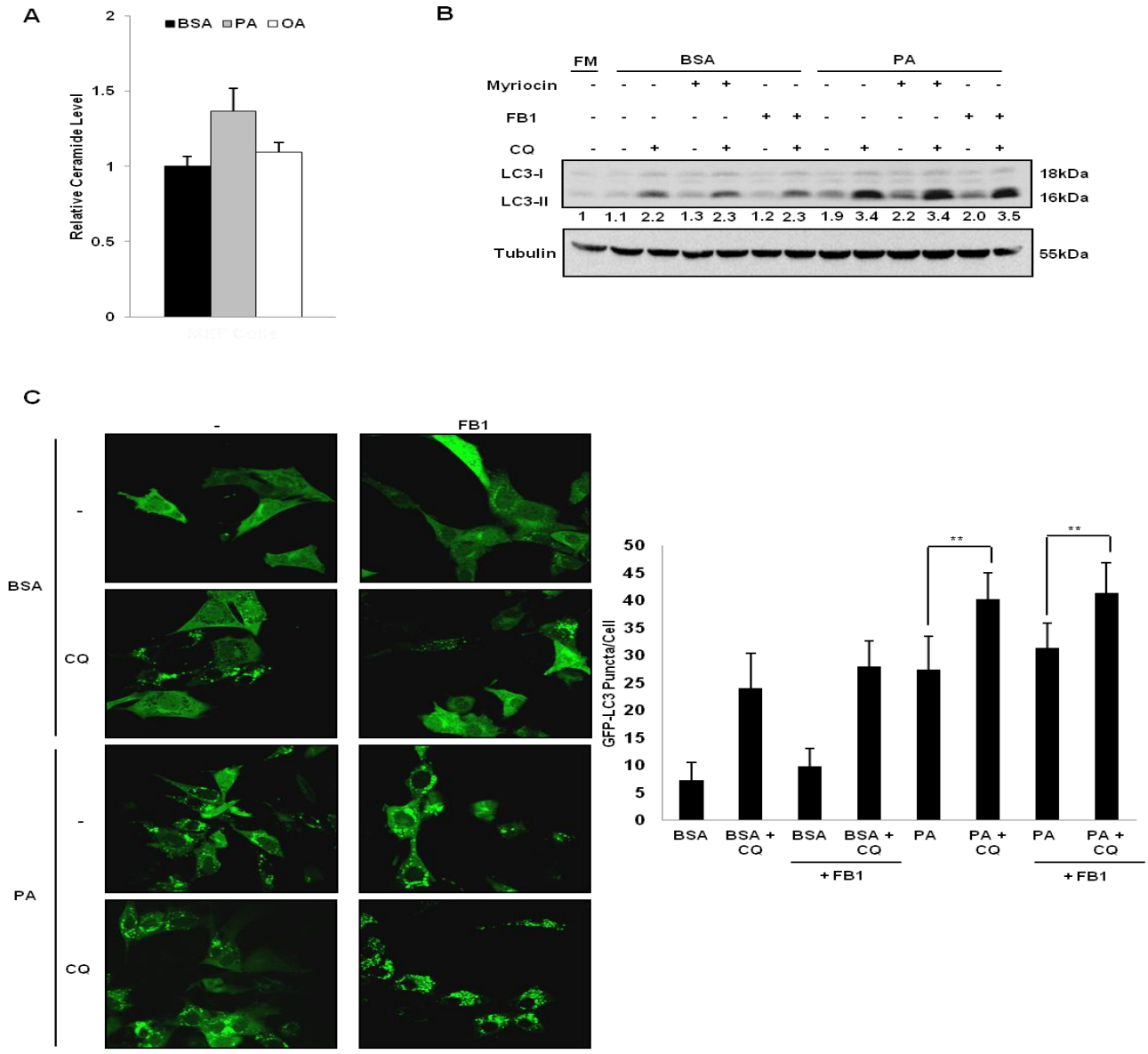


Figure 10. Autophagy induction by PA is not caused by intracellular accumulation of ceramide. (A) MEFs were treated with BSA, PA (0.25 mM) or OA (0.25 mM) for 4 hours and the total ceramide levels were then quantified. The relative ceramide levels of cells from the various treatments were calculated by normalizing to the ceramide levels of the cells treated with BSA. Data were presented as means ± S.D. of three independent experiments. (B) MEFs were treated with BSA or PA (0.25 mM) for 4 hours with or without the presence of either myriocin (1 μM) or FB1 (10 μM). CQ (10 μM) was added to the treated cells to observe the changes in the autophagic flux. (C) *Atg5* Tet-off MEFs stably expressing GFP-LC3 were treated with either BSA or PA (0.25 mM) for 4 hours with or without the presence of FB1 (10 μM) and the number of GFP-LC3 puncta/cell were quantified (**p<0.01, student's t-test).

As expected, we observed that treatment with either PA or OA resulted in a dose and time dependent increase in the relative levels of DAG and TAG compared to the BSA-treated control cells (Fig. 11A, left panel). Furthermore, although TAG levels in the PA-treated cells also increased significantly at treatment times up to 4 hours, we observed that the rate of increase in TAG level was not proportional to that of DAG with 0.25 mM of PA treatment (Fig. 11A, right panel).

On the other hand, the accumulation of DAG in the MEFs that were treated with different concentrations of OA were not statistically significant (Fig. 11B, left panel). Instead, we observed a significant time and dose-dependent increase in TAG accumulation in the cells treated with OA (Fig. 11B, right panel). To confirm the results from this part of the study, we treated the HepG2 cells with similar concentrations of PA or OA for the respective time points. Similar to the results observed for the MEFs, only the PA-treated HepG2 cells had significantly elevated levels of DAG accumulation by up to 4 hours of treatment but this was not seen in the OA-treated HepG2 cells (Fig. 12). Interestingly, PA treatment also resulted in a significant increase in the intracellular TAG levels by 4 hours (Fig. 12A, right panel) although the magnitude of increase was smaller when compared to the increase in TAG levels of the OA-treated HepG2 cells (Fig. 12B, right panel). There have been previous studies that have shown that the enzyme responsible for the conversion of DAG to TAG, diacylglycerol acyltransferase (DGAT), has a higher specificity for fatty acyl-CoAs that contain the 18C:1 chain derived from OA compared to other fatty acyl-CoAs that have the 16C:0 chain coming from PA (Coleman and Bell, 1976; Li et al., 2010). Therefore such a substrate specificity by DGAT could well explain the trends observed in our data where the OA-treated MEFs and HepG2 have a much

more elevated levels of intracellular TAG compared to that of DAG (Fig. 11B and 12B); while in cells treated with PA the accumulation of intracellular DAG was more significant than that of the increase in TAG levels (Fig. 11A and 12A).

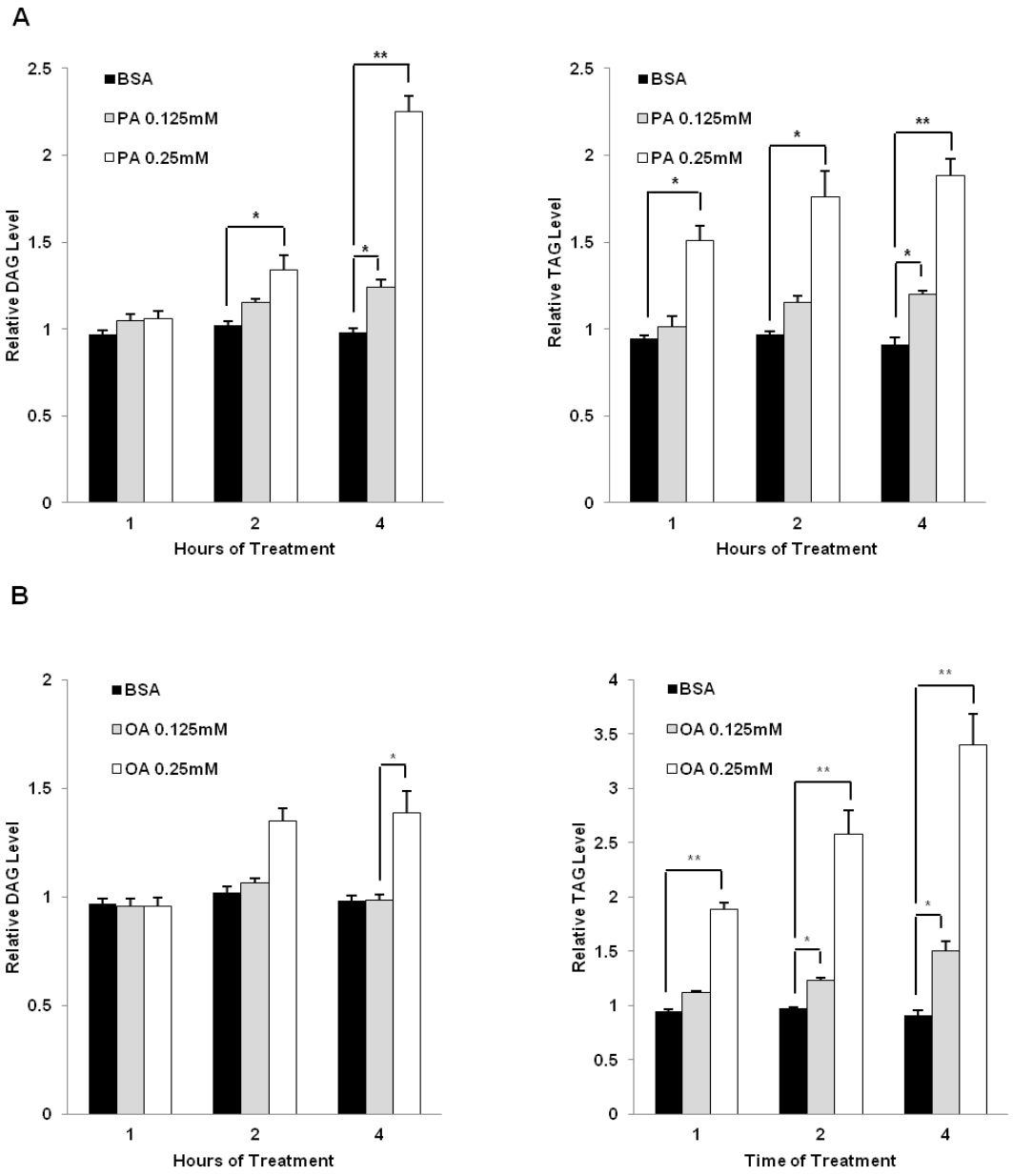


Figure 11. PA and OA treatment induce differential accumulation of intracellular DAG and TAG which is time and dose dependent in MEFs. (A) MEFs were treated with BSA and PA (0.125 mM and 0.25 mM) for 1, 2 and 4 hours. The total DAG (Left panel) and TAG (Right panel) levels were then quantified. The relative DAG and TAG levels were calculated by normalizing their respective levels in each treatment at different time points to the levels present in the control cells. (B) Cells were treated with BSA and OA (0.125 mM and 0.25 mM) for 1, 2 and 4 hours. The total DAG (Left panel) and TAG (Right panel) levels were then quantified. The relative DAG and TAG levels were calculated as described in (A) Data were presented as means ± S.D. of three independent experiments and Student's T-test were calculated between the BSA treated cells and either PA or OA treated cells at each respective time point (*$p<0.05$, **$p<0.01$, student's t-test).

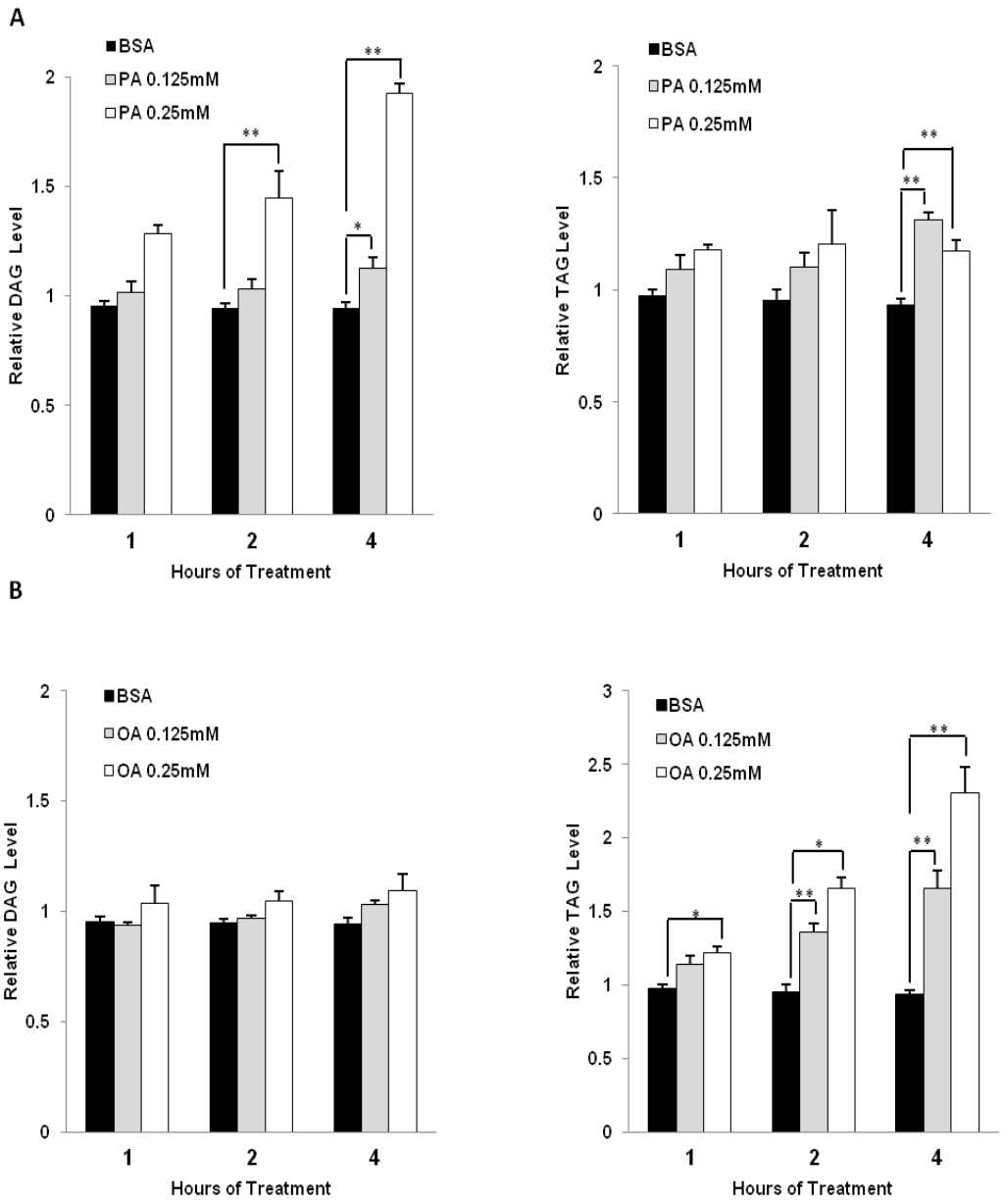
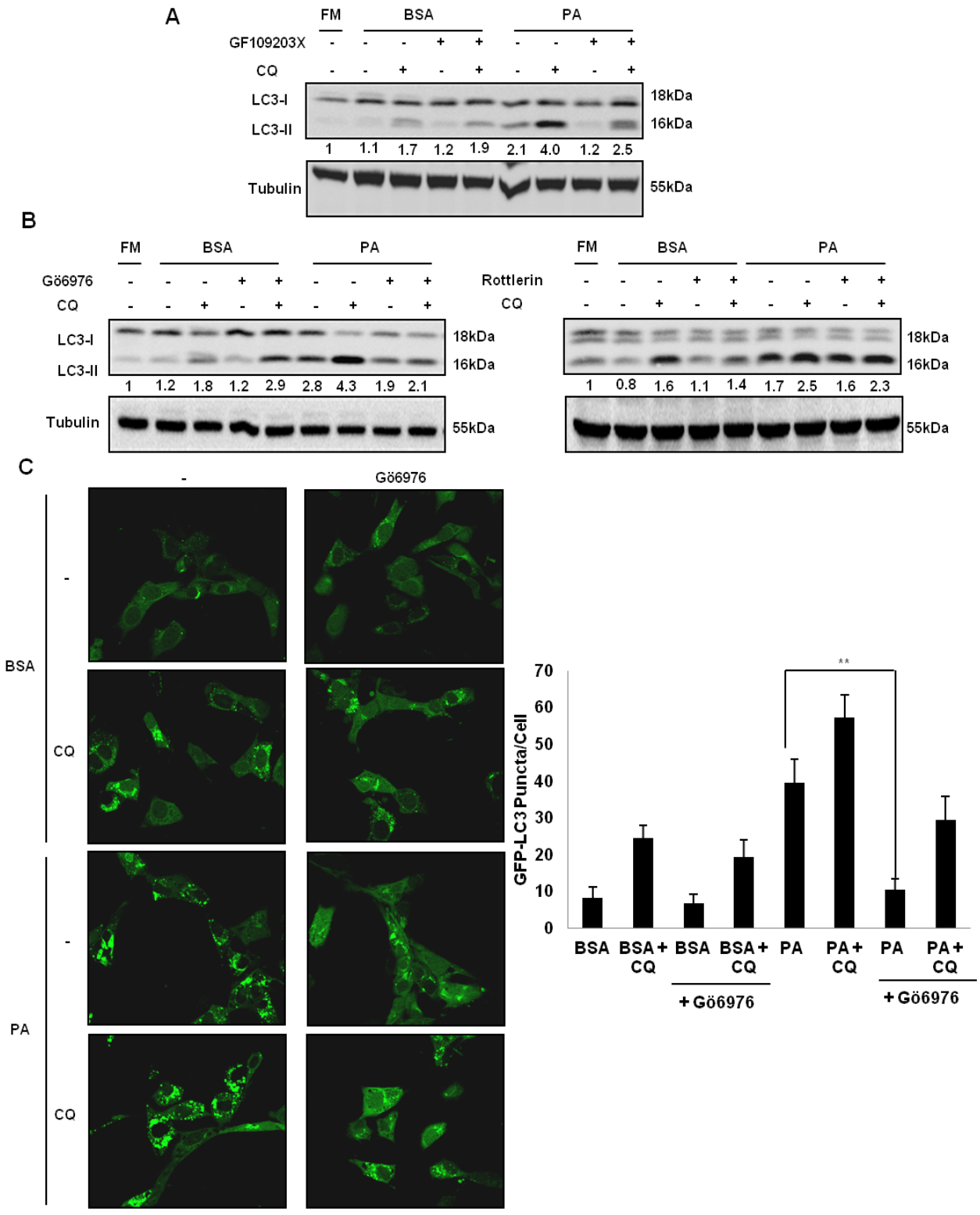


Figure 12. PA and OA treatment induce differential accumulation of intracellular DAG and TAG which is time and dose dependent in HepG2. (A) HepG2 cells were treated with BSA and PA (0.125 mM and 0.25 mM) for 1, 2 and 4 hours. The total DAG (Left panel) and TAG (Right panel) levels were then quantified. The relative DAG and TAG levels were calculated by normalizing their respective levels in each treatment at different time points to the levels present in the control cells. (B) Cells were treated with BSA and OA (0.125 mM and 0.25 mM) for 1, 2 and 4 hours. The total DAG (Left panel) and TAG (Right panel) levels were then quantified. The relative DAG and TAG levels were calculated as described in (A) Data were presented as means ± S.D. of three independent experiments and Student's T-test were calculated between the BSA treated cells and either PA or OA treated cells at each respective time point (*p<0.05, **p<0.01, student's t-test).

3.1.5 Inhibition of Protein Kinase C blocks autophagy induction caused by PA treatment

The results from Figures 11 and 12 suggest strongly that autophagy induction caused by PA treatment could be due to accumulation of intracellular DAG species in the cells Therefore we went on to try to elucidate possible the signaling pathways that could be affected by elevated levels of DAG accumulation in cells. One of the well known signaling pathways in which DAG plays an important role during signal transduction is that of the DAG-PKC signaling, where DAG serves as a natural agonist to recruit PKC proteins to the plasma membrane for activation (Newton, 2001; Takai et al., 1979). As described earlier, there are essentially 3 classes of PKC family comprising a total of 10 PKC isozymes in the mammalian system: classical, novel and atypical PKCs (Newton, 2001; Takai et al., 1979). Amongst these PKC members, some of them have already been described to play a role in autophagy induction downstream of several cellular stresses (Chen et al., 2008; Sakaki and Kaufman, 2008; Sakaki et al., 2008). To investigate whether PKC was involved in PA-induced autophagy, we first made use of different chemical inhibitors that targeted all members of PKCs or specific classes of the PKC family. Firstly, GF109203X, a general PKC inhibitor, was able to markedly reduce LC3-II levels in cells treated with PA with or without the presence of CQ (Fig. 13A). This observation suggested that inhibition of the PKC signaling pathway impairs PA-induced autophagy. Next, we tried to identify which class of PKC family was involved in PA-induced autophagy by testing the effects of two other chemical inhibitors, Gö6976 and Rottlerin, which inhibit the classical and novel classes of PKC respectively. As shown in Figure 13B (Left panel), the classical PKC inhibitor Gö6976 was found to reduce the

LC3-II level in PA-treated cells, similar to the effects observed with the use of GF109203X. In contrast, addition of the novel PKC inhibitor Rottlerin failed to suppress the autophagic flux induced by PA treatment (Fig. 13B, Right panel). We further confirmed the effect of Gö6976 by examining GFP-LC3 puncta formation in the MEFs that were stably expressing GFP-LC3. Addition of Gö6976 prevented the increase in the formation of GFP-LC3 punctas in the PA-treated cells, again suggesting that PA-induced autophagy requires the presence of active classical PKC members (Fig. 13C). Furthermore, we were able to observe a significant decrease in LC3-II conversion and autophagic flux when HepG2 cells were treated with PA in the presence of Gö6976 suggesting that the requirement of classical members of PKC for PA-induced autophagy is not cell type specific (Fig. 13D). All these findings indicate a possibility that members of the classical PKC are implicated in PA-induced autophagy. As the activation of the classical PKC members requires both the presence of calcium ions (Ca^{2+}) and DAG (Newton, 2001; Takai et al., 1979), we then investigated whether PA-induced autophagy is sensitive to the loss of free intracellular $Ca2^{+}$ ions in the cells. To this end, we made use of BAPTA-AM, an intracellular Ca^{2+} ion chelator (Sakaki et al., 2008). As expected, the addition of BAPTA-AM resulted in an overall decrease in autophagic flux observed in the PA-treated cells (Fig. 13E) suggesting that the free intracellular Ca^{2+} ions are involved in PA-induced autophagy. Overall, the results from this part of our study support the notion that the classical group of PKC is implicated in PA-induced autophagy via enhanced intracellular level of DAG and calcium ions.


A
FM
BSA
PA
GF109203X
CQ
LC3-I
LC3-II
18kDa
16kDa
1 1.1 1.7 1.2 1.9 2.1 4.0 1.2 2.5
Tubulin
55kDa
B
Gö6976
Rottlerin
1 1.2 1.8 1.2 2.9 2.8 4.3 1.9 2.1
1 0.8 1.6 1.1 1.4 1.7 2.5 1.6 2.3
C
BSA
PA
GFP-LC3 Puncta/Cell
70
60
50
40
30
20
10
0
**
BSA
BSA + CQ
BSA + CQ
PA
PA + CQ
PA + CQ
+ Gö6976
+ Gö6976

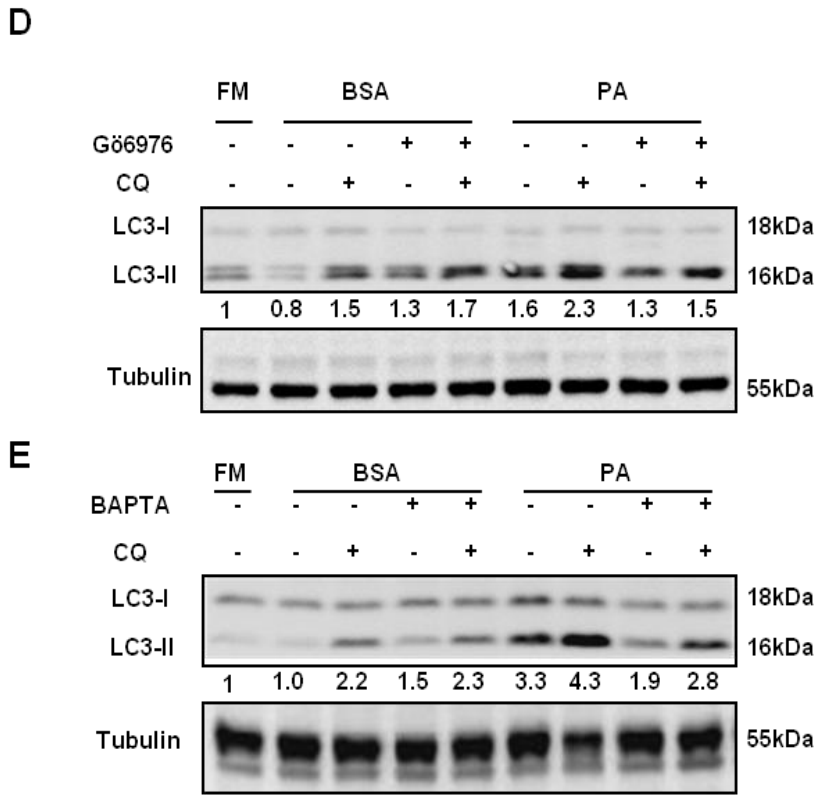


Figure 13. PA-induced autophagy requires the activation of PKC family. (A) MEFs were treated with BSA or PA (0.25 mM) for 4 hours with or without the presence of the general PKC inhibitor GF109203X (1 μM). CQ (10 μM) was added to the treated cells to determine the levels of autophagic flux. (B) MEFs were treated with BSA or PA (0.25 mM) for 4 hours with or without the presence of either the classical PKC inhibitor Gö6976 (1 μM) (Left panel) or the novel PKC inhibitor, Rottlerin (10 μM) (Right panel). CQ (10μM) was added to the treated cells to determine the levels of autophagic flux. (C) *Atg5* Tet-off MEFs stably expressing GFP-LC3 were treated with either BSA or PA (0.25mM) for 4 hours with or without the presence of classical PKC inhibitor Gö6976 (1 μM) and the number of GFP-LC3 puncta/cell were quantified (**$p<0.01$, student's t-test). (D) HepG2 cells were treated with BSA or PA (0.25 mM) for 4 hours with or without the presence of the classical PKC inhibitor Gö6976 (1 μM). CQ (10 μM) was added to the treated cells to determine the levels of autophagic flux. (E) MEFs were treated with BSA control or PA (0.25 mM) for 4 hours with or without the presence of BAPTA-AM (10 μM).

3.1.6 PKC-α is involved in the induction of autophagy in PA-treated cells

After establishing the role of classical PKCs in PA-induced autophagy, we then tried to identify the member of PKC within the classical group that is responsible for autophagy induction in PA-treated cells. There was indeed enhanced phosphorylated PKC-α in PA-treated cells comparing to the BSA-treated cells, while no evident changes in phosphorylation of PKC-α was observed in the OA-treated cells (Fig. 14A). As expected, a similar increase was found in cells treated with 12-*O*-tetradecanoylphorbol-13-acetate (TPA), which is known to activate members of the PKC family including PKC-α (Gschwendt et al., 1991). To further confirm the results obtained above, we further examined the activation of PKC-α during PA-treatment by measuring the phosphorylation levels of tyrosine residues from immuno-precipitated PKC-α protein. As shown in Figure 14B, we observed that PKC-α tyrosine phosphorylation level was significantly increased after PA treatment compared to the BSA treated cells. A similar increase in tyrosine phosphorylation was observed in the TPA-treated cells (Fig. 14B). Consistently, PA has a much stronger effect than OA in enhancing the PKC-α tyrosine phosphorylation level while this effect was comparable to the effect of TPA treatment (Fig. 14B). Finally, we confirmed the involvement of PKC-α in PA-induced autophagy via siRNA knock-down of PKC-α. An increase in autophagic flux was observed in the PA-treated cells that were transfected with the control scrambled siRNA (Fig. 14C). However, we observed that the increase in LC3-II conversion and autophagic flux was inhibited in the MEFs where PKC-α was knocked-down with the PKC-α siRNA (Fig. 14C). There, data presented in this part of our study clearly demonstrates that PKC-α

activation is the upstream signaling pathway that is essential for autophagy induction that is caused by PA stimulation.

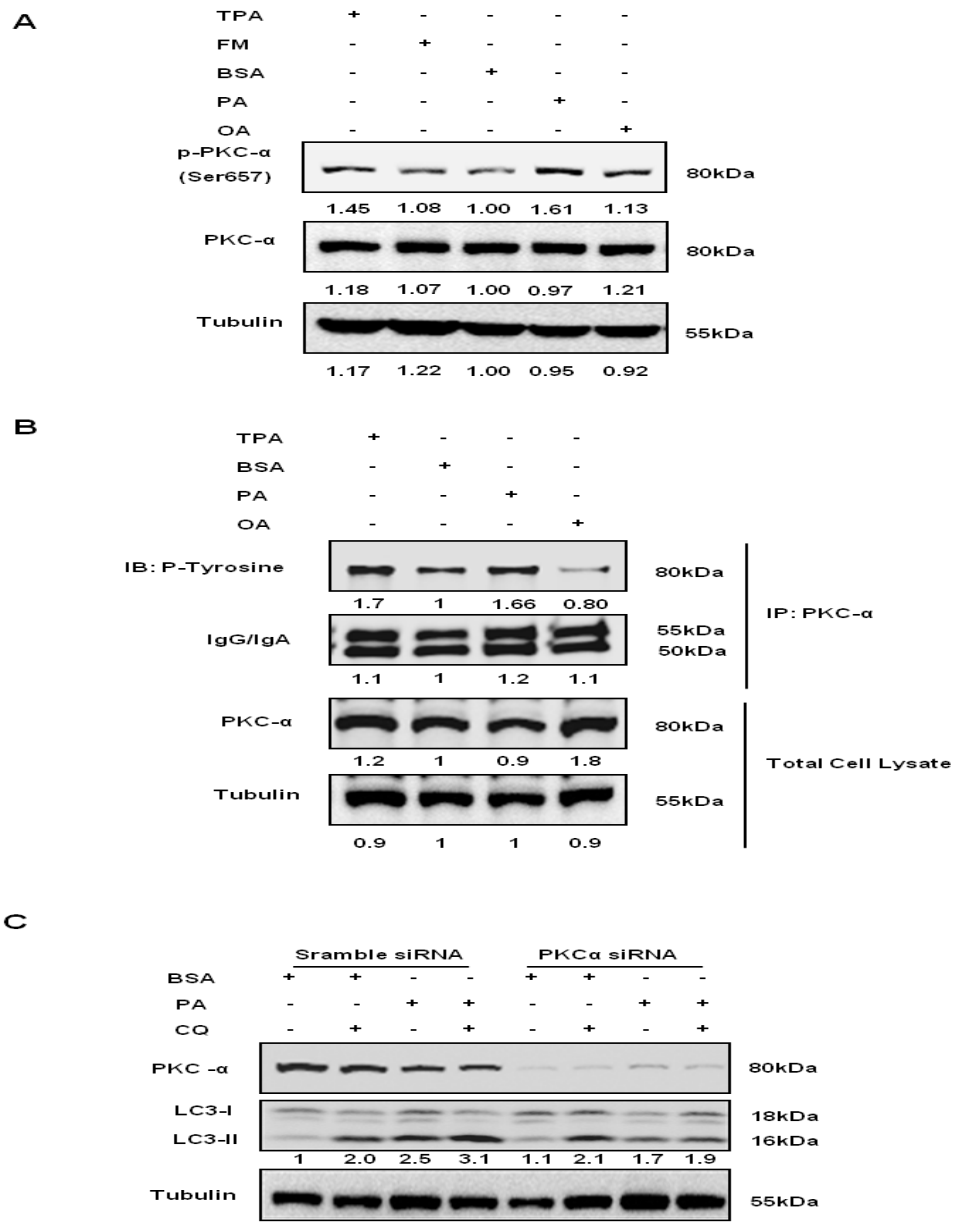


Figure 14. Activation of PKC-α is required for PA-induced autophagy. (A) MEFs were treated with BSA, PA (0.25 mM) or OA (0.25 mM) for 4 hours. Cells treated with TPA (100 nM) for 20 minutes were used as a positive control for activation of PKC-α. The respective lane intensity was quantified using as a fold change to the BSA treatment. (B) MEFs were treated with BSA, PA (0.25 mM) or OA (0.25 mM) for 4 hours and PKC-α was then immunoprecipitated. TPA (100 nM) was added to the cells for 20 minutes as a positive control for PKC- activation. The respective lane intensity was quantified as a fold change to the BSA control treatment. (C) PKC-α in MEFs was knocked-down with PKC-α siRNA while the control cells were transfected with scramble siRNA as described. The cells were then treated with either BSA or PA (0.25 mM) for 4 hours with or without the presence of CQ (10 μM).

3.1.7 DGAT1 knock-down does not affect autophagy levels upon OA treatment

As mentioned earlier, the substrate specificity of DGAT for fatty acyl-CoAs that contain the 18C:1 chain derived from OA compared to other fatty acyl-CoAs that have the 16C:0 chain coming from PA (Coleman and Bell, 1976; Li et al., 2010) could explain why the OA-treated MEFs and HepG2 have a much more elevated levels of intracellular TAG compared to that of DAG (Fig. 11B and 12B); while in cells treated with PA the accumulation of intracellular DAG was more significant than that of the increase in TAG levels (Fig. 11A and 12A). There are 2 isoforms of DGAT enzymes that have been identified in mammalian cells namely DGAT1 and DGAT2 (Coleman and Mashek, 2011). Although both enzymes contribute to TAG synthesis, there may still be functional difference between the 2 since $Dgat1^{-/-}$ mice are resistant to diet induced obesity (Smith et al., 2000) while $Dgat2^{-/-}$ mice dies shortly after birth (Stone et al., 2004). Previous studies have described that knockdown of DGAT1 alone is sufficient to cause an increase in DAG levels while inhibiting the formation of TAG (Listenberger et al., 2003; Listenberger et al., 2001). To further investigate whether DAG accumulation is indeed required for induction of autophagy upon FFAs stimulation, we therefore knocked-down DGAT1 in the MEFs as indicated (Fig. 15A). However, we observed only a very slight but not significant increase in autophagic levels of the cells treated with OA upon DGAT1 knockdown. Such observation thus suggests that both DGAT1 and DGAT2 play an important role in the efficient conversion of DAG to TAG in cells upon stimulation by exogenous source of PA. Additional experiments consisting of both DGAT1 and DGAT2 concurrently will be essential to confirm for the role of both enzymes in TAG formation and autophagic induction during OA and PA treatments in the cells.

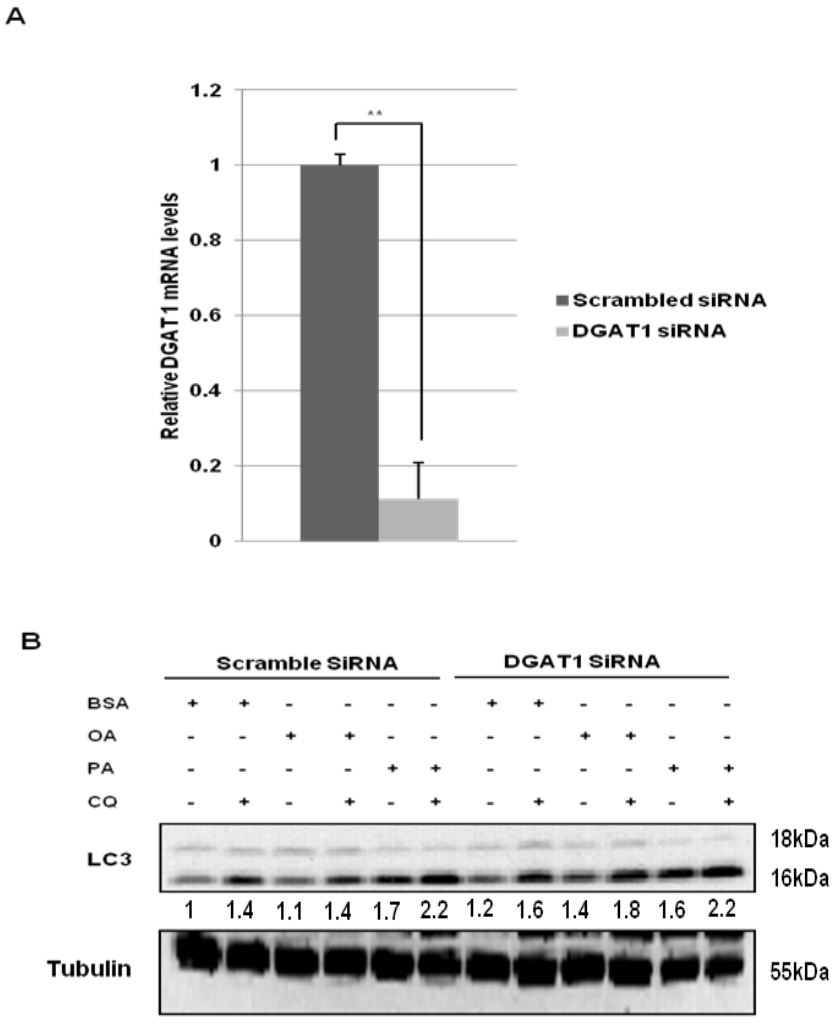
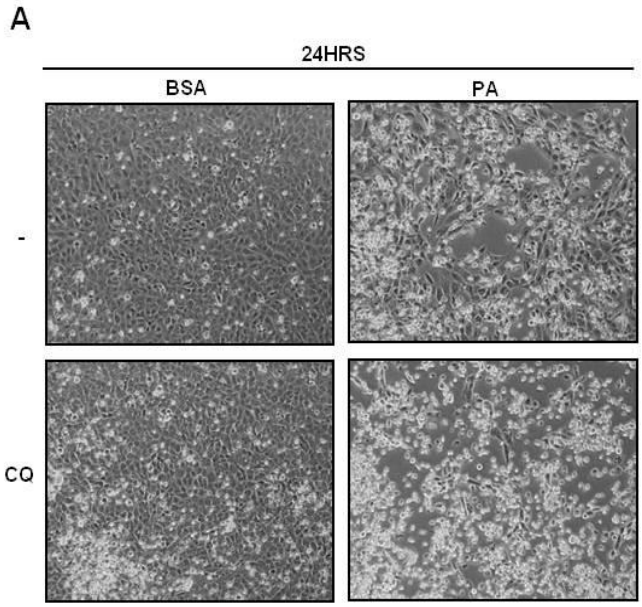


Figure 15. OA treatment does not induce autophagy in DGAT1 knockdown MEFs. (A) DGAT1 was knocked down in MEFS using DGAT-1 siRNA as described. Scrambled siRNA were used as a control for the knock down. Real-Time PCR was then used to determine the DGAT1 mRNA levels in the MEFs treated with scrambled and DGAT-1 siRNA. Relative DGAT1 mRNA levels were obtained by normalizing to the DGAT1 mRNA levels in the MEFs treated with scrambled siRNA (**$p<0.01$, student's t-test). (B) MEFS treated with scrambled siRNA as control or DGAT1 siRNA to knock down DGAT-1 protein were treated with either BSA, PA (0.25 mM) or OA (0.25 mM) for 4 hours. CQ (10 uM) was added to MEFs together with the respective treatments to measure the autophagic flux.

3.1.8 Autophagy induction protects cell against lipotoxic properties of PA

In the next part of the study, we aimed to investigate the physiological relevance of PA-induced autophagy. There have been many studies which have reported that PA is cytotoxic and is able to lead to lipoapoptosis upon chronic exposure to cells (Cunha et al., 2008; Listenberger et al., 2003; Listenberger et al., 2001; Maedler et al., 2001; Malhi et al., 2006; Schaffer, 2003). Using phase-contrast microscopy, we first observed that treatment with PA up to 24 hours resulted in a significant increase in the number of dead cells as compared to the BSA-treated cells (Fig. 16A). This observation was further validated using the propidium iodide live cell exclusion assay coupled with flow cytometry as shown in Figure 16B - C. Notably, we observed that the addition of CQ markedly enhanced cell death induced by PA in both assays (Fig. 16A-C). Furthermore, addition of CQ to the BSA treated cells for up to 24 hours did not induce any significant decrease in cell viability, suggesting that CQ alone is not cytotoxic to the cells (Fig. 16C). Since CQ is known to block autophagy by suppressing the lysosomal function, the observations reported thus indicate that PA-induced autophagy may serve as a pro-survival function to protect against PA-mediated lipotoxicity. To confirm that the above observations were not cell type specific, we repeated the treatments on the HepG2 cells. Indeed, we observed significant increase in cell death of the PA-treated HepG2 cells using both phase-contrast microscopy and PI staining when compared to BSA-treated cells (Fig. 16D-E). Furthermore, addition of CQ to the PA-treated HepG2 cells also resulted in further increase in cell death compared to those treated with PA alone (Fig. 16D-E). Therefore the observations in HepG2 cells are in agreement with our conclusion that PA-induced autophagy may play a cell survival role during PA-induced lipotoxicity.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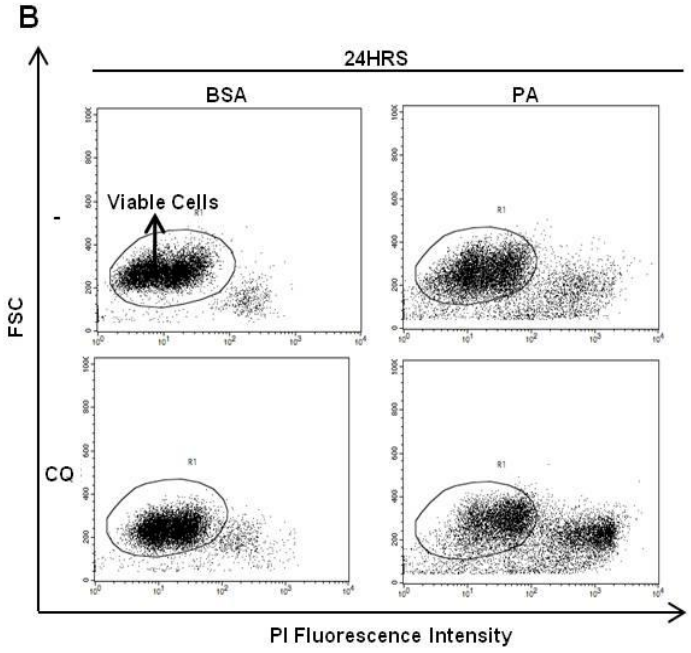
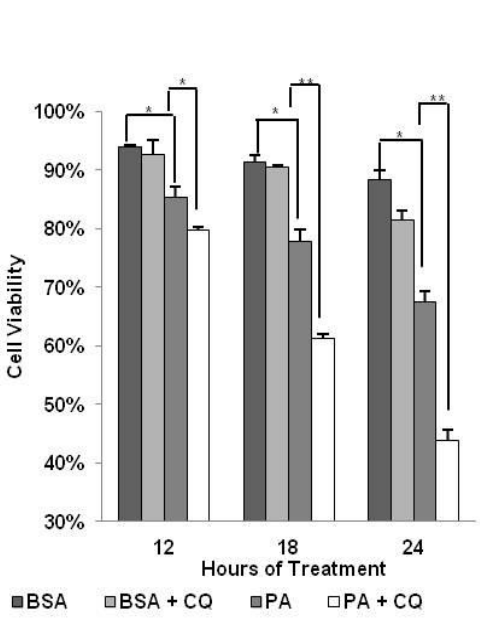
24HRS
BSA
PA
-
CQ


B
24HRS
BSA
PA
Viable Cells
-
CQ
FSC
PI Fluorescence Intensity


C
Cell Viability
100%
90%
80%
70%
60%
50%
40%
30%
12
18
24
Hours of Treatment
BSA
BSA + CQ
PA
PA + CQ

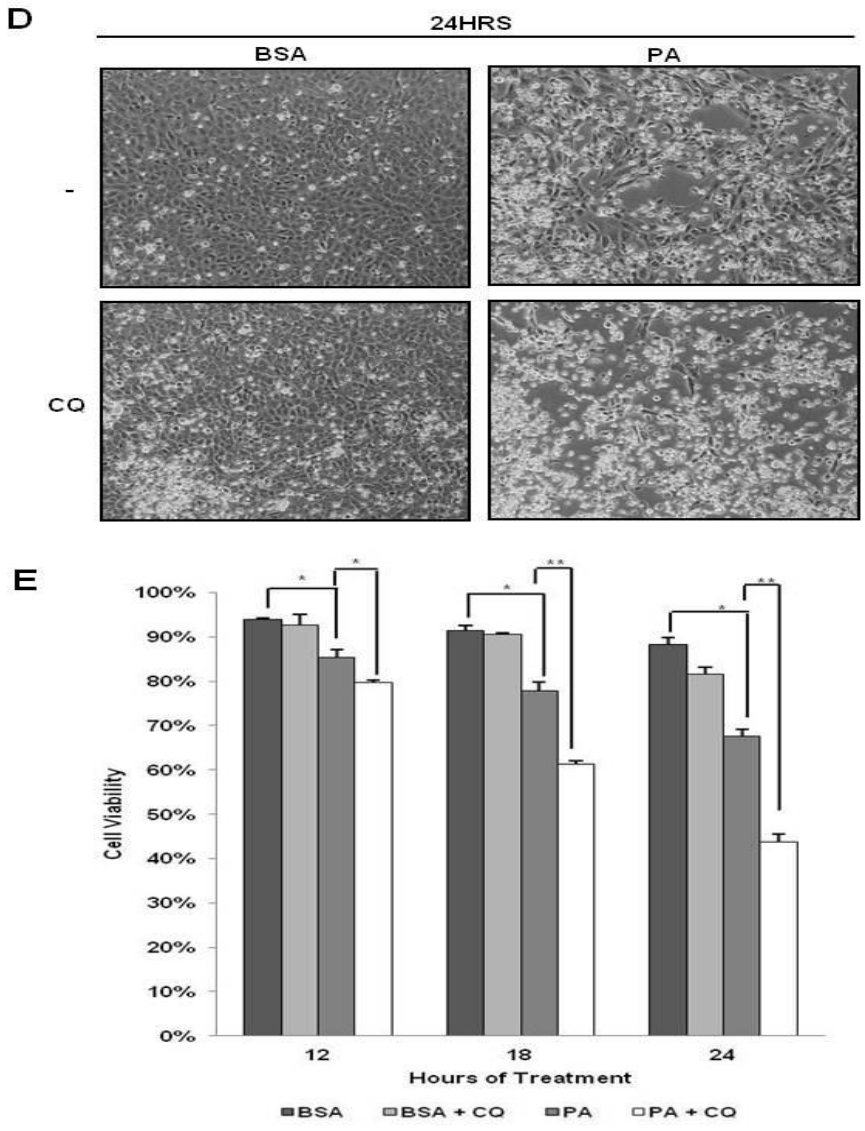
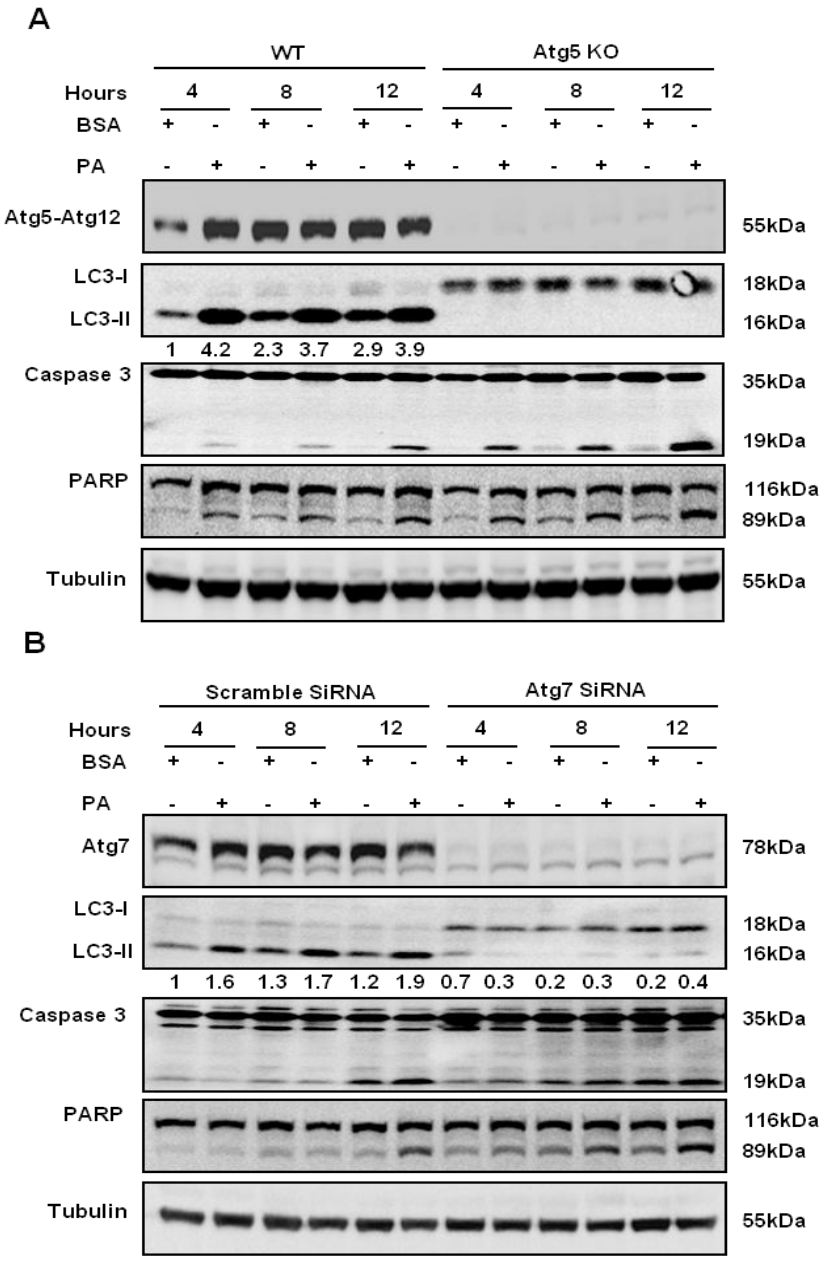


Figure 16. Autophagy acts as a cell survival mechanism against lipotoxicity caused by PA. (A) Cell morphology observed under a phase contrast microscopy (×200) of MEFs treated with either BSA or PA (0.25 mM) for 24 hours with or without the presence of CQ (10 μM). (B) Representative dot-plots of flow cytometry data of propidium iodide exclusion test. MEFs treated as described in (A). (C) Quantification of the cell viability data from panel B. Data were presented as means ± S.D. of three independent experiments. (*p<0.05, **p<0.01, student's t-test). (D) HepG2 cells were treated with either BSA or PA (0.25 mM) for 24 hours with or without the presence of CQ (10 μM) and the cell morphology was observed under a phase contrast microscopy (×200). (E) Cells were treated with BSA or PA (0.25 mM) with or without the addition of CQ (10 μM) for the time points indicated and then subjected to propidium iodide staining. Quantification of the cell viability data are presented as means ± S.D. of three independent experiments. (*p<0.05, **p<0.01, student's t-test).

To further confirm that autophagy plays a cell survival role in response to PA and to investigate the cell death mechanism involved, we compared PA-induced cell death between the WT and *Atg5* KO MEFs. There were significantly higher levels of the classic apoptotic markers, Caspase 3 and PARP cleavage in the PA-treated *Atg5* KO MEFs when compared to the PA-treated WT MEFs (Fig. 17A). The above observation suggested that the cells died via apoptosis upon chronic PA treatment and is in agreement with previous results that autophagy plays an important cell survival role in cells during times of lipotoxic stress. We then confirmed the above results by knocking down Atg7 in the MEFs. As shown in Figure 17B, Atg7 knock-down led to (i) complete suppression of LC3-II conversion induced by PA, and (ii) significant increase of apoptotic cell death, shown by an increase of both the classic apoptotic markers Caspase 3 and PARP cleavage. Similar results were obtained when we repeated the Atg7 knock down experiment on the HepG2 cells where we observed the suppression of LC3-II conversion in the Atg7 knock down cells coupled to the increased cleavage of Caspase 3 and PARP (Fig. 17C).

Since our earlier data establish the role of PKC-α in PA-induced autophagy, we investigated whether PKC-α knock-down would affect the lipotoxicity of PA. It was found that PKC-α knock-down first reduced the LC3-II protein level, being consistent with the earlier findings that the general and classical PKC inhibitors are capable of inhibiting PA-induced autophagy (Fig. 13A-C). More importantly, PKC-α knock-down also markedly enhanced both caspase 3 and PARP cleavage, thus further supporting the pro-survival function of autophagy induced by PA (Fig. 17D). Interestingly, we observed that reduction of the LC3-II level in PKC-α knock-down was not as effective as Atg7 knock-down in suppression of autophagy (LC3-II conversion), while the sensitization on

PA-induced apoptosis (Caspase 3 and PARP cleavage) was similar or even stronger (Fig. 17B and D). Such a discrepancy suggests the possibility that PKC-α may also mediate other pro-survival mechanisms, in addition to autophagy, to protect against PA-induced cytotoxicity.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WT
Atg5 KO
Hours
4
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8
12
BSA
PA
Atg5-Atg12
55kDa
LC3-I
LC3-II
18kDa
16kDa
1 4.2 2.3 3.7 2.9 3.9
Caspase 3
35kDa
19kDa
PARP
116kDa
89kDa
Tubulin
55kDa
B
Scramble SiRNA
Atg7 SiRNA
Hours
4
8
12
4
8
12
BSA
PA
Atg7
78kDa
LC3-I
LC3-II
18kDa
16kDa
1 1.6 1.3 1.7 1.2 1.9 0.7 0.3 0.2 0.3 0.2 0.4
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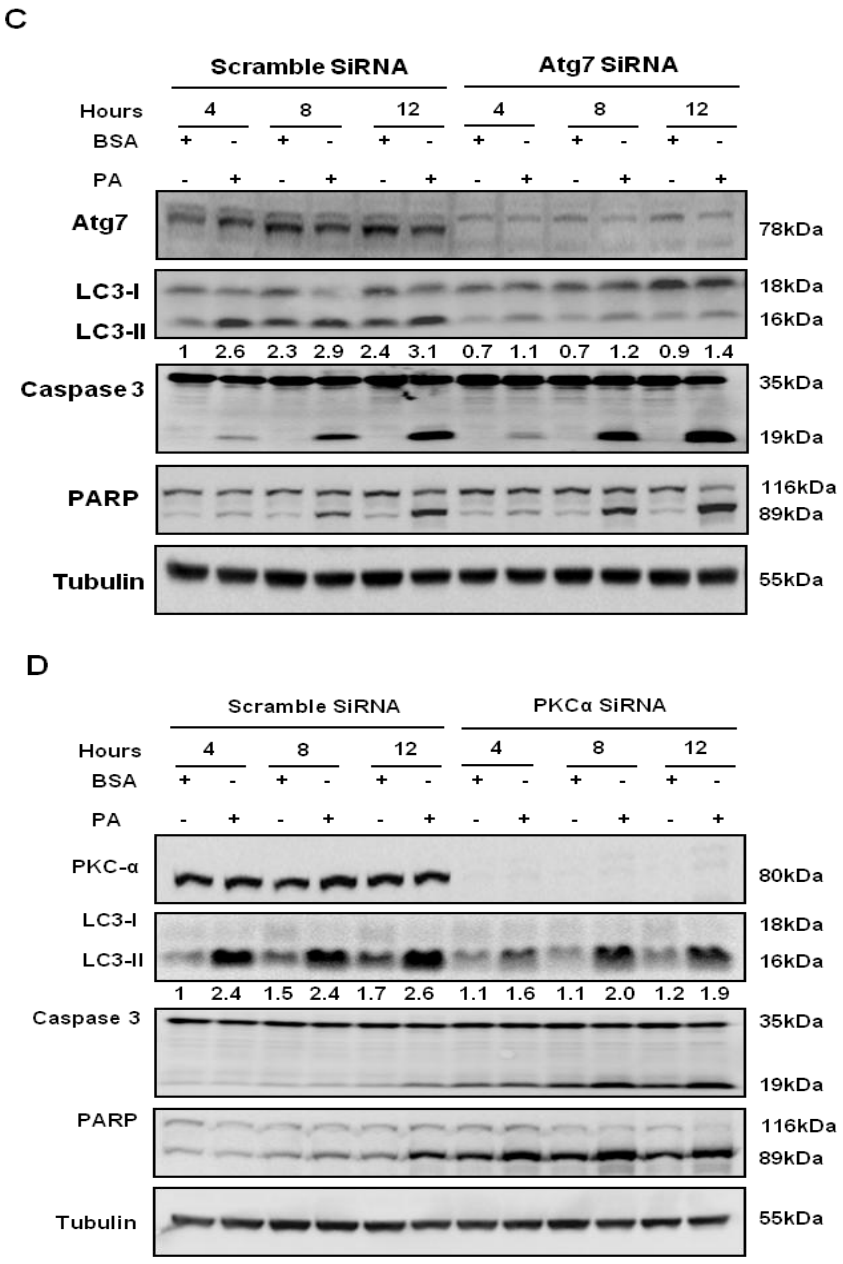


Figure 17. Autophagy acts as a cell survival mechanism against lipoapoptosis induced by PA treatment. (A) WT and *Atg5* KO MEFs were treated with either BSA or PA (0.25 mM) for the indicated time points (B) Atg7 was knocked-down with Atg7 siRNA and the cells were treated with either BSA or PA (0.25 mM) for the indicated time points. (C) Atg7 in HepG2 cells was knocked-down with Atg7 siRNA and the cells were treated with either BSA or PA (0.25 mM) for the indicated time points. (D) PKC-α in MEFs was knocked-down with PKC-α siRNA and the cells were treated with either BSA or PA (0.25 mM) for the indicated time points.

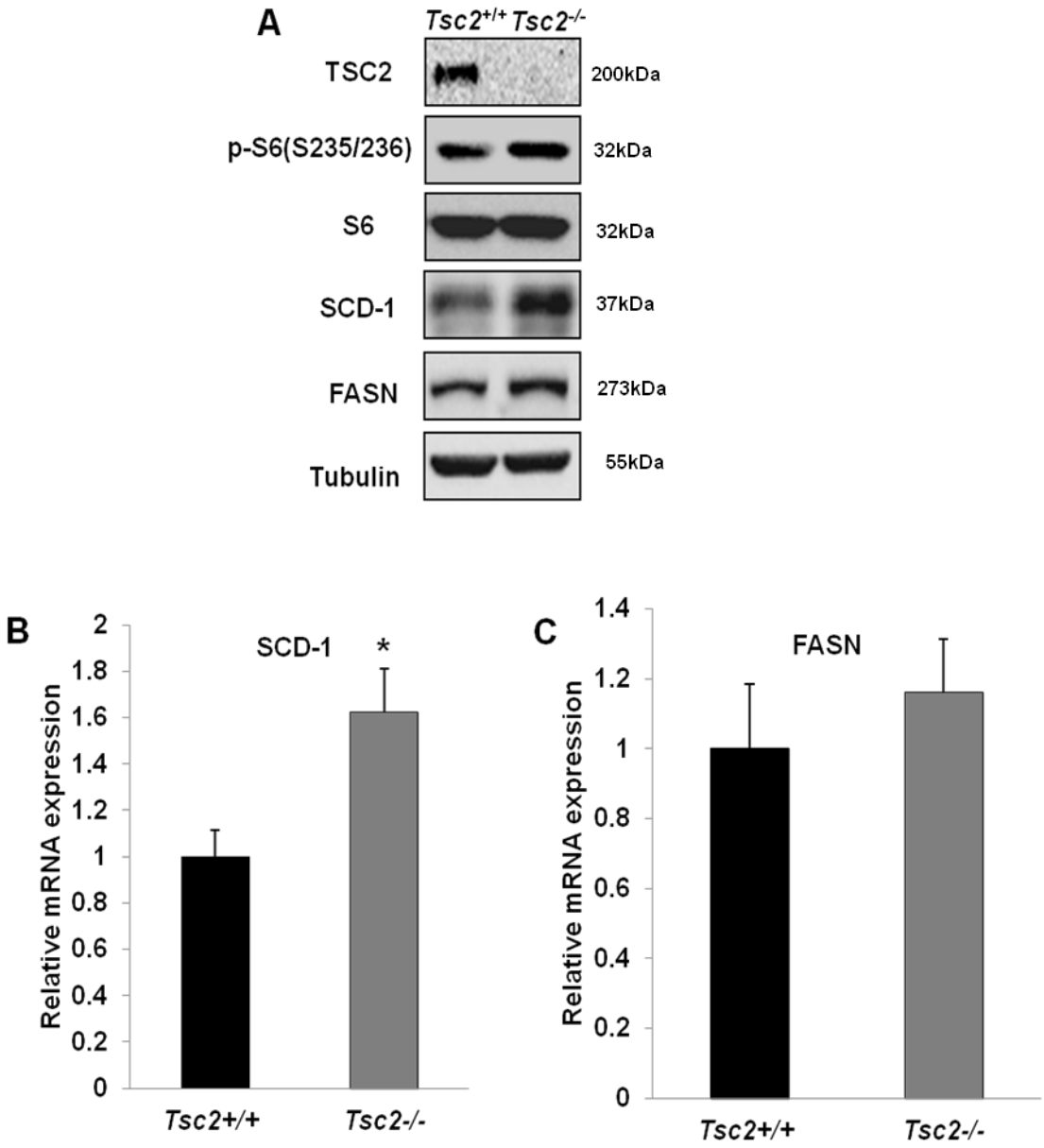
3.2 Critical role of SCD-1 in autophagy regulation via lipogenesis and lipid rafts-coupled Akt-FoxO1 signaling pathway

3.2.1 Elevated levels of lipogenic enzymes and lipid species in the *Tsc2*$^{-/-}$ MEFs

In the first part of the study, we have shown that stimulating cells with a lipotoxic environment through the addition of exogenous SFAs like PA can lead to the induction of autophagy while adding MUFAs such as OA does not affect the autophagic process. Therefore in the next part of the study, we set out to investigate how modulation of the levels of intracellular FFAs can affect the autophagic process. As described, cells deficient in the functional TSC1-TSC2 complex possess a constitutively activated MTORC1 signaling pathway that is independent of growth factors regulation and TSC patients normally develop multiple benign tumors at multiple organs (Bhaskar and Hay, 2007; Tee et al., 2002). A constitutively active MTORC1 signaling pathway has been shown to result in increased *de novo* lipogenesis in the cells via increased expression of lipogenic enzymes like FASN and SCD-1 which regulates the *de novo* synthesis of FAs in cells (Duvel et al., 2010; Peterson et al., 2011; Porstmann et al., 2005; Porstmann et al., 2008). Therefore, we decided to make use of the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs as our cellular model in this part of the study to investigate how alterations in intracellular levels of SFAs and MUFAs can affect the autophagic process in cells with normal versus hyperactivated MTORC1 signaling pathway.

In order to first understand the changes of lipid metabolism downstream of MTORC1, we compared the expression of lipogenic enzymes such as SCD-1 and FASN between the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs. Indeed, the SCD-1 protein level was highly elevated in the

Tsc2$^{-/-}$ MEFs when compared to the *Tsc2*$^{+/+}$ MEFs and a similar trend was observed for FASN, although to a much lesser extent (Fig. 18A). The mRNA levels of SCD-1, but not that of FASN, were also significantly elevated in the *Tsc2*$^{-/-}$ MEFs when compared to the *Tsc2*$^{+/+}$ MEFs (Fig. 18B and 18C, respectively). We then proceeded to compare the levels of different lipid species between the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs after lipid extraction. For the neutral lipids that were measured, the TAG levels in the *Tsc2*$^{-/-}$ MEFs were more than 2 fold higher than that in the *Tsc2*$^{+/+}$ cells (Fig. 18D), while the DAG levels were similar in both cell types (Fig. 18E). On the other hand, the total cholesterol levels in the *Tsc2*$^{-/-}$ MEFs were almost 1.7 fold higher than that in the *Tsc2*$^{+/+}$ cells (Fig. 18F), while no significant difference was observed for the cholesteryl esters (CE) (Fig. 18G). We then went on to measure and compare the levels of glycerophospholipids in the two different cell types. Of all the glycerophospholipids measured, we observed significantly higher levels of phosphatidylcholine (PC), phosphatidylinositol (PI), phosphatidylglycerol (PG) and PE in the *Tsc2*$^{-/-}$ MEFs (Fig. 19A-D, respectively), while a similar trend was found for phosphatidic acid (PhA) and phosphatidylserine (PS), although not statistically significant (Fig. 19E and 19F, respectively). Thus the data from this part of our study suggest that in *Tsc2*$^{-/-}$ MEFs with constitutive activation of MTORC1 there is increased expression of enzymes involved in *de novo* lipogenesis, leading to increased levels of various lipid species. This result is generally in agreement with previous studies that MTORC1 signaling pathway is a positive regulator for genes involved in *de novo* lipogenesis (Duvel et al., 2010; Laplante and Sabatini, 2009; Peterson et al., 2011).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p-S6(S235/236)
32kDa
S6
32kDa
SCD-1
37kDa
FASN
273kDa
Tubulin
55kDa
B
SCD-1
*
Relative mRNA expression
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Tsc2+/+
Tsc2-/-
C
FASN
Relative mRNA expression
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1.2
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Tsc2+/+
Tsc2-/-

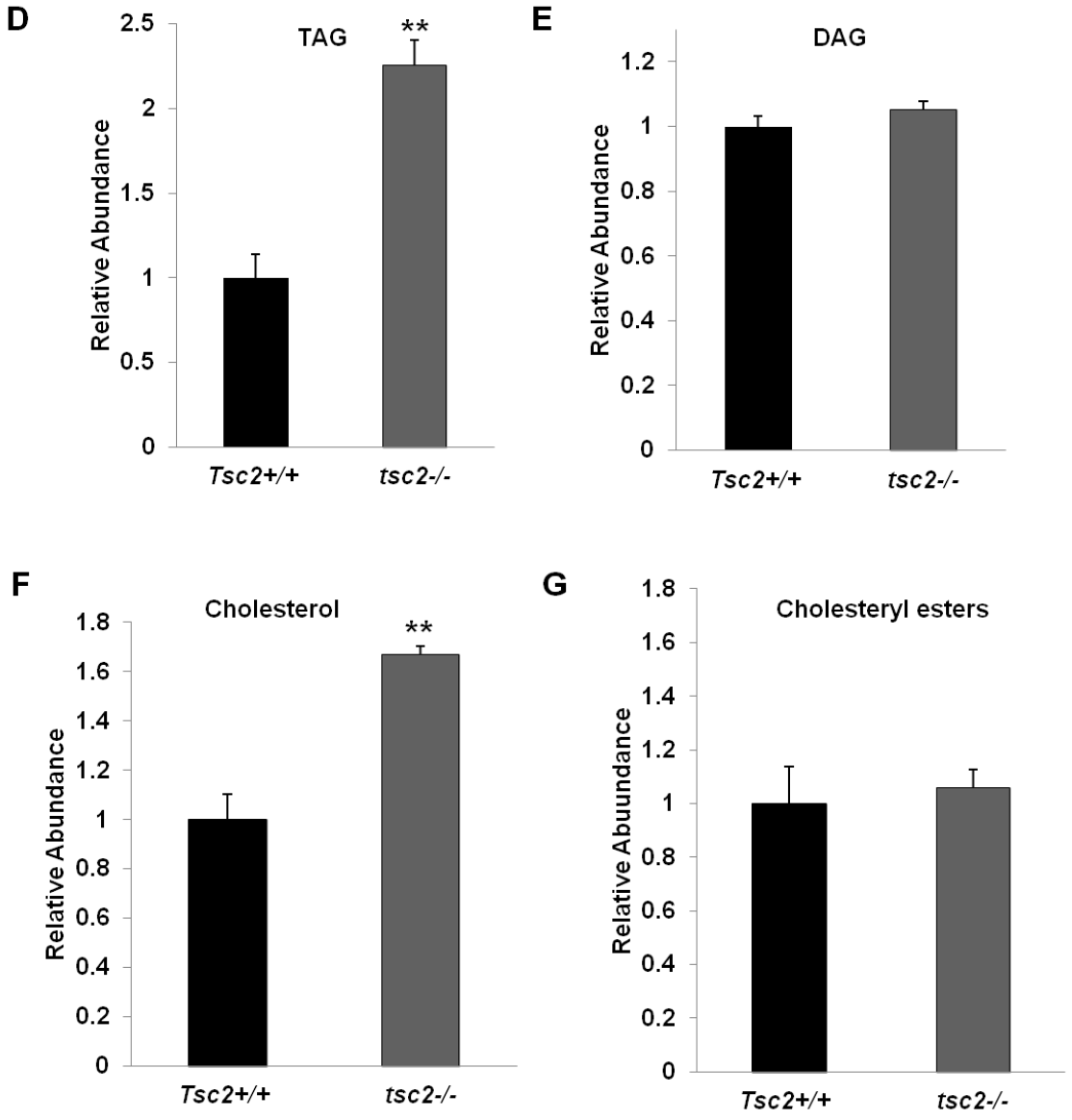
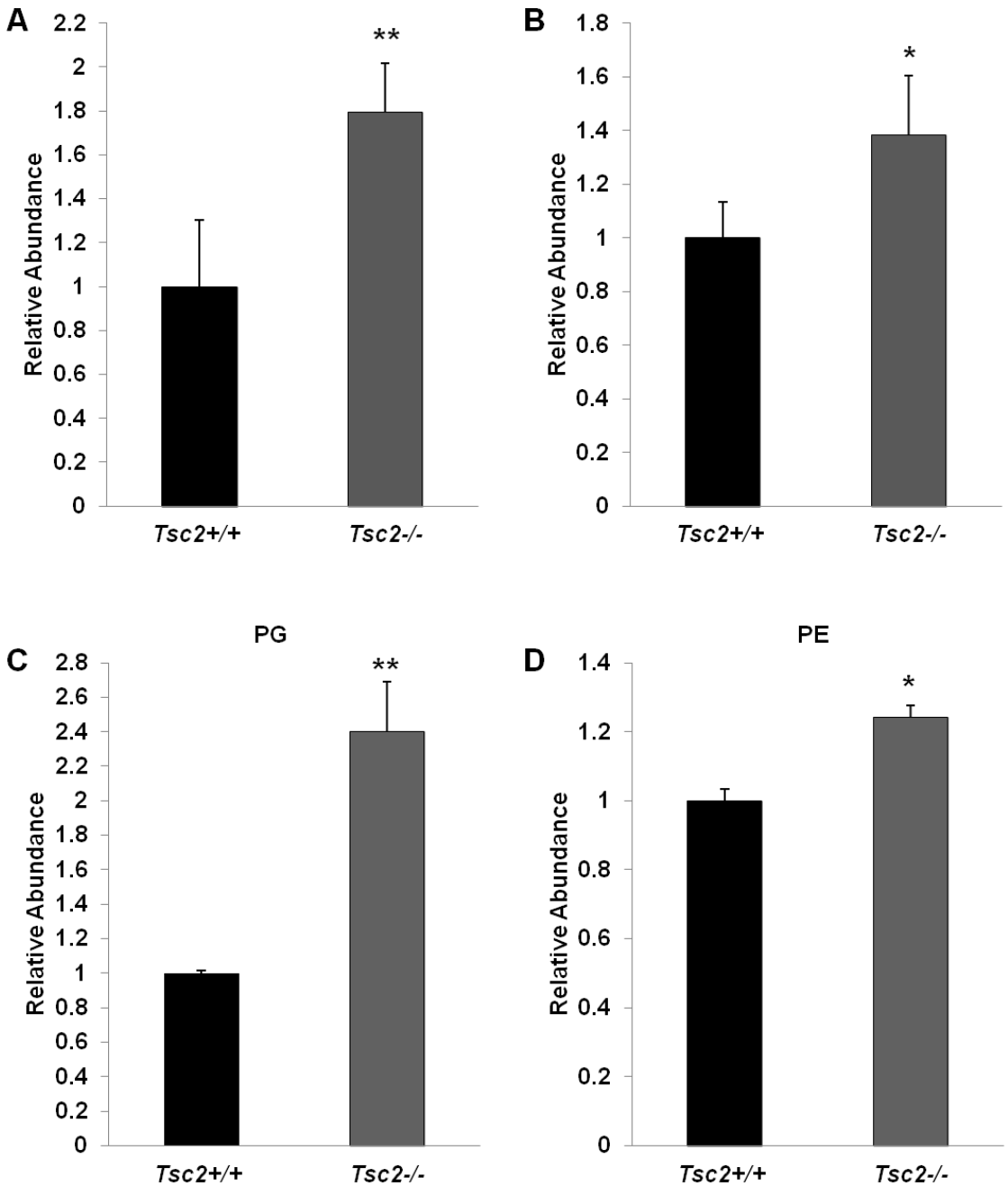


Figure 18: Enzymes involved in *de novo* lipogenesis pathways and neutral lipids are elevated in the *Tsc2*$^{-/-}$ MEFs. (A) Basal levels of FASN and SCD-1 in the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs. (B and C) mRNA levels of (B) *SCD-1* and (C) *FASN* were measured by qRT-PCR and presented as the mean ± SD relative to the levels in *Tsc2*$^{+/+}$ MEFs from three independent experiments (*$p<0.05$, student's t-test). (D-G) Lipid was extracted from the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs and the total levels of (D) triacylglycerol (E) diacylglycerol, (F) cholesterol and (G) cholesterol esters were measured as described and presented as the mean ± SD relative to the levels in *Tsc2*$^{+/+}$ MEFs from three independent experiments (**$p<0.01$, student's t-test).


A
Relative Abundance
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2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
Tsc2+/+
Tsc2-/-
B
Relative Abundance
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
*
Tsc2+/+
Tsc2-/-
PG
C
Relative Abundance
2.8
2.6
2.4
2.2
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
Tsc2+/+
Tsc2-/-
PE
D
Relative Abundance
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1.2
1
0.8
0.6
0.4
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0
*
Tsc2+/+
Tsc2-/-

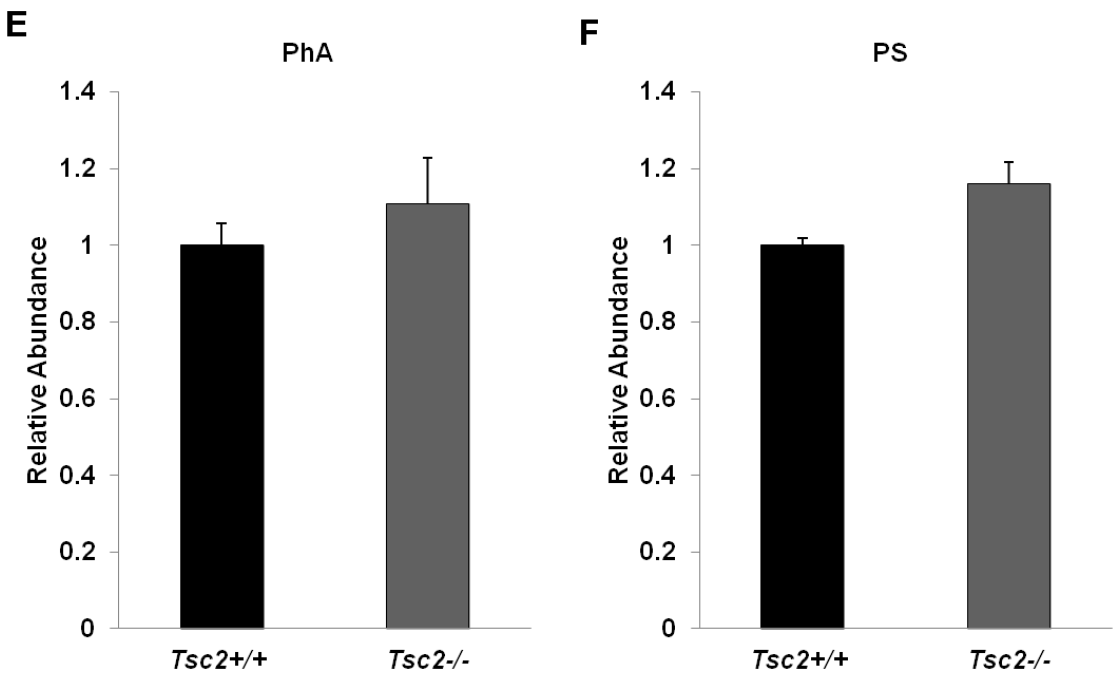
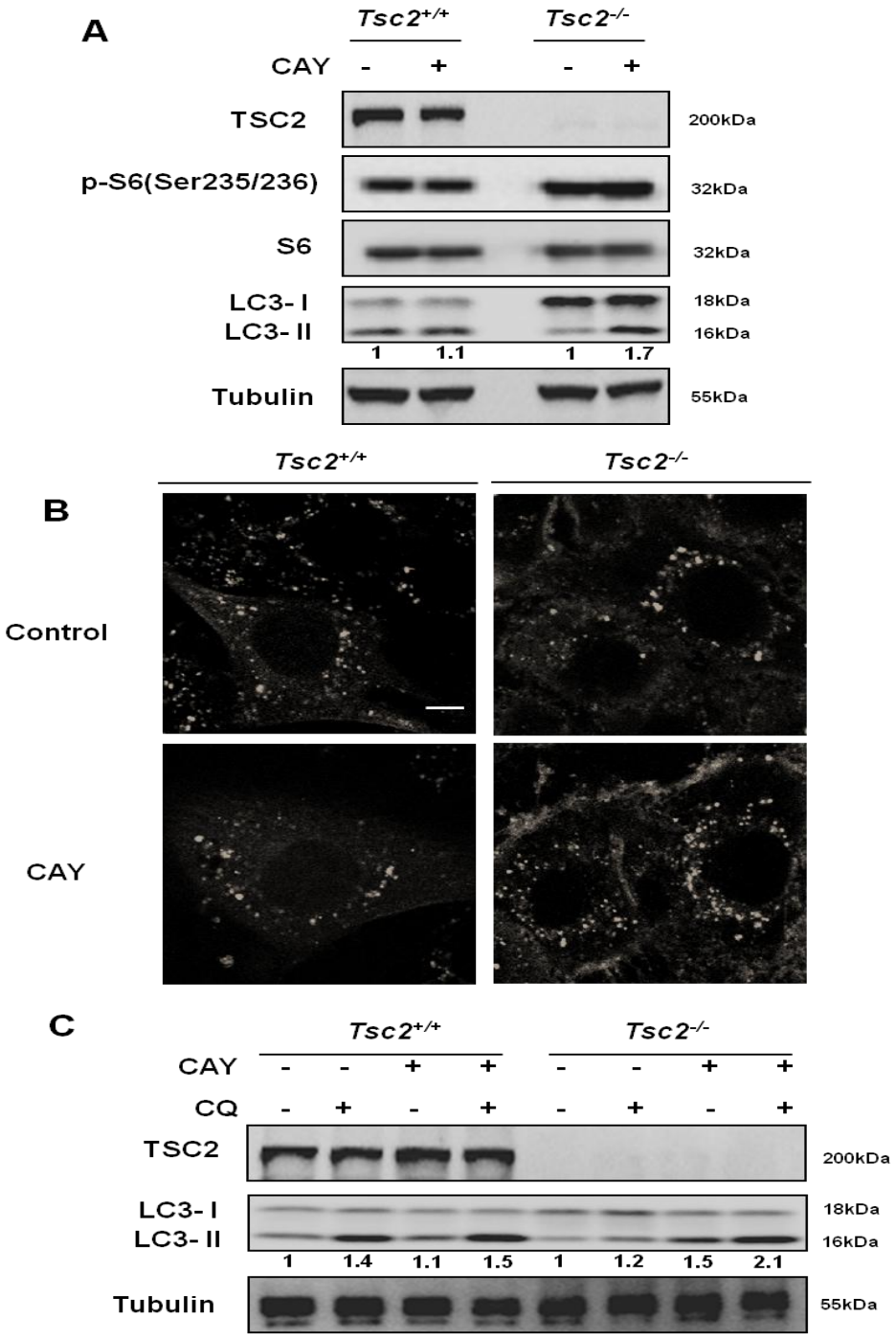


Figure 19: Various groups of glycerophospholipid species are elevated in the *Tsc2*$^{-/-}$ MEFs. Lipid was extracted from the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs and the total levels of (A) PC (B) PI (C) PG (D) PE (E) PhA and (F) PS were measured as described in the experimental procedure and presented as the mean ± SD relative to the levels in *Tsc2*$^{+/+}$ MEFs from three independent experiments (*$p<0.05$, **$p<0.01$, student's t-test).

3.2.2 Inhibition of SCD-1 enzymatic activity leads to induction of autophagy in the *Tsc2*$^{-/-}$ MEFs

Earlier studies have demonstrated that elevated SCD-1 expression and enzymatic activity is important for the proliferation of cancer cells (Minville-Walz et al., 2010; Morgan-Lappe et al., 2007; Scaglia et al., 2009). Furthermore, there is evidence linking loss of SCD-1 activity to the down-regulation of the PI3K-Akt-MTOR signalling pathway in different cancer cells (Fritz et al., 2010; Igal, 2010; Nashed et al., 2012). As the MTORC1 signaling pathway acts as a negative regulator of autophagy, we went on to investigate the effects of inhibition of the enzymatic activity of SCD-1 on autophagy in both *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs. Interestingly, inhibition of SCD-1 activity with a small molecule inhibitor CAY-10566 (CAY) for 24 hours resulted in an increase in LC3-I to LC3-II conversion in the *Tsc2*$^{-/-}$ MEFs, suggesting an increase in autophagic activity (Fig. 20A). This observation was further supported by the increase in the formation of GFP-LC3 puncta in the CAY-treated *Tsc2*$^{-/-}$ MEFs with transient transfection of the GFP-LC3 plasmid (Fig. 20B). We further determined the autophagic flux by using CQ to block autophagosome degradation. As shown in Figure 20C, addition of CQ markedly increased LC3-II conversion in the *Tsc2*$^{-/-}$ MEFs treated with the SCD-1 inhibitor. To show that the increase in autophagic flux translated into an increase in the degradative ability of the autolysosomes, we next used a lysosomal substrate DQ-BSA. Inhibition of SCD-1 with CAY was able to increase the degradation of DQ-BSA in the *Tsc2*$^{-/-}$ MEFs as evidenced by the increase in fluorescence intensity due to the cleavage of the DQ-BSA substrate (Fig. 20D). On the other hand, *Tsc2*$^{-/-}$ MEFs have previously been


A
Tsc2+/+
Tsc2-/-
CAY
-
+
-
+
TSC2
200kDa
p-S6(Ser235/236)
32kDa
S6
32kDa
LC3- I
LC3- II
18kDa
16kDa
1
1.1
1
1.7
Tubulin
55kDa
B
Tsc2+/+
Tsc2-/-
Control
CAY
C
Tsc2+/+
Tsc2-/-
CAY
CQ
TSC2
200kDa
LC3- I
LC3- II
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1.5
2.1
Tubulin
55kDa

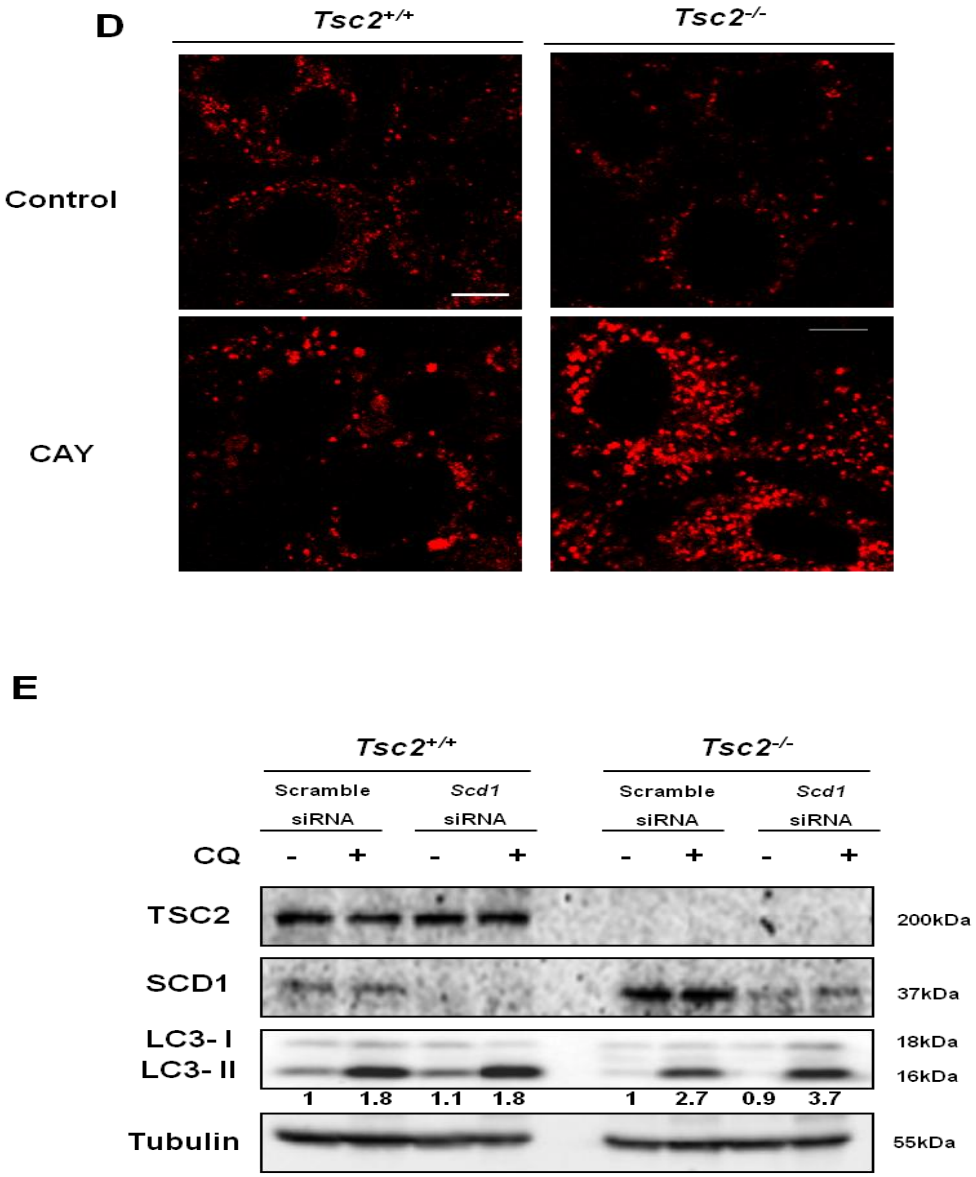
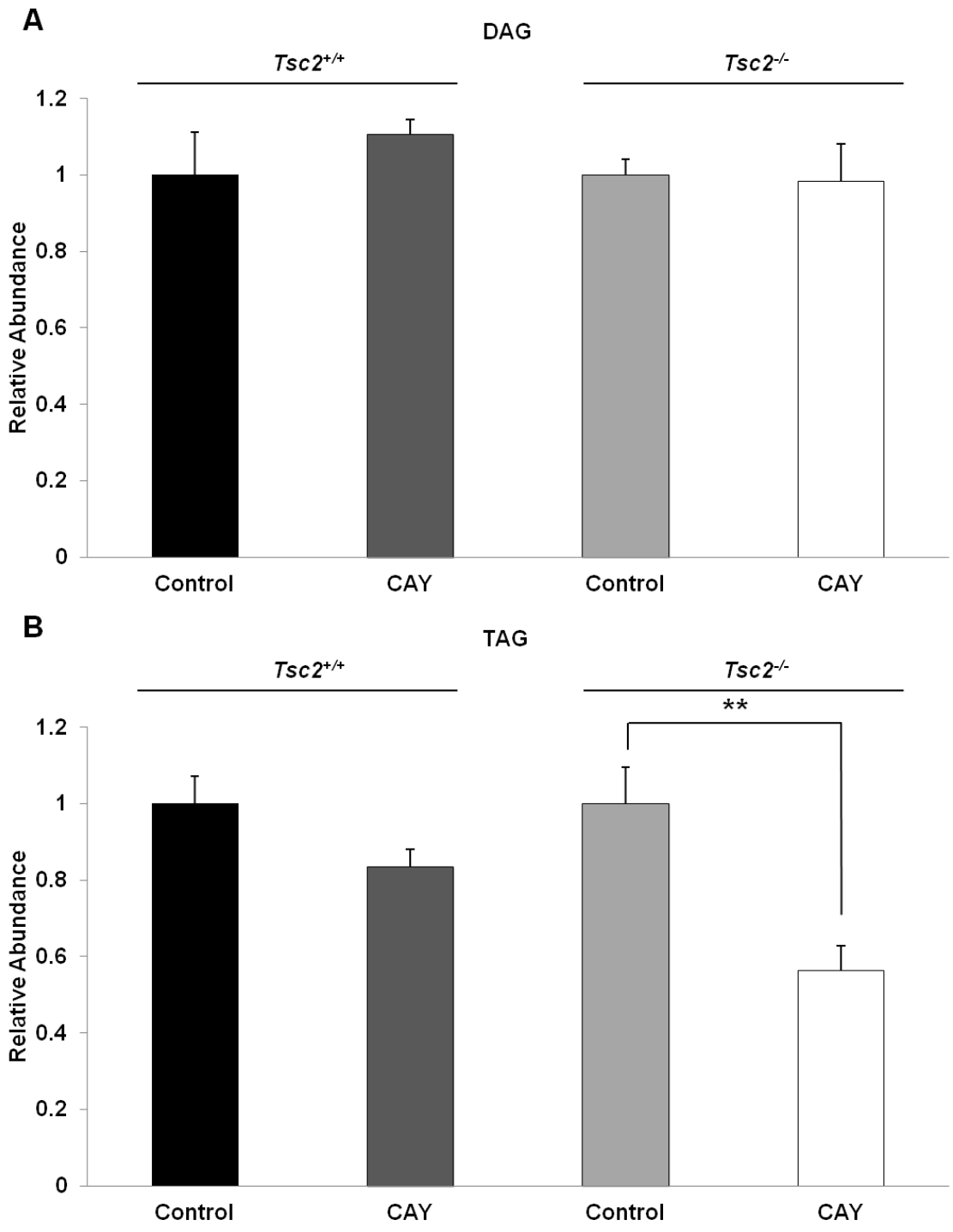


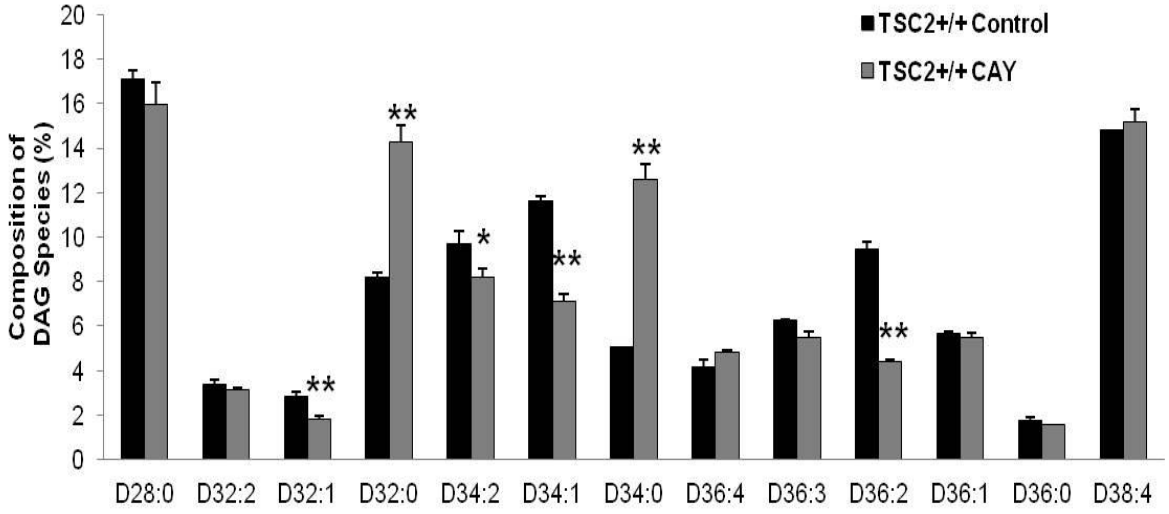
Figure 20: Inhibition of SCD-1 enzymatic activity by CAY induces autophagy that is independent of MTORC1 inhibition in the *Tsc2*$^{-/-}$ MEFs. (A) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 hours and the cell lysates were collected to measure their respective autophagy levels. (B) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were transfected with the GFP-LC3 plasmid and subjected to treatment with CAY (10 µM) for 24 hours. GFP-LC3 punctas formation was then visualized using confocal microscopy and images were taken as described. Scale Bar: 10µm. (C) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) alone, CQ (10 µM) or concurrently with both inhibitors for 24 hours for measurement of autophagic flux. (D) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 hours. DQ^{TM} Red BSA (10 µg/ml) was added for the last hour of treatment and visualized using confocal microscopy. Scale Bar: 10 µm. (E) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were either transfected with scrambled or SCD-1 siRNA as described and the cells were treated with or without CQ (10µM) for 24 hours to determine the levels of autophagic flux.

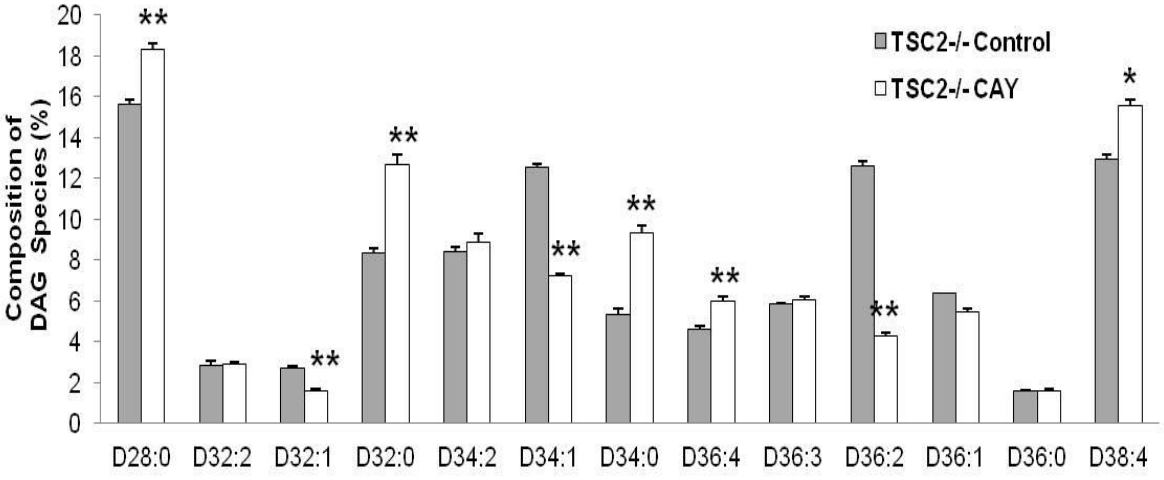
shown to have lower basal levels of autophagy compared to the *Tsc2*$^{+/+}$ MEFs due to its relatively high levels of MTORC1 activity (Ng et al., 2011). Consistently, we observed increased number of GFP-LC3 puncta and higher DQ-BSA fluorescence intensity in the non-treated *Tsc2*$^{+/+}$ MEFs when compared to the corresponding *Tsc2*$^{-/-}$ MEFs (Fig. 20A-D).

In order to confirm the data from the chemical inhibitor, we next transiently knocked down the expression of SCD-1 in both the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs and investigated the changes in autophagic flux. Consistently, knockdown of SCD-1 led to increased autophagic flux only in the *Tsc2*$^{-/-}$ MEFs (Fig. 20E). Interestingly, SCD-1 inhibition did not affect the total DAG levels in both *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs, while total TAG levels were decreased significantly only in the CAY-treated *Tsc2*$^{-/-}$ MEFS when the lipids from the treated cells were extracted and subjected to mass spectrometry analysis (Fig. 21A-B). Further analysis of the compositions of the neutral lipids in cells treated with CAY showed similar decrease in unsaturated fatty acids and increase in saturated fatty acids in both cells (Fig. 21C-D). These results thus suggest that the small molecule inhibitor CAY is equally effective in inhibiting SCD-1 enzymatic activity in both *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs and the differences observed with autophagy induction are not due to differential response of the cells to the SCD-1 inhibitor.


A
DAG
Tsc2+/+
Tsc2-/-
Relative Abundance
1.2
1
0.8
0.6
0.4
0.2
0
Control
CAY
Control
CAY
B
TAG
Tsc2+/+
Tsc2-/-
**
Relative Abundance
1.2
1
0.8
0.6
0.4
0.2
0
Control
CAY
Control
CAY

C


TSC2+/+ Control
TSC2+/+ CAY
Composition of DAG Species (%)
0
2
4
6
8
10
12
14
16
18
20
D28:0
D32:2
D32:1
D32:0
D34:2
D34:1
D34:0
D36:4
D36:3
D36:2
D36:1
D36:0
D38:4
**
*


TSC2-/- Control
TSC2-/- CAY
Composition of DAG Species (%)
0
2
4
6
8
10
12
14
16
18
20
D28:0
D32:2
D32:1
D32:0
D34:2
D34:1
D34:0
D36:4
D36:3
D36:2
D36:1
D36:0
D38:4
**
*

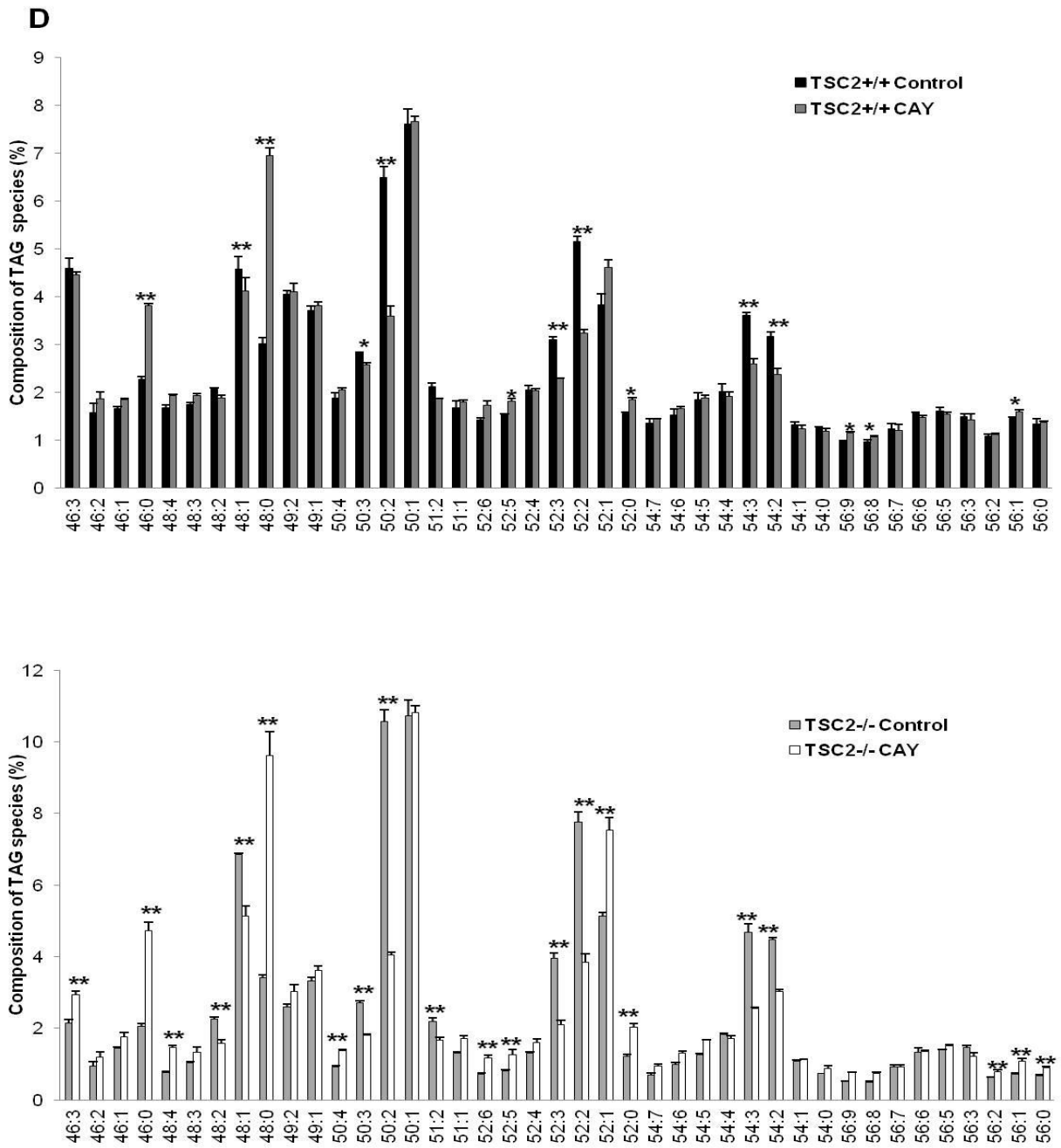


Figure 21: Inhibition of SCD-1 activity by CAY results in decrease of unsaturated fatty acid chains in DAG and TAG. *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 hours. Cells were then subjected to lipid extraction and the (A) total DAG levels and (B) total TAG levels were measured. The same samples were also analyzed for (C) composition of DAG species and (D) the composition of TAG species. Data is presented as the mean ± SD relative to the levels of control cells from three independent experiments (*p<0.05, **p<0.01, student's t-test).

To further verify the involvement of TSC2 in determining the susceptibility to SCD-1 inhibition, we utilized a pair of *Tsc2*$^{-/-}$ MEFs that were stably reconstituted with either an empty vector (+EV) or *TSC2* (+TSC2) (Huang et al., 2008). As expected, the cells reconstituted with TCS2 protein had decreased levels of MTORC1 activity as evidenced by the lower levels of p-S6 compared to the cells reconstituted with the empty vector (Fig. 22A). Furthermore, the expression of SCD-1 protein levels was decreased in the TSC2 reconstituted cells, being consistent with the earlier finding for the higher level of SCD-1 expression in *Tsc2*$^{-/-}$ MEFs (Fig. 20A and Fig. 22A). Consistently, upon treatment with the SCD-1 inhibitor, there was no induction of autophagy observed in the cells reconstituted with TSC2 protein (Fig. 22B). Therefore, data from this part of our study demonstrate that inhibition of the enzymatic activity of SCD-1 is able to selectively induce autophagy in cells deficient of TSC2 protein expression.

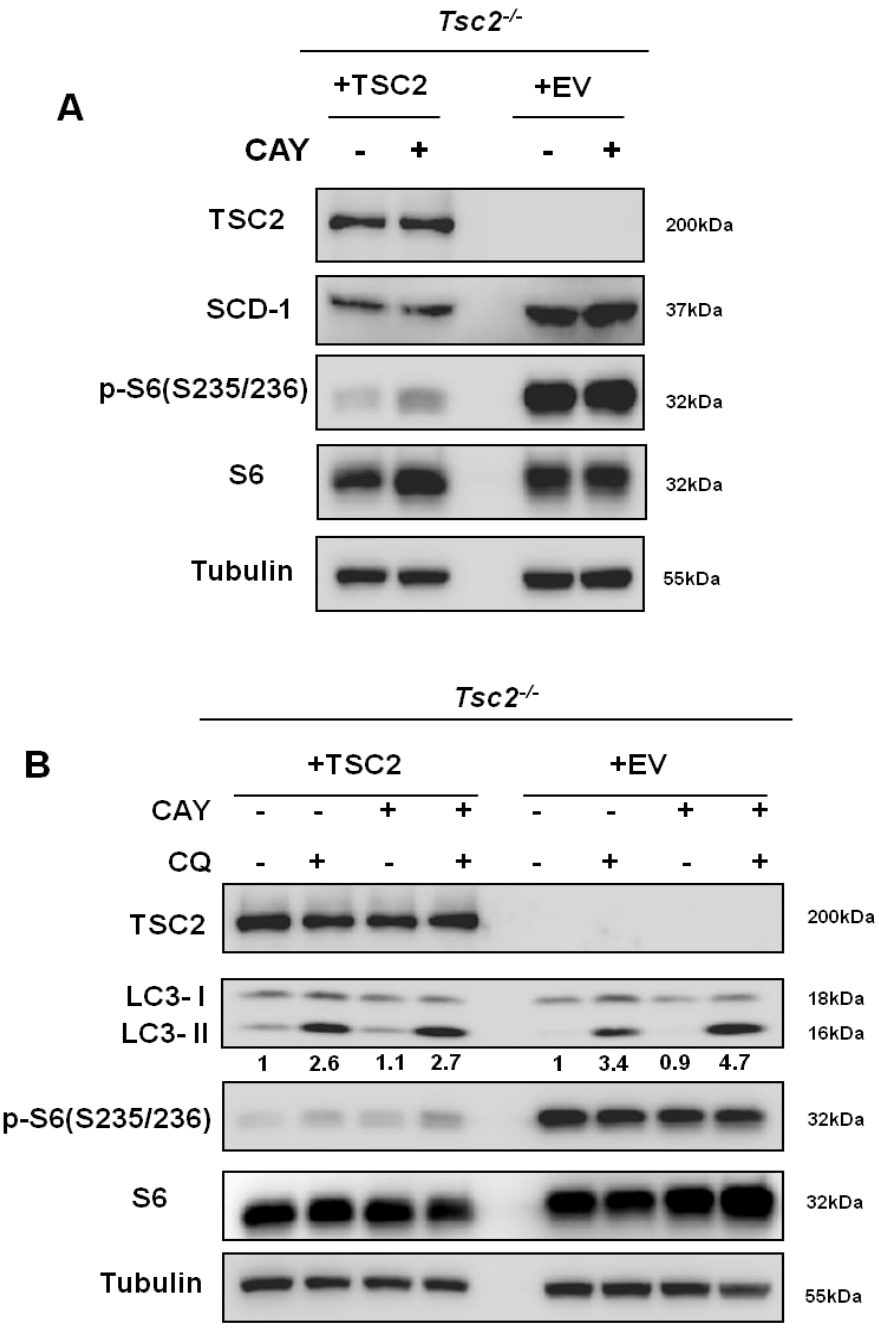


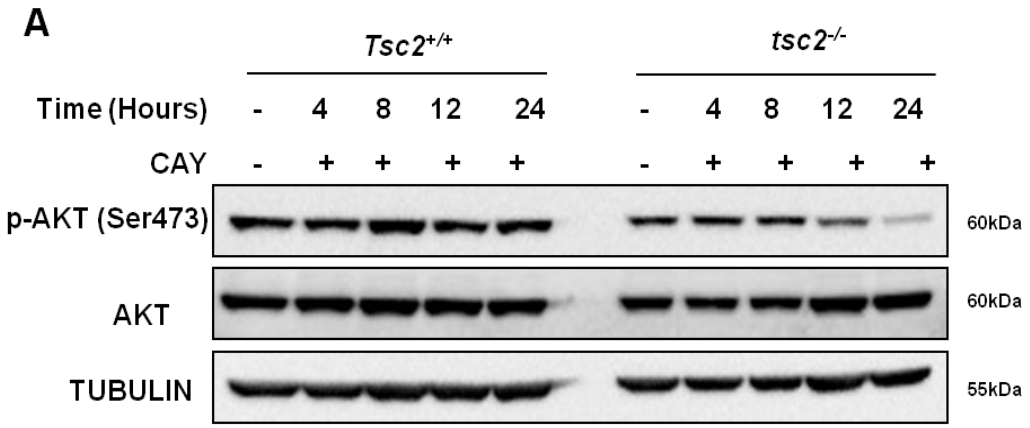
Figure 22: Induction of autophagy by inhibition of SCD-1 enzymatic activity can be rescued with reconstitution of TSC2 protein into *Tsc2*$^{-/-}$ MEFs. (A) *Tsc2*$^{-/-}$ MEFs reconstituted with TSC2 protein (+TSC2) or an empty vector (+EV) were treated with CAY (10 µM) for 24 hours. (B) *Tsc2*$^{-/-}$ MEFs reconstituted with TSC2 protein (+TSC2) or an empty vector (+EV) were treated with CAY (10 µM) alone, CQ (10 µM) or concurrently with both inhibitors for 24 hours for measurement of autophagic flux.

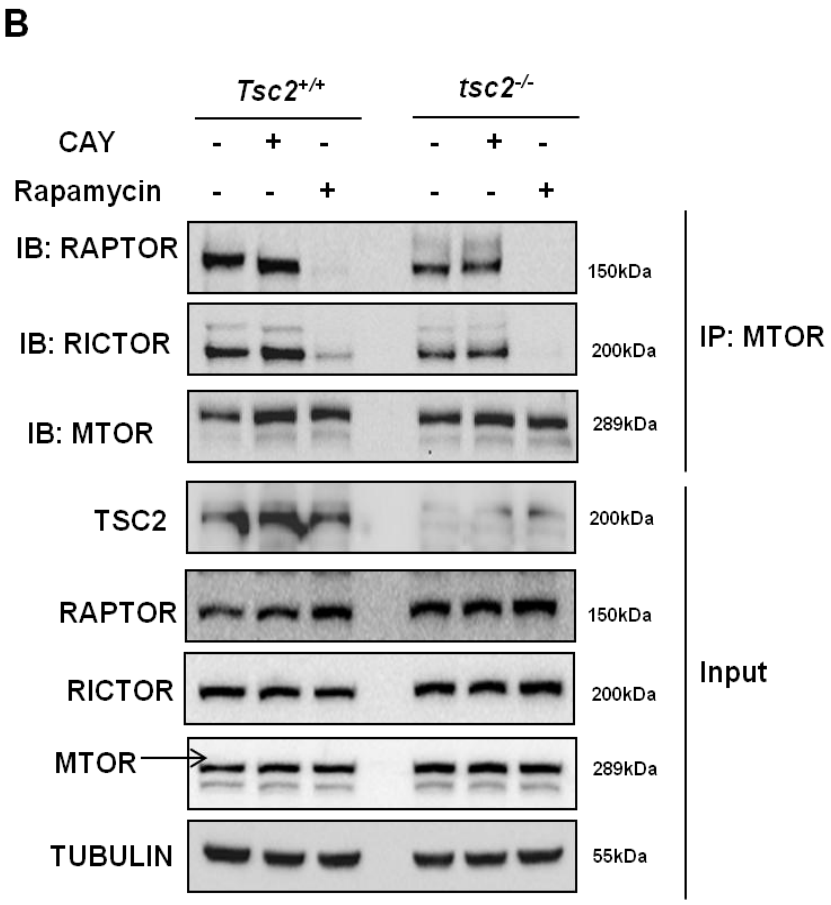
3.2.3 Inhibition of SCD-1 blocks phosphorylation and activation of Akt without affecting MTORC2

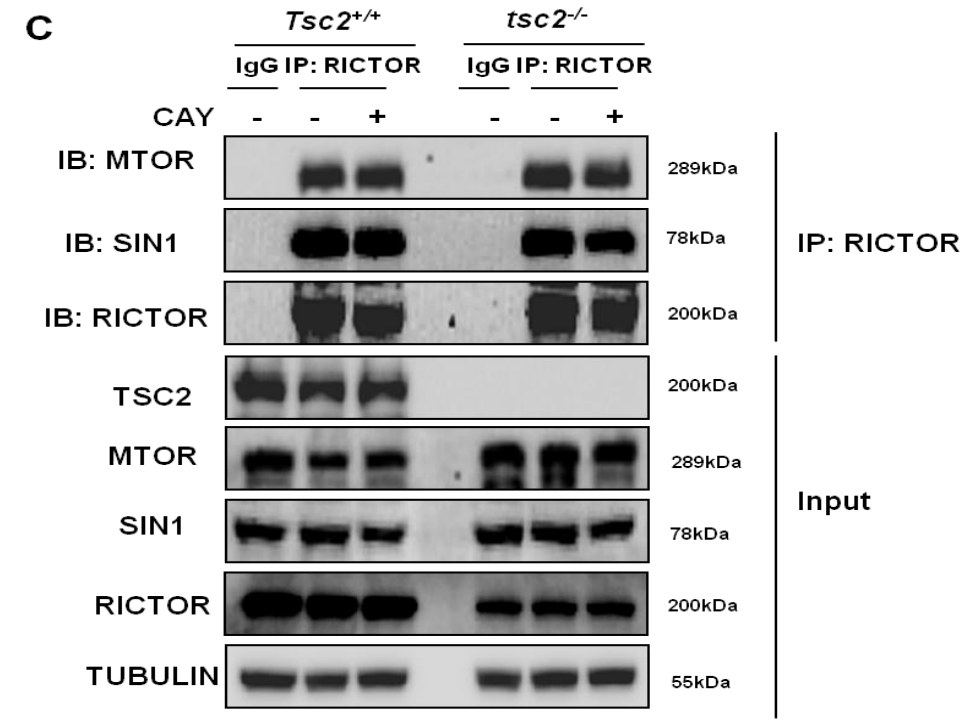
The earlier data showed that CAY failed to change the phosphorylation levels of S6 protein in both *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs (Fig. 21A, Fig. 22A-B), suggesting that autophagy induced by SCD-1 inhibition in the *Tsc2*$^{-/-}$ MEFs was independent of MTORC1 regulation. However, we did observe a time-dependent decrease of phosphorylation of Akt-S473 in the *Tsc2*$^{-/-}$ MEFs, but not in the *Tsc2*$^{+/+}$ cells when SCD-1 activity was inhibited (Fig. 23A). It is known that MTORC2 is the kinase responsible for phosphorylation of Akt-S473 (Sarbassov et al., 2005). Moreover, it was recently reported that disruption of lipid homeostasis in cells could affect the association between MTOR and Rictor, a vital component of MTORC2 and thus inhibit Akt activation (Zhang et al., 2012). Therefore, we examined whether MTORC2 assembly was impaired following inhibition of SCD-1 enzymatic activity. As shown in Figure 23B, the interactions of MTOR with Raptor or Rictor were not affected upon inhibition of SCD-1 with CAY in both *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs, suggesting that both MTORC1 and MTORC2 formation was not affected adversely by SCD-1 inhibition. In contrast, rapamycin markedly reduced the interaction between MTOR and Raptor or Rictor, being consistent with an earlier report that prolonged treatment with rapamycin disrupted both MTORC1 and MTORC2 formation (Sarbassov et al., 2006). To further confirm the effect of SCD-1 inhibition on MTORC2, we detected MTORC2 formation by co-immunoprecipitation with an antibody against Rictor. Similarly, association of Rictor with essential components of MTORC2 including MTOR and Sin-1 proteins were not affected upon inhibition of SCD-1 enzymatic activity in both cell types (Fig. 23C).

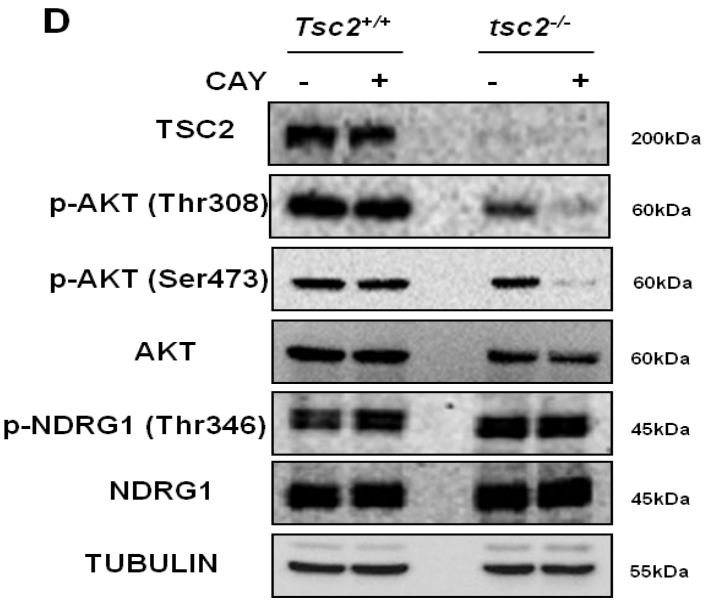
Phosphorylation of N-myc downstream-regulated gene (NDRG) protein at T346 has also been shown to be MTORC2-dependent (Garcia-Martinez and Alessi, 2008). Consistently, CAY did not alter the phosphorylation of T346 on NDRG protein in either the *Tsc2*$^{-/-}$ MEFs (Fig. 23D). Therefore, it is believed that neither the kinase activity of MTORC2 nor the complex assembly was affected by inhibition of SCD-1 enzymatic activity. We further investigated whether phosphorylation of T308 on the Akt protein mediated by PDK-1 was affected by inhibition of SCD-1 enzymatic activity in the *Tsc2*$^{-/-}$ cells. Indeed we observed that CAY caused a loss of phosphorylation at T308 on Akt protein in the *Tsc2*$^{-/-}$ MEFs, but not in the *Tsc2*$^{+/+}$ cells, being consistent with the trends that were observed in the changes of phosphorylation of Akt-S473 (Fig. 23D). To further confirm the effects of loss of SCD-1 activity on Akt activation, we investigated the phosphorylation on Akt with SCD-1 knockdown using siRNA. In agreement with the results from the chemical inhibitor, loss of SCD-1 resulted in decrease of phosphorylation at both S308 and T473 sites on Akt protein only in the *Tsc2*$^{-/-}$ MEFs (Fig. 23E). To further confirm the above conclusions, we then tried to inhibit SCD-1 activity in the cells reconstituted with TSC2 protein to verify the effects of SCD-1 inhibition on MTORC2 formation. Consistently, we also observed that treatment with CAY did not affect the formation of MTORC2 in the cells reconstituted with TSC2 protein (Fig. 24A-B). Furthermore, CAY treatment also did not affect the phosphorylation of NDRG protein on T346 in the TCS2 reconstituted MEFs (Fig. 24C). Notably, TSC2 protein reconstitution was able to prevent the loss of phosphorylation on the T308 and S473 sites of the Akt protein by SCD-1 inhibition (Fig. 24C). These observations thus help to confirm that that

inhibition of Akt protein phosphorylation occurred only in cells deficient for TSC2 protein expression.


A
Tsc2+/+
tsc2-/-
Time (Hours)
- 4 8 12 24 - 4 8 12 24
CAY
- + + + + - + + + +
p-AKT (Ser473)
60kDa
AKT
60kDa
TUBULIN
55kDa


B
Tsc2+/+
tsc2-/-
CAY
- + - - + -
Rapamycin
- - + - - +
IB: RAPTOR
150kDa
IB: RICTOR
200kDa
IB: MTOR
289kDa
IP: MTOR
TSC2
200kDa
RAPTOR
150kDa
RICTOR
200kDa
MTOR
289kDa
TUBULIN
55kDa
Input


C
Tsc2+/+
tsc2-/-
IgG
IP: RICTOR
IgG
IP: RICTOR
CAY - - + - - +
IB: MTOR
289kDa
IB: SIN1
78kDa
IP: RICTOR
IB: RICTOR
200kDa
TSC2
200kDa
MTOR
289kDa
Input
SIN1
78kDa
RICTOR
200kDa
TUBULIN
55kDa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tsc2-/-
CAY - + - +
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200kDa
p-AKT (Thr308)
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60kDa
AKT
60kDa
p-NDRG1 (Thr346)
45kDa
NDRG1
45kDa
TUBULIN
55kDa

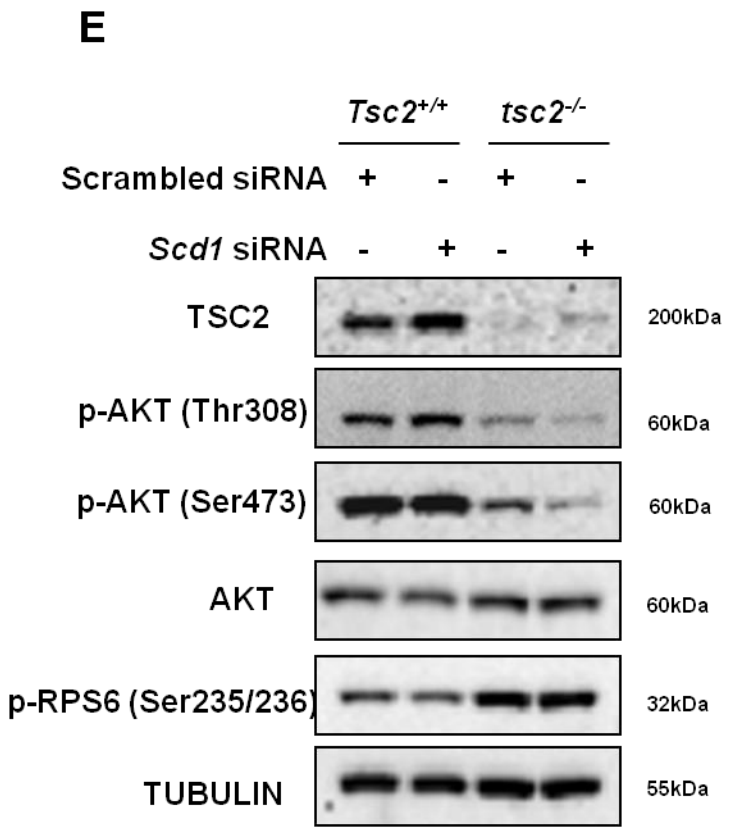
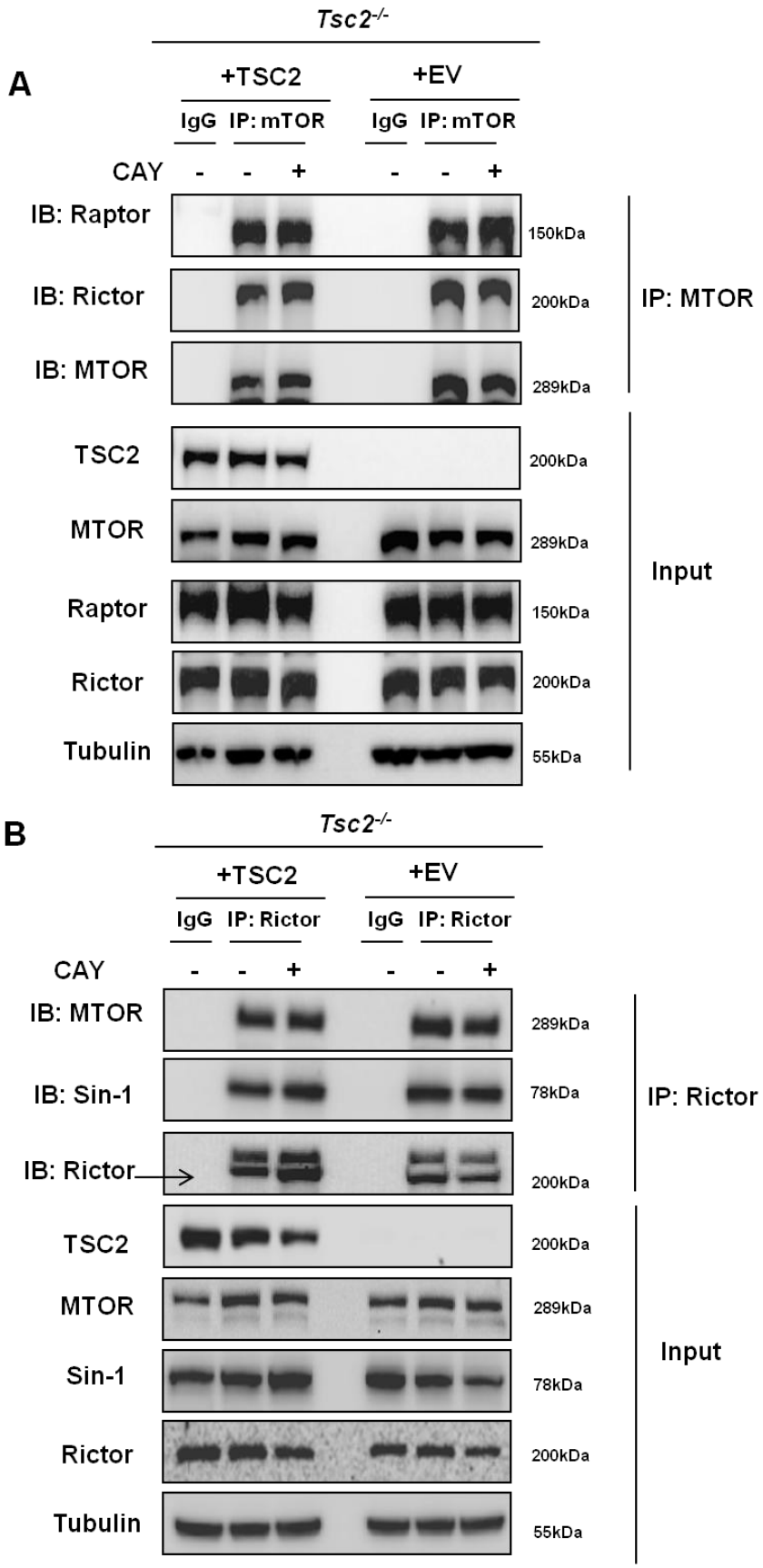


Figure 23: Inhibition of SCD-1 enzymatic activity does not affect MTORC2 formation and activity but inhibits Akt protein activation. (A) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for the different time points as indicated and cell lysates were collected for immunoblot analysis. (B) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) or rapamycin (100 nM) for 24 hours. Co-immunoprecipitation using an anti-MTOR antibody was then carried out as described. (C) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 hours. Co-immunoprecipitation using an Anti-Rictor antibody was then carried out carried out as described. (D) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 hours and the cell lysates were collected for immunoblot analysis. (E) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were either transfected with scrambled or SCD-1 siRNA as described before the cells were collected for analysis using immunoblotting.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Tsc2-/-
+TSC2
+EV
IgG
IP: mTOR
IgG
IP: mTOR
CAY
- - + - - +
IB: Raptor
IB: Rictor
IB: MTOR
150kDa
200kDa
289kDa
IP: MTOR
TSC2
MTOR
Raptor
Rictor
Tubulin
200kDa
289kDa
150kDa
200kDa
55kDa
Input
B
Tsc2-/-
+TSC2
+EV
IgG
IP: Rictor
IgG
IP: Rictor
CAY
- - + - - +
IB: MTOR
IB: Sin-1
IB: Rictor
289kDa
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Tubulin
200kDa
289kDa
78kDa
200kDa
55kDa
Input

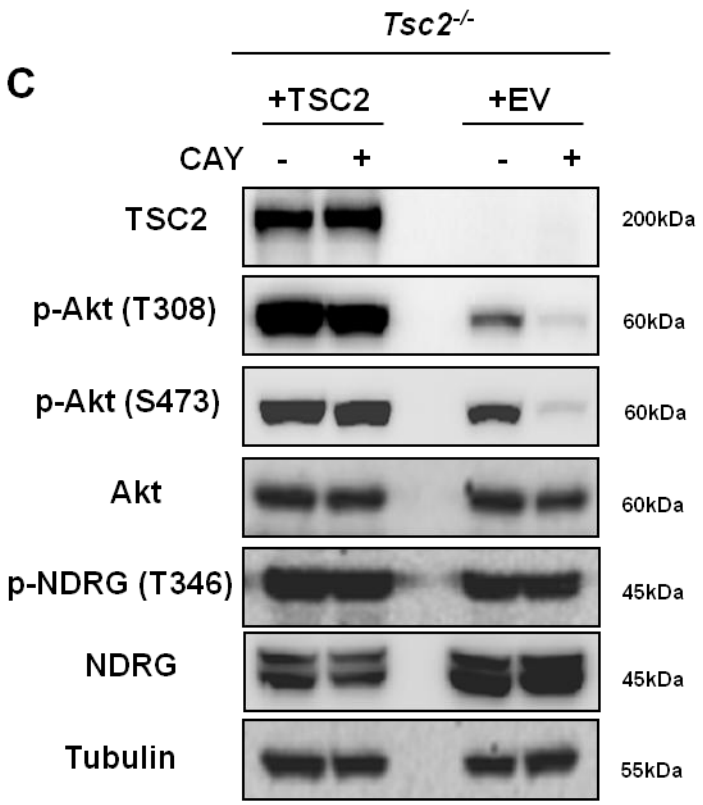


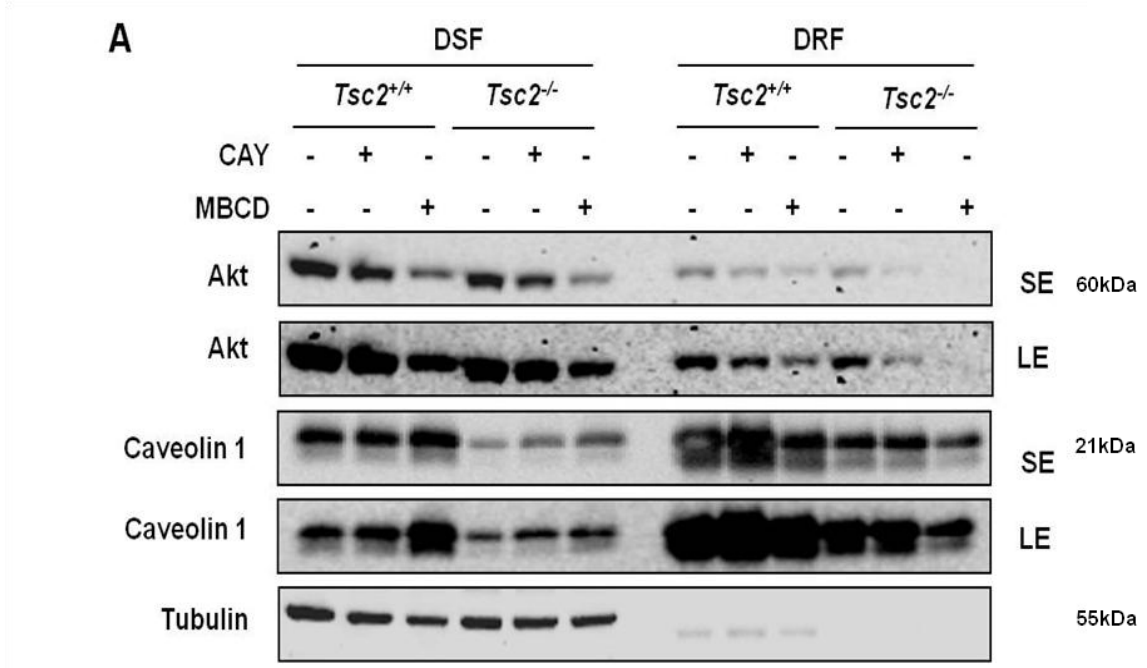
Figure 24: Inhibition of Akt protein activation in the *Tsc2*$^{-/-}$ MEFs can be rescued by the reconstitution of TSC2 protein into the *Tsc2*$^{-/-}$ MEFs. (A) Reconstituted *Tsc2*$^{-/-}$ MEFs (+TSC2 and +EV) were treated with CAY (10 µM) or rapamycin (100 nM) for 24 hours. Co-immunoprecipitation using an anti-MTOR antibody was then carried out. (B) Reconstituted *Tsc2*$^{-/-}$ MEFs (+TSC2 and +EV) were treated with CAY (10 µM) for 24 hours. Co-immunoprecipitation using an Anti-Rictor antibody was then carried out. (C) Reconstituted *Tsc2*$^{-/-}$ MEFs (+TSC2 and +EV) were treated with CAY (10 µM) for 24 hours and the cell lysates were collected for immunoblot analysis.

3.2.4 Involvement of lipid raft in regulation of Akt following inhibition of SCD-1 in *Tsc2*$^{-/-}$ MEFs

It has previously been reported that Akt is recruited to plasma membrane for activation and this process requires the presence of functional lipid rafts (Calay et al., 2010; Zhuang et al., 2002). Therefore, we then went on to examine the role of lipid rafts in Akt activation caused by SCD-1 inhibition in the *Tsc2*$^{-/-}$ MEFs by separating the treated cells into detergent resistant fractions (DRF) containing lipid rafts and the detergent soluble fractions (DSF). Firstly, we observed that SCD-1 inhibition by CAY led to a much more evident reduction of total Akt protein levels in the DRF containing lipid rafts in the *Tsc2*$^{-/-}$ MEFs in comparison to the *Tsc2*$^{+/+}$ cells (Fig. 25A). Methyl-β-cyclodextrin (MBCD) was used as a positive control to disrupt lipid rafts via cholesterol depletion and both *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs treated with it showed depletion of total Akt protein from the DRF (Fig. 25A). Secondly, we also observed that *Tsc2*$^{-/-}$ MEFs contained much lower level of caveolin-1 protein (Fig. 25A-B). Caveolin-1 is one of the key components in lipid rafts (Simons and Toomre, 2000) and notably, the caveolin-1 protein level was restored in the TSC2 reconstituted cells (Fig. 25B), indicating the possible functional connection between TSC2 and caveolin-1. Meanwhile, CAY increased caveolin-1 level in the detergent soluble fractions (DSF) of the *Tsc2*$^{-/-}$ MEFs, but not in the *Tsc2*$^{+/+}$ MEFs, suggesting that *Tsc2*$^{-/-}$ MEFs are more susceptible to the disruption of lipid raft upon inhibition of SCD-1 (Fig. 25A). In contrast, MBCD treatment exerted similar effects in both cells (Fig. 25A). Next, we visualized lipid rafts by using the fluorescence tagged Cholera Toxin Subunit B (CTxB) which binds to lipid raft enriched GM1 ganglioside (Yamaguchi et al., 2009). As shown in Figure 25C, *Tsc2*$^{-/-}$ MEFs had a much weaker

basal fluorescence in comparison to the *Tsc2*$^{+/+}$ cells, being consistent with the earlier observations that *Tsc2*$^{-/-}$ MEFs possess lower level of caveolin-1 protein (Fig. 25A-B). Treatment with CAY further decreased the CTxB staining in the *Tsc2*$^{-/-}$ MEFs, while no significant changes were observed in the *Tsc2*$^{+/+}$ cells (Fig. 25C). Consistently, MBCD was able to effectively disrupt CTxB staining in both cell types with similar efficacy and cholesterol is known to be an essential component of lipid rafts (Simons and Toomre, 2000). Accordingly, the loss of lipid raft staining could be rescued by cholesterol replenishment in the *Tsc2*$^{-/-}$ MEFs treated with CAY, suggesting that lipid rafts disruption caused by SCD-1 inhibition is likely mediated by cholesterol depletion (Fig. 25C). To confirm, we then measured the intracellular cholesterol levels and found that treatment with CAY caused a significant decrease (about 30%) in cholesterol levels in the *Tsc2*$^{-/-}$ MEFs, while no changes were found in the *Tsc2*$^{+/+}$ cells (Fig. 25D). Interestingly, other lipid components of lipid rafts including sphingolipids like sphingomyelin and ceramide were equally affected by CAY treatment in both cell types (Fig. 25E-F respectively).

Since we observed a significant decrease in cholesterol levels in the *Tsc2*$^{-/-}$ MEFs upon SCD-1 inhibition (Fig. 25D), we therefore decided to further investigate the effects of cholesterol repletion on the CAY treated *Tsc2*$^{-/-}$ MEFs. Indeed, cholesterol repletion was able to prevent the loss of Akt protein from the DRF caused by MBCD treatment proving that functional lipid raft is required for Akt recruitment (Fig. 26A). More importantly, the loss of Akt protein from the DRF of the *Tsc2*$^{-/-}$ MEFs caused by CAY could be reversed significantly by cholesterol replenishment (Fig. 26A), suggesting that the loss of Akt protein from the DRF after SCD-1 inhibition is due to disruption of lipid rafts on the membrane. Analysis of the whole cell lysates showed that cholesterol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DSF
DRF
Tsc2+/+
Tsc2-/-
Tsc2+/+
Tsc2-/-
CAY
MBCD
Akt
Akt
Caveolin 1
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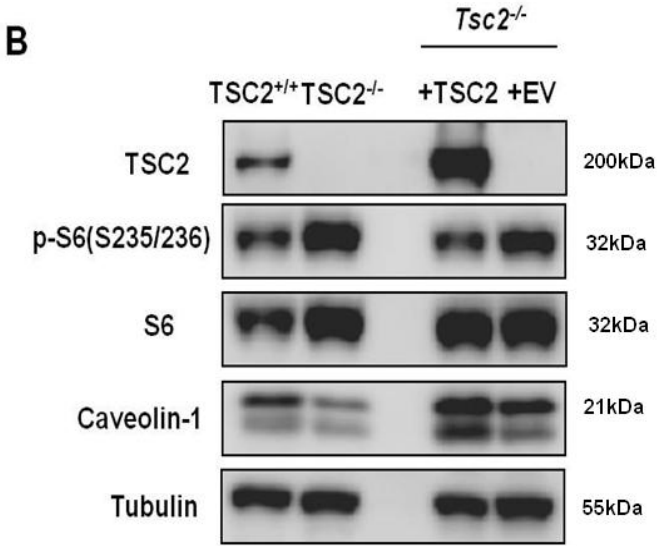
Tubulin
SE
LE
SE
LE
60kDa
21kDa
55kDa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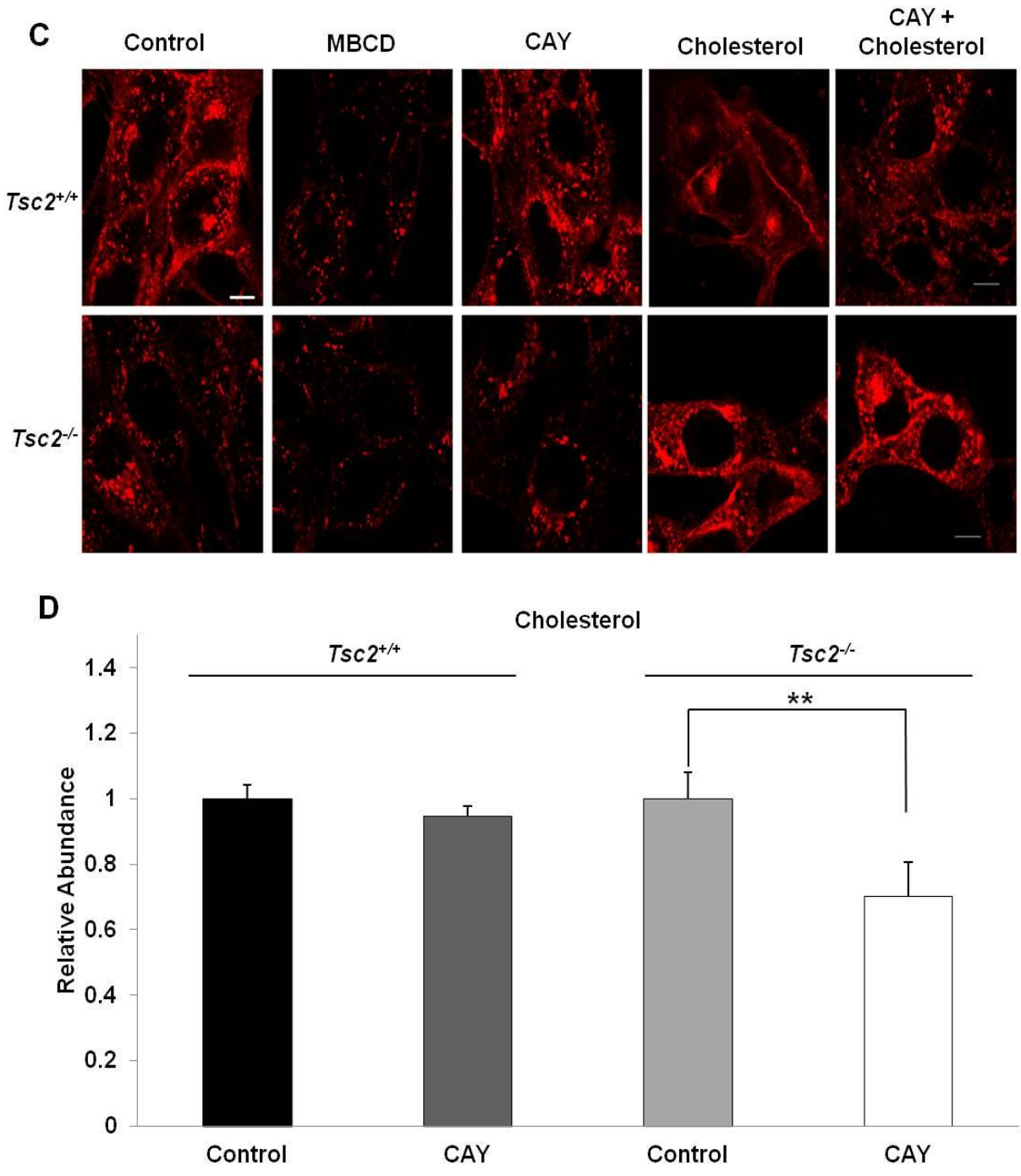
TSC2+/+ TSC2-/-
+TSC2 +EV
TSC2
p-S6(S235/236)
S6
Caveolin-1
Tubulin
200kDa
32kDa
32kDa
21kDa
55kDa


C
Control
MBCD
CAY
Cholesterol
CAY + Cholesterol
Tsc2+/+
Tsc2-/-
D
Cholesterol
Tsc2+/+
Tsc2-/-
**
Relative Abundance
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Control
CAY
Control
CAY

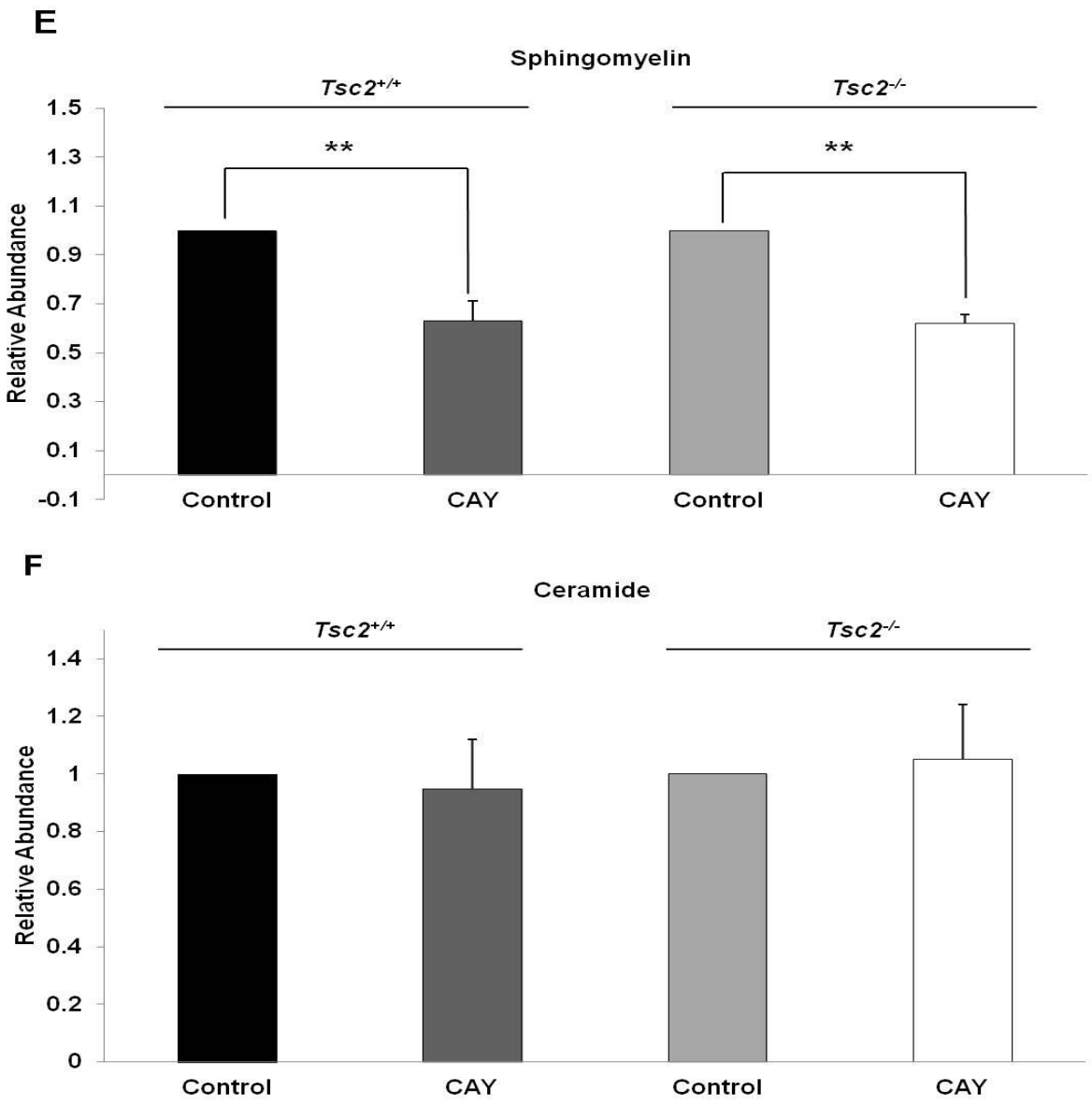
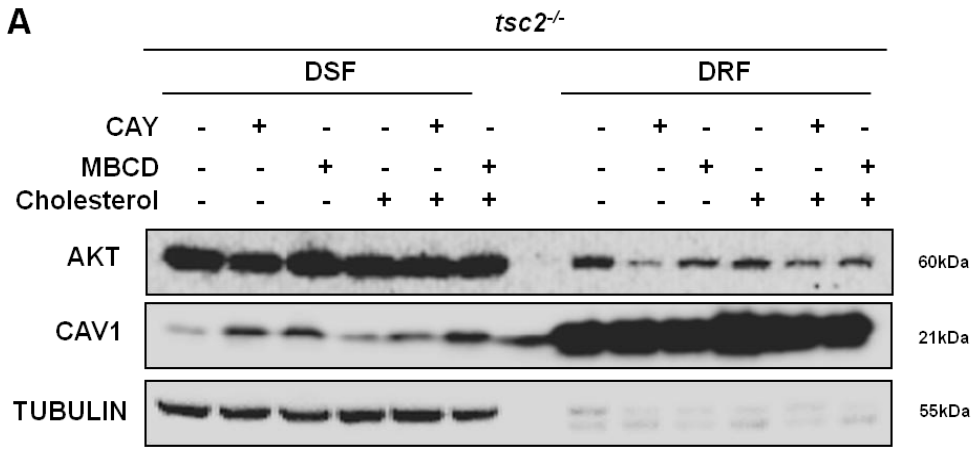
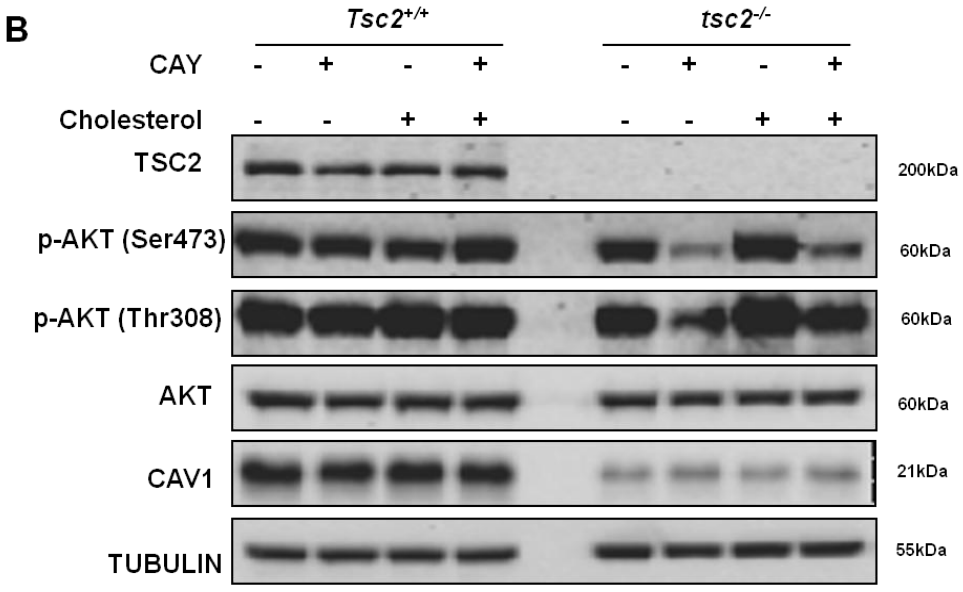
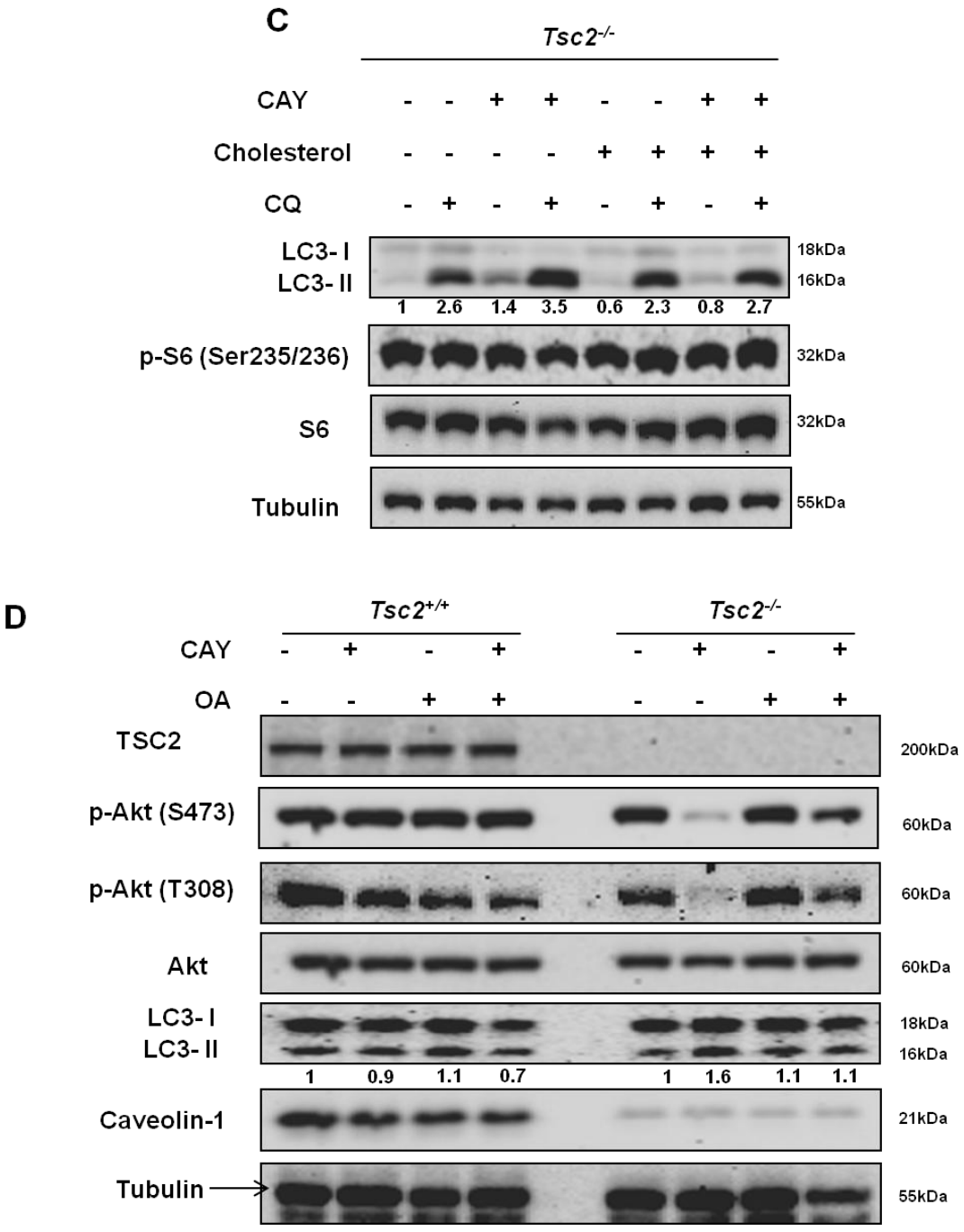


Figure 25: Inhibition of SCD-1 enzymatic activity disrupts lipid rafts and reduces Akt recruitment to plasma membrane in *Tsc2*$^{-/-}$ MEFs. (A) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with either CAY (10 µM) for 24 hours or MBCD (5 mM) for 2 hours. The DSF and DRF were then separated as described. (B) Basal levels of caveolin-1 are shown for the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs and the reconstituted *Tsc2*$^{-/-}$ MEFs (+TSC2 and +EV). (C) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 hours or MBCD (5 mM) for 2 hours. Water soluble cholesterol (CHO, 15 µg/ml) was added to a set of CAY treated cells for the last 2 hours. Lipid rafts were then stained using CTxB and visualized using confocal microscopy. Scale Bar: 10 µm. (D-F) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 hours. Lipid extraction was carried out and the total levels of (D) cholesterol, (E) sphingomyelin and (F) ceramides were measured as described and presented as the mean ± SD relative to the levels of control cells from three independent experiments (**$p<0.01$, student's t-test).

replenishment partly restored phosphorylation of Akt (T308 and S473 sites) in *Tsc2*$^{-/-}$ MEFs treated with CAY (Fig. 26B). Finally, we tested the changes of autophagic flux in *Tsc2*$^{-/-}$ MEFs with either cholesterol repletion. As shown in Figure 26C, cholesterol repletion indeed reduced the autophagic flux level measured by the LC3-II level in *Tsc2*$^{-/-}$ MEFs treated with CAY, without affecting MTORC1 activity. We further tried to investigate the effects of supplementing the medium with OA which is one of the major products of SCD-1 catalyzed desaturation process. We observed that similar to cholesterol repletion, the addition of OA to CAY treated *Tsc2*$^{-/-}$ MEFs was able to prevent total loss of phosphorylation on T308 and S473 sites of the Akt protein when compared to the cells treated with CAY alone (Fig. 26D). Furthermore, the increase in autophagic flux that was observed in cells treated with CAY alone could also be inhibited when the medium was supplemented with OA (Fig. 26E). Taken together, the data from this part of our study thus suggest that Akt protein recruitment to the lipid rafts is impaired during inhibition of SCD-1 activity due to disruption of lipid rafts formation. Furthermore, the loss of cholesterol or unsaturated fatty acid production caused by SCD-1 inhibition may contribute to the disruption of lipid rafts and impaired Akt activation and autophagy induction in the *Tsc2*$^{-/-}$ MEFs.


A
tsc2-/-
DSF
DRF
CAY
MBCD
Cholesterol
AKT
CAV1
TUBULIN
60kDa
21kDa
55kDa


B
Tsc2+/+
tsc2-/-
CAY
Cholesterol
TSC2
p-AKT (Ser473)
p-AKT (Thr308)
AKT
CAV1
TUBULIN
200kDa
60kDa
60kDa
60kDa
21kDa
55kDa


C
Tsc2-/-
CAY - - + + - - + +
Cholesterol - - - - + + + +
CQ - + - + - + - +
LC3- I
LC3- II
18kDa
16kDa
1 2.6 1.4 3.5 0.6 2.3 0.8 2.7
p-S6 (Ser235/236)
32kDa
S6
32kDa
Tubulin
55kDa
D
Tsc2+/+
Tsc2-/-
CAY - + - + - + - +
OA - - + + - - + +
TSC2
200kDa
p-Akt (S473)
60kDa
p-Akt (T308)
60kDa
Akt
60kDa
LC3- I
LC3- II
18kDa
16kDa
1 0.9 1.1 0.7 1 1.6 1.1 1.1
Caveolin-1
21kDa
Tubulin
55kDa

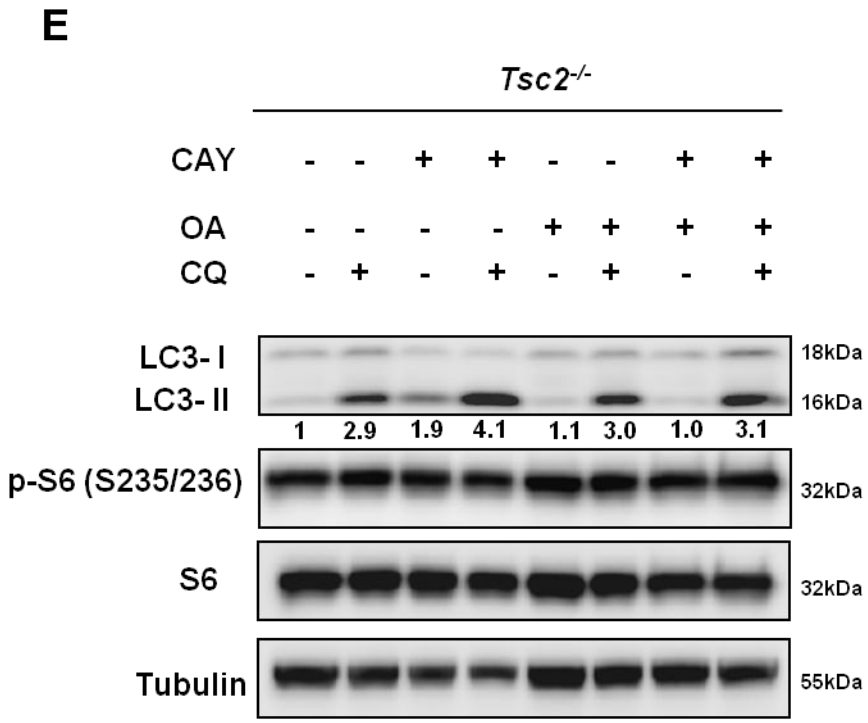
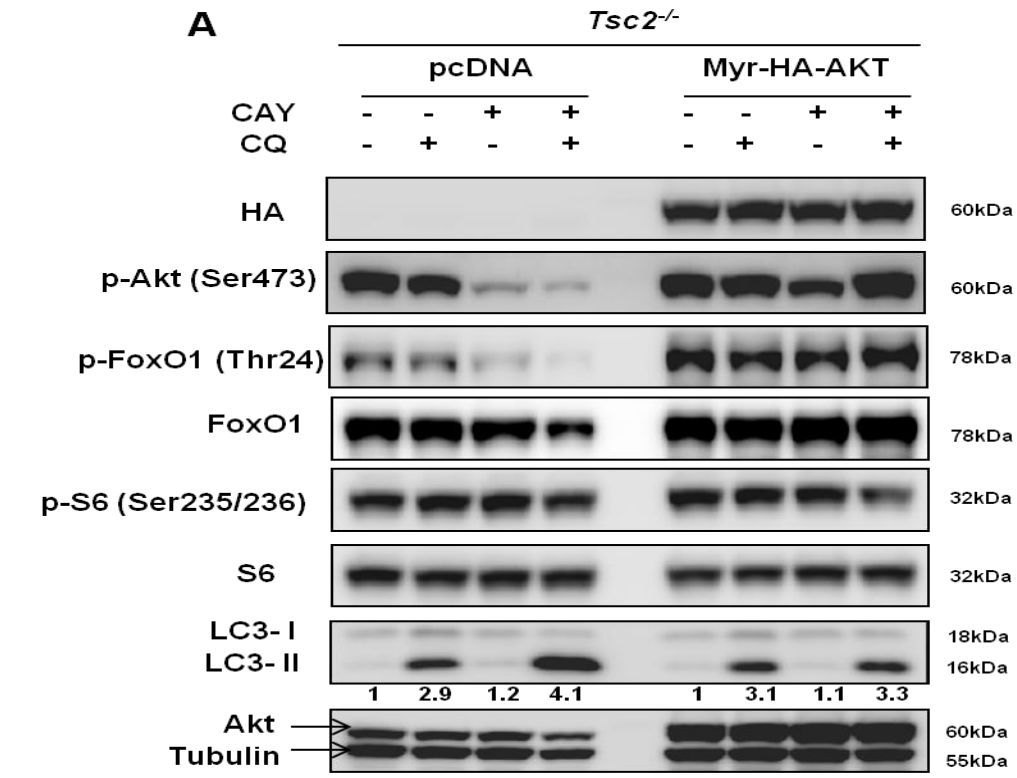
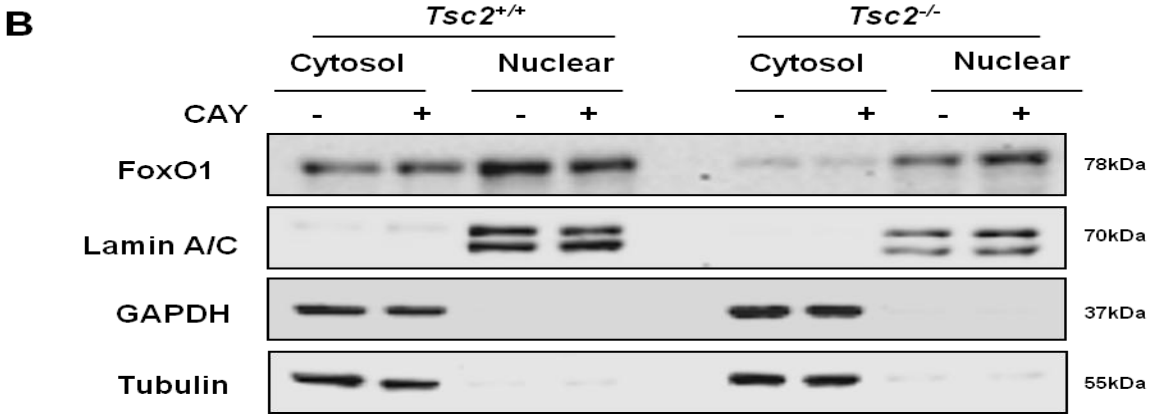


Figure 26: Repletion of cholesterol or OA prevents autophagy induction in SCD-1 inhibited cells. (A) *Tsc2*$^{-/-}$ MEFs were treated with either CAY (10 µM) for 24 hours or MBCD (5 mM) for 2 hours. Water soluble cholesterol (CHO, 15 µg/ml) was added concurrently to the treated cells for the last 2 hours as a form of cholesterol repletion. The DSF and DRF were then separated as described. (B) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with either CAY (10 µM) for 24 hours or water-soluble cholesterol (CHO, 15 µg/ml) for 2 hours. Water soluble cholesterol (CHO, 15 µg/ml) was added concurrently to CAY treated cells for the last 2 hours as a form of cholesterol repletion. (C) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated as described in (B). Additionally, CQ (10 µM) was added to the cells to measure the autophagic flux levels. (D) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) alone, OA (200 µM) alone or concurrently with both for 24 hours and cells were collected for immunoblot analysis. (E) *Tsc2*$^{-/-}$ MEFs were treated as described in (D). Additionally, CQ (10 µM) was added to the cells to measure the autophagic flux levels.

3.2.5 Autophagy induction in the Tsc2$^{-/-}$ MEFs upon SCD-1 inhibition is FoxO1-dependent

The results that have been reported thus far suggest that autophagy induction by the inhibition of SCD-1 enzymatic activity is dependent on Akt, but not MTORC1. Downstream substrates of Akt include the family of FoxO transcription factors, some of which have been implicated in autophagy induction (Sengupta et al., 2009; Xu et al., 2011; Zhao et al., 2010; Zhou et al., 2012). To further investigate the involvement of Akt-FoxO1 signaling axis in autophagic induction caused by inhibition of SCD-1 activity, we then transfected the *Tsc2*$^{-/-}$ MEFs with the constitutively active myristoylated form of Akt (Myr-Akt) (Murga et al., 1998). Transfection of Myr-Akt into the *Tsc2*$^{-/-}$ MEFs had no effect on the p-S6 levels, being consistent with the notion that regulation of MTOR activity in these cells is independent of Akt activity (Fig. 27A). CAY treatment in the *Tsc2*$^{-/-}$ MEFs led to loss of phosphorylation of S473 on Akt and also T24 on FoxO1 only in the pcDNA transfected control cells, but not in cells with that over-expressed Myr-Akt (Fig. 27A). Akt is responsible for the phosphorylation of T24 on FoxO1 and this phosphorylation has been shown to inhibit the transcriptional activity of FoxO1 (Burgering and Kops, 2002). Consistently, over-expression of Myr-Akt prevented the loss of phosphorylation of S473 on Akt and subsequently T24 on FoxO1 upon inhibition of SCD-1 activity (Fig. 27A). Furthermore, Myr-Akt expression prevented the increase of autophagic flux induced by CAY in the *Tsc2*$^{-/-}$ MEFs (Fig. 27A). Thus our results support a notion that autophagy induction caused by SCD-1 suppression in *Tsc2*$^{-/-}$ MEFs could be mediated by the activation of the FoxO1 transcription factor downstream of Akt inhibition. When Akt is inactivated, the hypo-phosphorylated FoxO1 translocates to the


A
Tsc2-/-
pcDNA
Myr-HA-AKT
CAY
CQ
HA
p-Akt (Ser473)
p-FoxO1 (Thr24)
FoxO1
p-S6 (Ser235/236)
S6
LC3- I
LC3- II
1 2.9 1.2 4.1
1 3.1 1.1 3.3
Akt
Tubulin
60kDa
60kDa
78kDa
78kDa
32kDa
32kDa
18kDa
16kDa
60kDa
55kDa


B
Tsc2+/+
Tsc2-/-
Cytosol
Nuclear
Cytosol
Nuclear
CAY
FoxO1
Lamin A/C
GAPDH
Tubulin
78kDa
70kDa
37kDa
55kDa

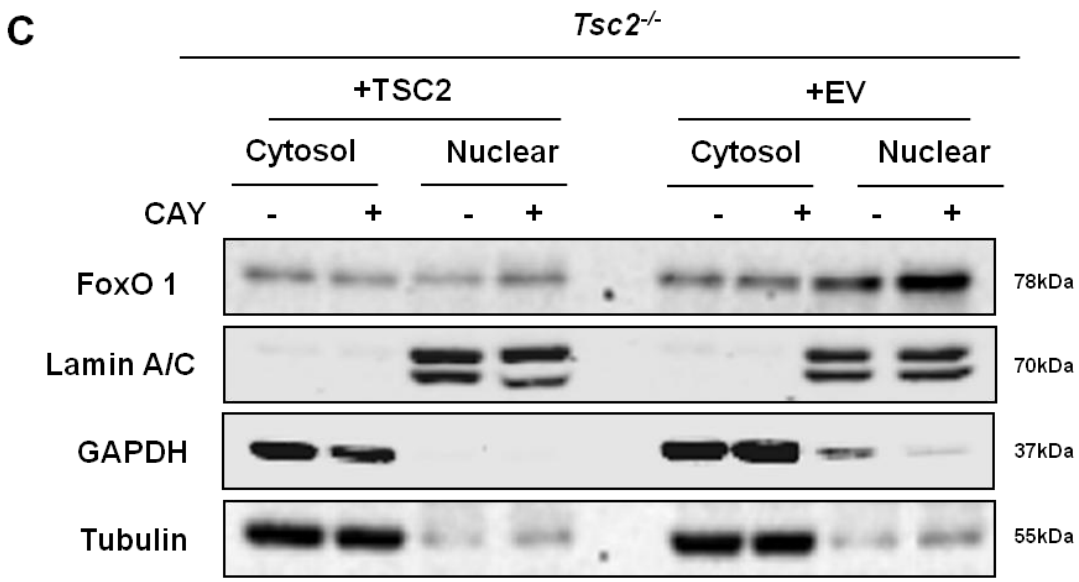
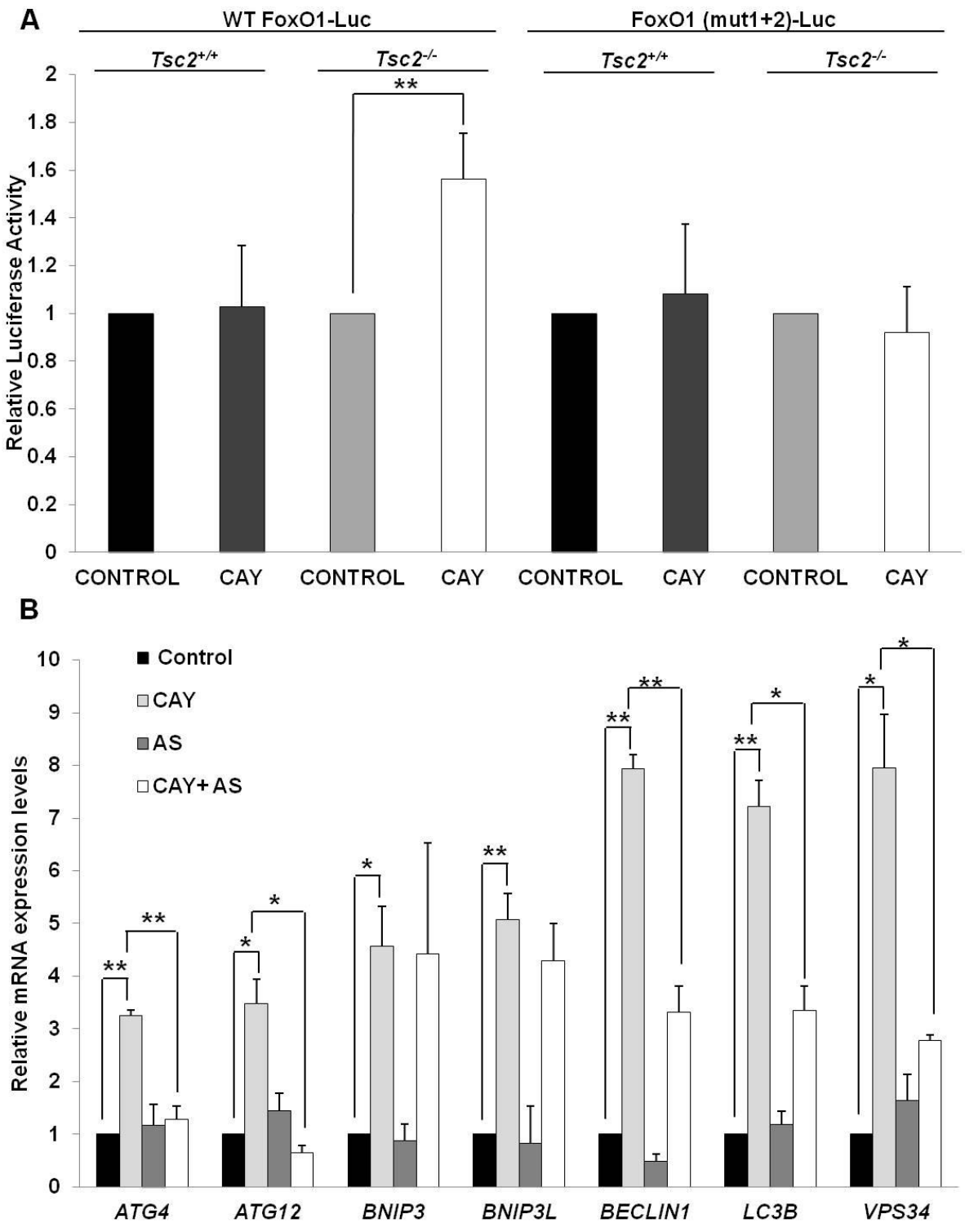


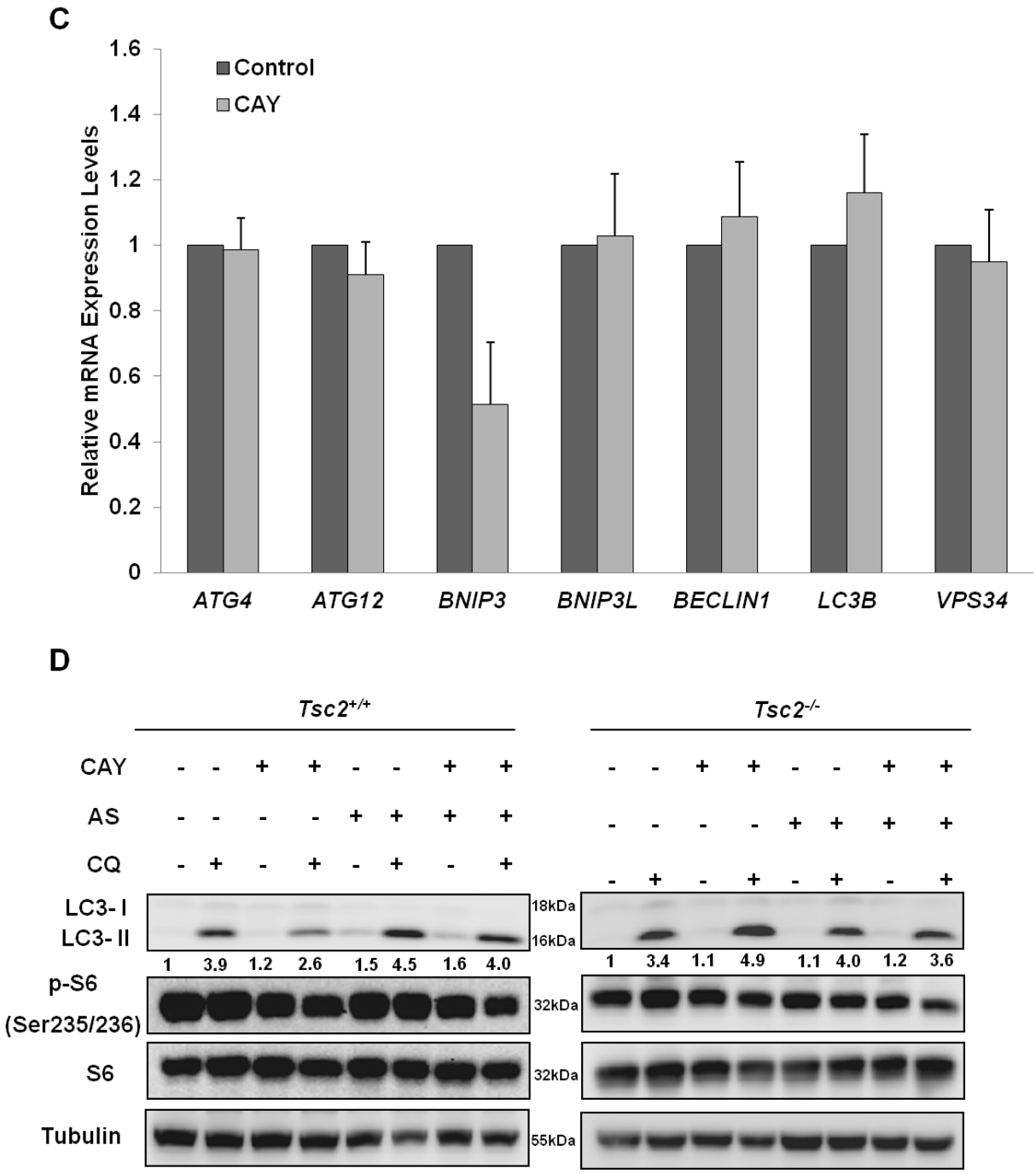
Figure 27: Activation of FoxO1 is required for autophagy induction caused by SCD-1 inhibition in the *Tsc2*$^{-/-}$ MEFs. (A) *Tsc2*$^{-/-}$ MEFs were transfected with either pcDNA as control or HA-Myr-Akt as described and the cells were then treated with CAY (10 µM) alone, CQ (10 µM) alone or concurrently with both inhibitors for 24 hours for measurement of autophagic flux. (B) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 hours and then the cytosol and nuclear fraction was separated as described. (C) Reconstituted *Tsc2*$^{-/-}$ MEFs (+TSC2 and +EV) were treated with CAY (10 µM) for 24 hours and the cytosol and nuclear fraction was separated as described.

nucleus. Consistently, CAY increased accumulation of FoxO1 protein in the nuclear fraction of *Tsc2*$^{-/-}$ MEFs, but not in the *Tsc2*$^{+/+}$ cells (Fig. 27B). Consistently, FoxO1 nuclear translocation was reversed by the reconstitution of TSC2 protein (Fig. 27C).

Since FoxO1 can act as a transcription factor after it is has translocated into the nucleus, we next tried to investigate whether nuclear translocation of FoxO1 after SCD-1 inhibition can indeed lead to increased transcriptional activity. We first transfected *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs with the two different luciferase constructs, WT FoxO1-luc and FoxO1(mut 1+2)-luc before subjecting the cells to CAY treatment. Indeed, we observed a significant increase in the FoxO1 transcriptional activity in the CAY treated *Tsc2*$^{-/-}$ MEFs as measured by the luciferase assay (Fig. 28A). As expected, there was no increase in luciferase activity in the cells transfected with the FoxO1(mut 1+2)-luc construct which contains mutations in the consensus sequence recognized by FoxO1 (Al-Mubarak et al., 2009). Our data thus further support the notion that suppression of SCD-1 by CAY increases FoxO1 transcriptional activity. In agreement with previous results where phosphorylation of FoxO1 was not affected in the CAY *Tsc2*$^{+/+}$ MEFs, we observed no changes in the luciferase activity of the CAY treated *Tsc2*$^{+/+}$ MEFs that were transfected with the WT FoxO1-luc construct (Fig. 28A). FoxO proteins have been previously reported to control the expression of genes that are involved in controlling the autophagic process (Sengupta et al., 2009; Xu et al., 2011; Zhao et al., 2010; Zhou et al., 2012). Therefore we then went on further to examine the mRNA levels of some FoxO1 target genes in the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs treated with CAY. In particular, we observed significant increases in mRNA levels of genes like *ATG4*, *ATG12*, *BECLIN1*, *BNIP3*, *BNIP3L*, *LC3B* and *VPS34* in CAY-treated *Tsc2*$^{-/-}$ MEFs (Fig. 28B), while no significant

changes in the mRNA levels of these genes were observed in the *Tsc2*$^{+/+}$ MEFs when treated with the same inhibitor (Fig. 28C). Moreover, a known FoxO1 protein inhibitor AS1842856 (AS) (Nagashima et al., 2010) was able to significantly suppress the activating effect of CAY on the expression of the target genes, except for *BNIP3* and *BNIP3L* (Fig. 28B). Consistently, AS was able to block the increase in autophagic flux caused by CAY treatment in the *Tsc2*$^{-/-}$ MEFs (Fig. 28D, left panel), while no changes in the autophagic flux of the *Tsc2*$^{+/+}$ MEFs was found (Fig. 28D, right panel). To support the data from the FoxO1 chemical inhibitor, we carried out experiments with FoxO1 knock-down using siRNA in the *Tsc2*$^{-/-}$ MEFs. As shown in Fig. 28E, FoxO1 knockdown effectively reduced the autophagic flux caused by CAY treatment. Therefore, the above results provide strong evidence suggesting that autophagy induction in CAY-treated *Tsc2*$^{-/-}$ MEFs is dependent on activation of FoxO1 downstream of impaired Akt activation due to disruption of lipid rafts in the cells.


A
WT FoxO1-Luc
FoxO1 (mut1+2)-Luc
Tsc2+/+
Tsc2-/-
Tsc2+/+
Tsc2-/-
Relative Luciferase Activity
2
1.8
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
**
CONTROL
CAY
CONTROL
CAY
CONTROL
CAY
CONTROL
CAY
B
Control
CAY
AS
CAY+AS
Relative mRNA expression levels
10
9
8
7
6
5
4
3
2
1
0
ATG4
ATG12
BNIP3
BNIP3L
BECLIN1
LC3B
VPS34


C
Control
CAY
Relative mRNA Expression Levels
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
ATG4
ATG12
BNIP3
BNIP3L
BECLIN1
LC3B
VPS34
D
Tsc2+/+
Tsc2-/-
CAY
AS
CQ
LC3- I
LC3- II
18kDa
16kDa
1 3.9 1.2 2.6 1.5 4.5 1.6 4.0
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p-S6
(Ser235/236)
32kDa
S6
32kDa
Tubulin
55kDa

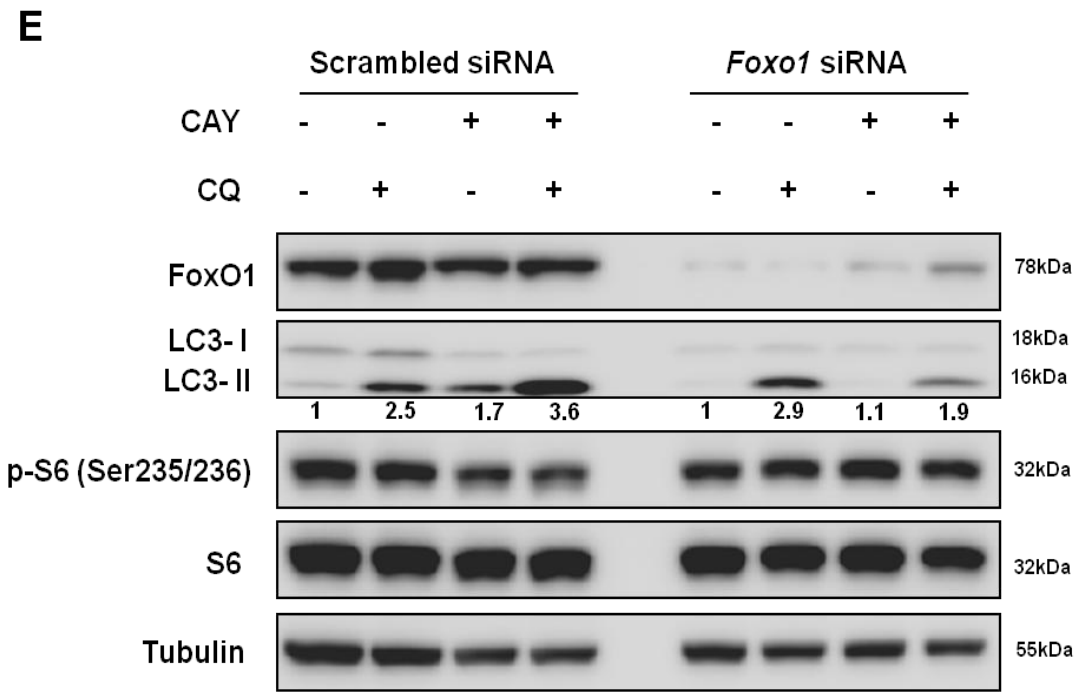
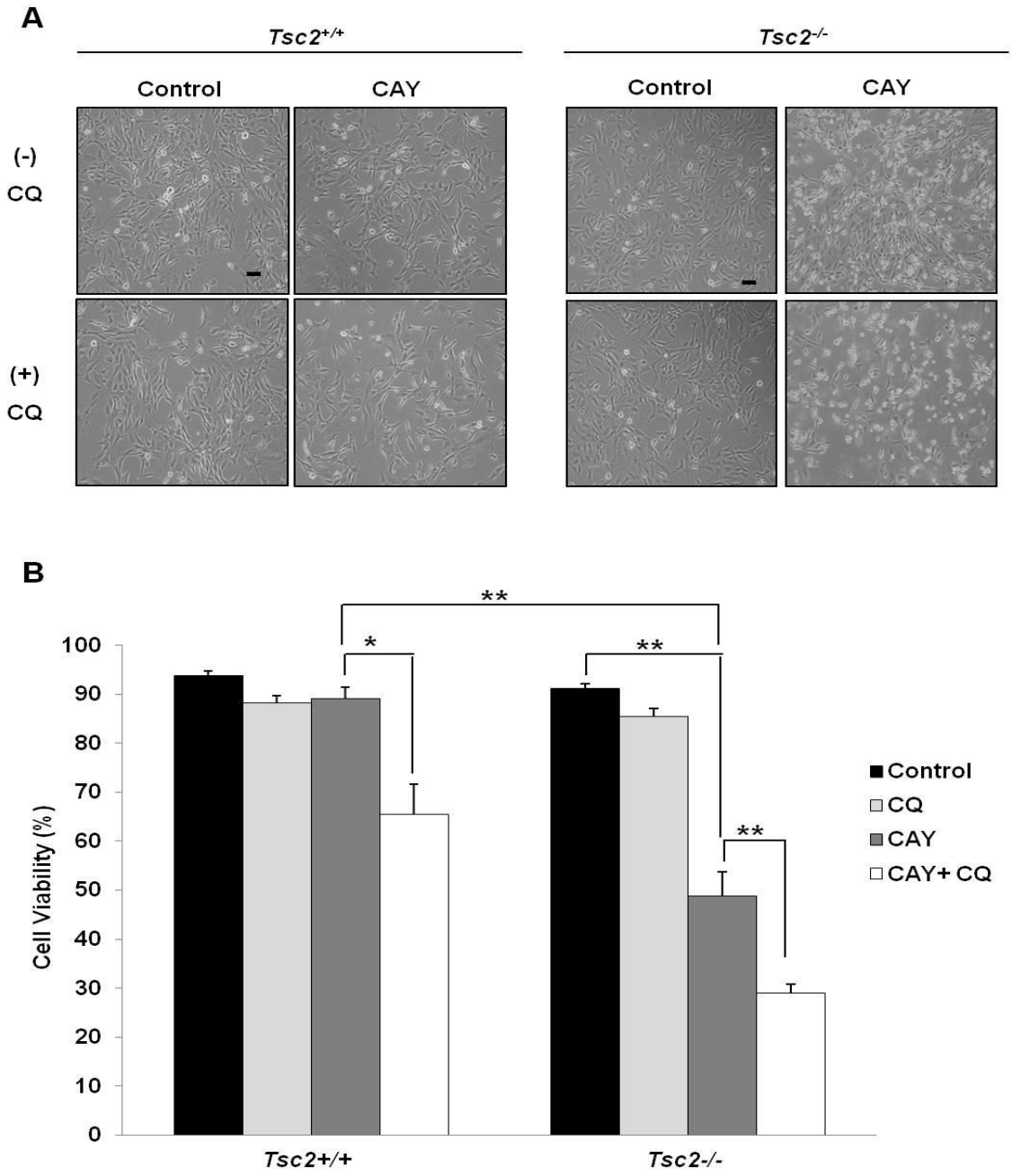


Figure 28: Activation of FoxO1 transcriptional activity is required for autophagy induction upon SCD-1 inhibition in *Tsc2*$^{-/-}$ MEFs. (A) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were transfected with either WT FoxO1-luc or FoxO1(mut 1+2)-luc vector together with *Renilla* luciferase vector as internal control as described. The cells were then treated with CAY (10 μM) for 24 hours before the luciferase activity was determined. Relative luciferase activity was presented as the mean ± SD relative to the levels of the control cells from three independent experiments (**p<0.01, student's t-test). (B) *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 μM) alone, AS (250 nM) alone or concurrently with both inhibitors for 24 hours. mRNA levels of various genes involved in the autophagy process were then measured by qRT-PCR and presented as the mean ± SD relative to the levels in the control cells from three independent experiments (*p<0.05, **p<0.01, student's t-test) (C) *Tsc2*$^{+/+}$ MEFs were treated with CAY (10 μM) for 24 hours and mRNA levels of various genes involved in the autophagy process was then measured by qRT-PCR and presented as the mean ± SD relative to the levels in the control cells from three independent experiments. (D) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 μM) alone, AS (250 nM) alone or concurrently with both inhibitors for 24 hours with or without the presence of CQ (10 μM) to measure the autophagic flux. (E) Scrambled or FoxO1 siRNA was transfected into *Tsc2*$^{-/-}$ MEFs as described and the cells were then treated with CAY (10 μM) alone, CQ (10 μM) alone or with both inhibitors concurrently for 24 hours to measure the autophagic flux.

3.2.6 $Tsc2^{-/-}$ MEFs are more sensitive to SCD-1 inhibition and autophagy promotes cell survival

SCD-1 enzymatic activity and the MUFAs it produces have been shown to be critical for cell proliferation and suppression of SCD-1 is cytotoxic to numerous cancer cell types that have been tested (Igal, 2010; Scaglia et al., 2005; Scaglia and Igal, 2005). Here we compared the cytotoxic effect of CAY between *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs. As shown in Figure 29A, *Tsc2*$^{-/-}$ MEFs were found to be much more sensitive to CAY-mediated cytotoxicity than *Tsc2*$^{+/+}$ MEFs. Furthermore, addition of CQ together with CAY resulted in further increase cell death (Fig. 29A), suggesting that autophagy is a cell survival mechanism in the *Tsc2*$^{-/-}$ MEFs. Consistent results were obtained when cell viability was quantified using propidium iodide live cell exclusion (Fig. 29B). The above results are in agreement with previous studies that cells with hyperactivated growth signaling pathways are more dependent on *de novo* lipogenesis for survival and thus more susceptible to stress factors (Hess et al., 2010; Igal, 2010; Menendez and Lupu, 2007). We further examined the form of cell death induced by SCD-1 inhibition. There was an evident increase of both caspase 3 and PARP cleavage, which are all classical markers of apoptosis, upon treatment with CAY for up to 48 hrs (Fig. 29C). Finally, a long term colony formation assay was carried out to investigate the long term effect of SCD-1 inhibition. CAY treatment significantly decreased the long term cell viability of the *Tsc2*$^{-/-}$ MEFs and the effects could be significantly enhanced by addition of CQ, while *Tsc2*$^{+/+}$ MEFs were much less susceptible to the treatment of CAY and/or CQ (Fig. 29D). Taken together, results from this part of our study suggest that autophagy mediated by SCD-1 inhibition represents an important pro-survival mechanism.


A
Tsc2+/+
Tsc2-/-
Control
CAY
Control
CAY
(-)
CQ
(+)
CQ
B
**
*
**
**
100
90
80
70
60
50
40
30
20
10
0
Cell Viability (%)
Control
CQ
CAY
CAY+ CQ
Tsc2+/+
Tsc2-/-

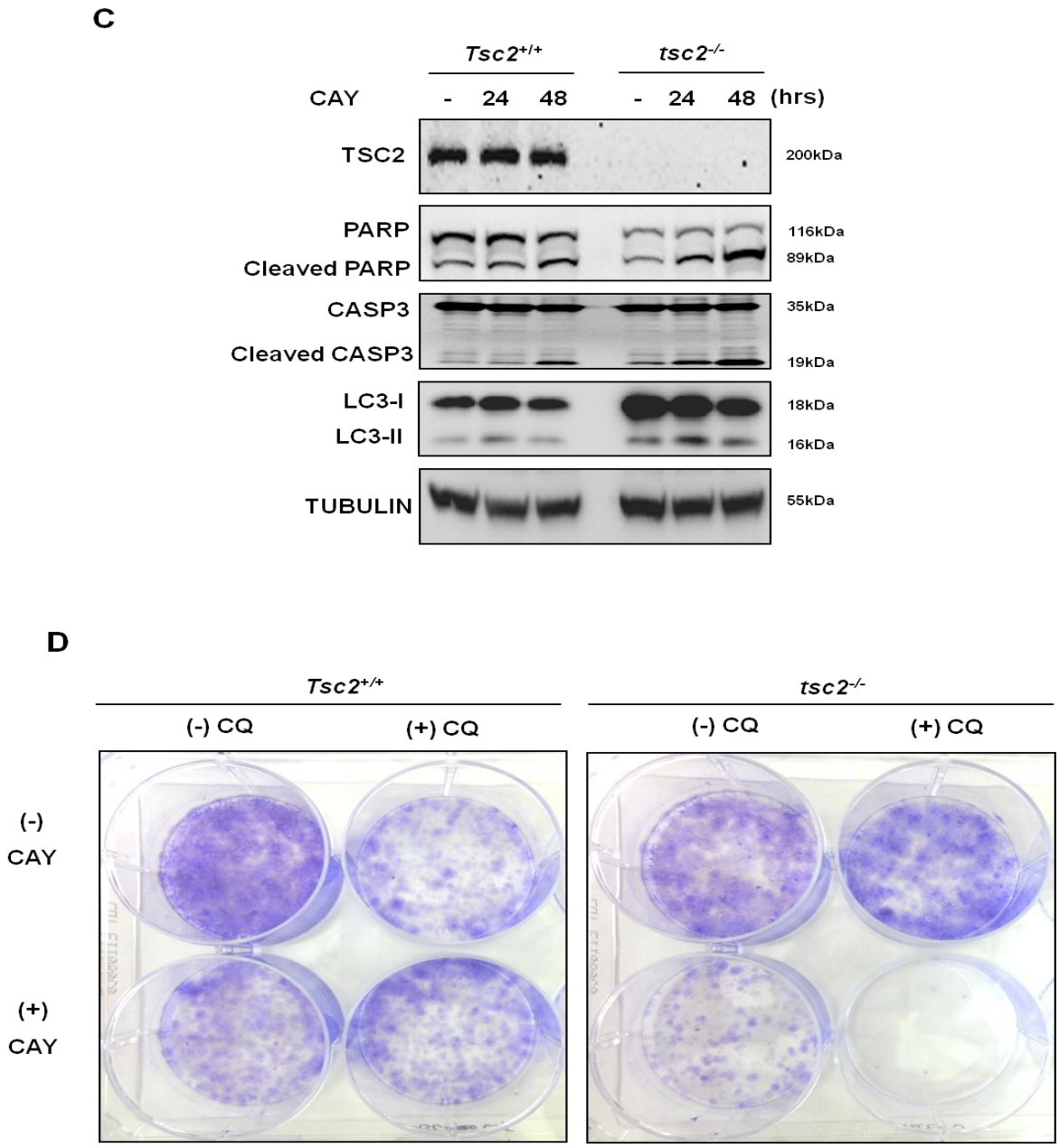


Figure 29: *Tsc2*$^{-/-}$ MEFs are more susceptible to cell death induced by SCD-1 inhibition and autophagy acts as a cell survival mechanism. (A) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) alone, CQ (10 µM) alone or with both inhibitors concurrently for 48 hours. The cell morphology was then observed using phase contrast microscope. Scale Bar: 200 µm. (B) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated as described in (A) above and cell viability was quantified using the propidium iodide live cell exclusion assay. The results are presented as the mean ± SD from three independent experiments (*$p<0.05$, **$p<0.01$, student's t-test) (C) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated with CAY (10 µM) for 24 and 48 hours and cell lysates were then collected for immunoblot analysis. (D) *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs were treated as described in (A) and then re-plated into 6-well plates for the long term cologenic survival assay.

4. General Discussion and Conclusions

4.1 Autophagy induction in cells by PA but not OA

At present, it is known that autophagy is able to regulate lipid metabolism through lipophagy (Settembre et al., 2013; Singh et al., 2009a), while it is still controversial whether lipid species like FFA can regulate autophagic activity. In this study we provide evidence showing that autophagy can be induced by the SFA, PA, but not by the MUFA, OA (Fig. 6 and 7). Our findings are generally consistent with earlier reports that FFA such as PA is capable of inducing autophagy in pancreatic ß-cells and also a variety of cancer cell lines (Choi et al., 2009; Ebato et al., 2008; Shen et al., 2012). On the other hand, it has been reported that both PA and OA treatment prevented fusion of autophagosome and lysosome and thus inhibits autophagy (Koga et al., 2010). Another recently published paper reported that only OA but not PA was capable of inducing autophagy in hepatocytes (Mei et al., 2011). There are several possible explanations for the conflicting results that have been reported so far. One possibility is that the effects of different FFAs like PA and OA on autophagy are tissue and cell type specific. Furthermore, other factor such as concentration and duration of FFAs, as well as the concentration of glucose present in the medium have been found to be responsible for different outcomes observed (Komiya et al., 2010; Las et al., 2011; Martino et al., 2012; Mei et al., 2011; Shen et al., 2012). More importantly, the FFAs that were used for treatment *in vitro* need to be conjugated to fatty acid free BSA in order for them to be taken up by the cells. However, detailed review of the literature on the methods used for preparation of the BSA-FFA complexes reveal that there are drastic differences in the ways these complexes are prepared, and this may be one of the major factors resulting in

the conflicting effects regarding the abilities of FFAs to induce or inhibit autophagy (Choi et al., 2009; Las et al., 2011; Martino et al., 2012; Mei et al., 2011; Singh et al., 2009a). Throughout our study, we have prepared our fatty acid complexes according to a well-established method that has been used in many other studies (Listenberger and Brown, 2007; Listenberger et al., 2003; Listenberger et al., 2001). Furthermore, we have consistently treated the cells in FBS-free medium to make sure that no other lipids are present in the medium used for culturing of the cells and ensure that the effects we have observed on autophagic activity is truly due to PA treatment.

4.2 *De Novo* Ceramide biosynthesis is not involved in PA-induced autophagy

In the first part of the study, we also attempted to identify the molecular mechanisms underlying PA-induced autophagy, with several important findings. Firstly, although an increase in total ceramide levels were observed in the PA treated cells, this increase was minimal and not statistically significant (Fig. 10A). Furthermore, we conclusively excluded the involvement of ceramides in PA-induced autophagy, as the inhibition of ceramides synthesis by myriocin or FB1 is unable to block autophagy induction (Fig. 10B-C). Previous reports have shown that treatment with C_2 and C_6 ceramides are able to induce autophagy in human cancer cells via inhibition of Akt and MTORC1 activity (Pattingre et al., 2009; Scarlatti et al., 2004). One possible explanation for this discrepancy is the fact that the type of ceramide species generated in their study after treatment with the short chain ceramides is different from those accumulated in PA-treated cells. In our study, the type of ceramide metabolites found in PA-treated cells consisted of mainly the Cer(d18:1/16:0) (N-(palmitoyl)-ceramide) species since the main

substrate used was PA made up of 16 carbon aliphatic chain. Furthermore, it is important to note that the other studies mentioned did not investigate specifically the types of ceramide species that were generated intracellularly upon C_2 and C_6 ceramides treatment (Pattingre et al., 2009; Scarlatti et al., 2004). Thus autophagy induction could have been a direct response to the short chain ceramides without the cells metabolizing them into the long chain ceramide species. Furthermore, another important thing to note is that the short chain C_2 and C_6 ceramides that were used for treatment are not normally present in the body under physiological conditions (Pattingre et al., 2009; Scarlatti et al., 2004). On the other hand our observations here are consistent with an earlier report that ceramide accumulation only plays a nominal role in affecting cellular homeostasis observed in cells treated with PA (Listenberger et al., 2001). In this study, PA treatment induced cytotoxicity was independent of ceramide synthesis, thus suggesting that ceramide accumulation upon PA treatment does not play a significant role in the subsequent cellular responses (Listenberger et al., 2001).

4.3 PA-induced autophagy is independent of MTORC1 regulation and mediated by DAG-PKCα signaling pathway

The other important finding in our study shows that PA-induced autophagy is independent of MTORC1 regulation as PA treatment fails to inhibit MTORC1 activity as observed by the unaltered levels of p-70S6K and p-S6 (Fig. 8 and 9). It has been reported that high levels of FFA leads to constant activation of MTOR activity, a process related to the development of diseases such as diabetes and obesity (Muoio and Newgard, 2008). Furthermore, it was shown that PA has the ability to induce activation of MTORC1 via another member of the PKC family, PKC-δ (Wang et al., 2010). Taken together, it is

obvious that PA-induced autophagy is not mediated via suppression of MTORC1 activity. As described earlier on, there have been recent studies that have started to propose the presence of non-canonical autophagy which is independent of MTORC1 regulation. The PKC family has been reported in a number of studies to be able to induce autophagy upon different cellular stresses without affecting MTORC1 activity. These studies have specifically identified members of the PKC family including PKC-δ and PKC-θ to be involved in autophagy induction (Chen et al., 2008; Sakaki and Kaufman, 2008; Sakaki et al., 2008; Shahnazari et al., 2010). In our study, we have identified a particular member of the classical PKC family, PKC-α as an important mediator in PA-induced autophagy, based on the following observations: (i) general and typical PKC inhibitors are able to block PA-induced autophagy (Fig. 13); (ii) PA, but not OA, induces PKC-α activation (Fig. 14A-B); and (iii) siRNA knock-down of PKC-α significantly reduced PA-induced autophagy (Fig. 14C). To our knowledge, this is the first time any study has identified PKC-α as an upstream regulator of autophagy. Similar to the recent report highlighting the importance of DAG in promoting anti-bacterial autophagy via promoting autophagosome formation and activation of PKC-δ (Shahnazari et al., 2010), results from our study have also indicated the importance of intracellular DAG. DAG acts as the main stimulus for induction of autophagy via activation of PKC-α, based on the common understanding that DAG is required for activation of classical PKC (Newton, 2001). Previous studies have demonstrated that DGAT, the enzyme responsible for conversion of DAG to TAG has a higher specificity for the 18C:1-CoA substrate derived from OA compared to the 16C:0-CoA substrate derived from PA (Coleman and Bell, 1976; Li et al., 2010) and this could be the reason why OA treatment resulted in more significant

increase in total TAG levels but much lesser accumulation of DAG compared to PA (Fig. 11 and 12).

At present, we have preliminary data showing that OA treatment after the knock-down of DGAT1 can ultimately lead to induction of autophagy as well (Fig. 15). This piece of evidence thus suggests that DAG accumulation in the OA-treated cells due to the loss of DGAT1 is critical for activating the autophagic process, most probably through activation of PKC-α as we have shown in the PA-treated cells. Results from this study also raise an interesting possibility that DAG accumulation in the cells has undesirable effects on the cellular homeostasis and the induction of autophagy via PKC activation may serve the purpose of alleviating such adverse effects. The activation of various members of the PKC family by accumulation of intracellular DAG induced by PA treatment has been well studied (Benoit et al., 2009; Eitel et al., 2003; Yu et al., 2002). These may help us understand the fact that lack of DAG accumulation in OA-treated cells did not lead to PKC activation and thus no autophagy induction.

Future studies are needed to elucidate how PKC-α leads to the induction of autophagy in the PA-treated cells. This will involve identifying the downstream targets of PKC-α that can lead to autophagy induction. There are many examples of kinases like AMPK that activates autophagy by phosphorylating and activating ULK1 (Egan et al., 2011; Kim et al., 2011) and JNK that phosphorylates Bcl-2 to induce autophagy by preventing its interaction with Beclin-1 (Wei et al., 2008a; Wei et al., 2008b). Thus PKC-α could also be acting as a kinase for some protein that is critical for autophagic activity. Furthermore, we will have to investigate the intracellular localization of the accumulated DAG upon PA and OA treatment. This would enable us to find out whether the accumulated DAG

can be found on the autophagosome membrane as a way of promoting autophagosome formation as has been shown in the previous study depicting the role of DAG in anti-bacterial autophagy (Shahnazari et al., 2010). As shown in our study, the activation of PKC-α is dependent on DAG activation and it will be important to examine whether PKC-α is recruited by DAG to locations near the isolation membrane where it can exert its kinase activity on substrates that will lead to enhanced autophagic activity during PA treatment.

4.4 Autophagy is an important cell survival mechanism for cells against lipotoxicity caused by PA

After establishing the role of DAG-PKC in PA-mediated autophagy, we next examined the role of autophagy in PA-mediated lipotoxicity. Lipotoxicity has been reported in many studies to be the main contributor to the progression of various diseases associated with excess lipid accumulation in the body such as obesity, diabetes and the metabolic syndrome (Brookheart et al., 2009; Chavez and Summers, 2010). Common dietary long chain saturated FFAs like PA are especially damaging to cells due to their ability to induce lipoapoptosis upon chronic treatment with high doses to simulate a lipotoxic condition while MUFAs like OA generally have no effects on cell viability (Shimabukuro et al., 1998a; Unger, 2003; Unger and Orci, 2002). Several factors such as increased ROS production (Piro et al., 2002) and induction of ER stress (Borradaile et al., 2006) upon treatment with SFAs have been implicated as the mechanisms of actions leading to lipotoxicity. On the other hand, it has been well established that autophagy generally serves as an important cell survival mechanism under various stress conditions, such as starvation, oxidative stress, DNA damage (Levine and Klionsky, 2004; Mizushima et al.,

2008). Thus it is no surprise for us to observe an increase in autophagic activity in the cells as a way to maintain cellular homeostasis and cell survival in response to the cytotoxic PA treatment. Data from this part of our study reveal that PA-induced autophagy play a pro-survival function and suppression of autophagy by either CQ or knock-down of Atg7 (Fig. 16 and 17 respectively) markedly enhanced PA-induced apoptosis. At present, it is still not clear how autophagy protects against PA-mediated cell death. One possibility remaining to be tested is that autophagy is involved in degrading and clearing the accumulated DAG from the cells via lipases that are present in the lysosomes. Earlier study has shown that autophagy plays an important role in lipid metabolism by affecting the intracellular levels of neutral lipids in the cells (Singh et al., 2009a). Thus, it is possible that autophagy can play a role in regulation of intracellular levels of DAG in order to promote cell survival in times of exogenous FFA stress. Such a hypothesis is supported by an earlier study that increased TAG accumulation and decreased levels of DAG in cells by over-expression of DGAT1 helped to prevent PA-mediated cell death (Listenberger et al., 2003). Therefore, an important experiment that should be done in the future will be to investigate whether excessive DAG accumulation in DGAT knocked-down cells upon PA treatment. This is important to prove that DAG species are the main cause of cell death in cells during lipotoxicity and thus autophagy induction might be a general cellular response to the cytotoxic effects of excess DAG accumulation in the cells.

The results of this part of the study suggest targeting autophagy as potential therapeutic strategies for lipotoxicity-related diseases. It will certainly be interesting to investigate whether induction of autophagy in disease models like obesity and diabetes can rescue

the cells from cell death induced by lipotoxicity. Taken together, as summarized in Figure 30, here we reveal in the first part of our study a novel mechanism in regulating PA-induced autophagy during times of lipotoxicity: PA promotes the accumulation of intracellular DAG, which in turn activates PKC-α as an upstream signaling mechanism for autophagy. Moreover, such inducible autophagy plays an important pro-survival role in mitigating PA-induced apoptosis and lipotoxicity.

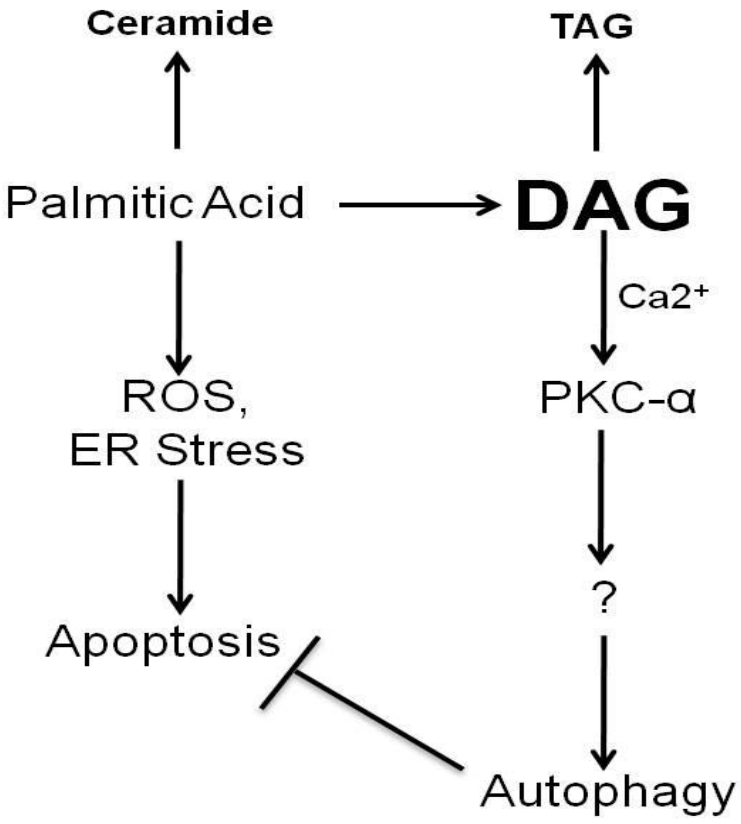


Figure 30. Summary of the proposed signalling pathways involved in PA-mediated autophagy and its pro-survival role in PA-induced apoptosis and lipotoxicity.

4.5 Inhibition of SCD-1 activity leads to autophagy induction in the *Tsc2*$^{-/-}$ MEFs

In the second part of our study, we tried to investigate if modulating the endogenous levels of SFAs and MUFAs in cells can lead to changes in autophagic activity. Specifically, we tried to use cells with constitutively active MTORC1 signaling pathway so as to investigate how cells with high rate of *de novo* lipogenesis would response to changes in endogenous levels of FFAs compared to cells not driven to have high rates of lipogenesis. Data from our first part of the study has conclusively shown that stimulation with exogenous SFA like PA could lead to autophagy induction via accumulation of DAG and activation of PKC-α while the MUFA, OA had no such effect. Thus we wanted to examine the roles of SCD-1 in lipogenesis and autophagy via suppression of SCD-1 by using both a chemical inhibitor and genetic knockdown to increase endogenous SFAs.

SCD-1 is essential in cells for the *de novo* synthesis of MUFAs that act as the building blocks for all major lipid classes, and play an important role in maintenance of membrane fluidity in cells (Hodson and Fielding, 2012; Ntambi and Miyazaki, 2003). Recently, SCD-1 has been identified as a potential cancer therapeutic target (Igal, 2010; Morgan-Lappe et al., 2007). We confirmed in our cellular model that *Tsc2*$^{-/-}$ MEFs indeed had higher mRNA and protein levels of SCD-1 which contributes to the increased lipogenesis in the cells compared to the *Tsc2*$^{+/+}$ MEFs (Fig. 18 and 19), supporting most of the earlier studies on the positive relationship between MTORC1 signaling and increased *de novo* lipogenesis biosynthesis (Duvel et al., 2010; Peterson et al., 2011; Porstmann et al., 2005; Porstmann et al., 2008). One key observation from this part of our study is that induction of autophagy was observed only in the *Tsc2*$^{-/-}$ MEFs but not in the *Tsc2*$^{+/+}$ MEFs when

SCD-1 activity was inhibited either with a chemical inhibitor or via siRNA knock-down (Fig. 20). This result suggests that cells with high levels of *de novo* lipogenesis (due to constitutive activation of MTORC1) are more susceptible to SCD-1 inhibition. This is in contrast to the results from the first part of our study as exogenous PA stimulation induces autophagy via accumulation of DAG and activation of PKC-α. Furthermore, although we did observe a decrease in MUFAs and increase in SFAs in both *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs upon inhibition of SCD-1 (Fig. 21C and 21D), we did not observe any significant changes in DAG levels in these cells (Fig. 21A). The observations here thus suggest that we are not able to significantly increase intracellular levels of DAG by modulating endogenous levels of SFAs. More importantly, the lack of DAG accumulation even in the *Tsc2*$^{-/-}$ MEFs suggests that some other signaling pathways are involved in the regulation of autophagy induction upon SCD-1 inhibition.

4.6 SCD-1 inhibition contributes to loss of cholesterol and disruption of lipid raft structures in the *Tsc2*$^{-/-}$ MEFs

One important finding from the second part of our study is that SCD-1 inhibition does not affect MTORC1 activity in the *Tsc2*$^{-/-}$ MEFs (Fig. 20 and 22), suggesting that autophagy induction we observed in the *Tsc2*$^{-/-}$ MEFs was independent of the canonical MTORC1 regulation. Instead, we demonstrated that SCD-1 inhibition reduced phosphorylation of Akt at 2 critical sites, T308 and S473 specifically in the *Tsc2*$^{-/-}$ MEFs (Fig. 23D-E and Fig. 24C). Additionally, we did not observe any disruption of the formation of MTORC2 which is responsible for phosphorylation of Akt at S473 (Fig. 23B-C and Fig. 24A-B) and this suggests to us that maybe the recruitment of Akt to the plasma membrane is affected upon SCD-1 inhibition in the *Tsc2*$^{-/-}$ MEFs.

The full activation of Akt in cells has been shown to be dependent on the presence of cholesterol-enriched lipid rafts on the plasma membrane. Furthermore, lipid raft disruption by the depletion of cholesterol resulted in loss of Akt recruitment to the plasma membrane, suppression of Akt activation and eventually cell death (Calay et al., 2010; Hill et al., 2002; Zhuang et al., 2002). Lipid rafts are microdomains consisting of cholesterol and sphingolipids like ceramide and sphingomyelin that are enriched on the exoplasmic leaflet of the membrane bilayer (Simons and Toomre, 2000). Caveolin-1 is an important protein in lipid rafts as it forms a subclass of plasma membrane lipid rafts termed caveolae by binding to cholesterol (Simons and Ikonen, 2000). Furthermore, caveolin-1 is also known to have the ability to regulate cholesterol levels in cells (Parton and Simons, 2007). It was previously reported that TSC2 binds to caveolin-1 protein and the loss of TSC2 was shown to lead to displacement of caveolin-1 from the plasma membrane, leading to decreased lipid raft formation (Jones et al., 2004). In this study, we observed lower level of caveolin-1 in the *Tsc2*$^{-/-}$ MEFs compared to the *Tsc2*$^{+/+}$ cells, and this caveolin-1 level could be restored in the TSC2 reconstituted MEFs (Fig. 25B and Fig. 26B). Such observations thus indicate that lipid rafts structure and function are impaired in *Tsc2*$^{-/-}$ MEFs. Our lipid profiling data showed that cholesterol depletion and subsequent disruption of lipid rafts was only found in the *Tsc2*$^{-/-}$ MEFs with SCD-1 inhibition (Fig. 25C-D). Therefore, it is highly possible that the impaired lipid rafts in the *Tsc2*$^{-/-}$ MEFs make these cells more susceptible to SCD-1 inhibition. It has been previously shown that the depletion of SCD-1 leads to decreased levels of cholesterol and TAG in mice (Miyazaki et al., 2000), while another study also showed that SCD-1 negatively regulates the efflux of cholesterol from cells (Sun et al., 2003). Therefore, the

Tsc2$^{-/-}$ MEFs with decreased levels of caveolin-1 are more susceptible to the depletion of cholesterol caused by SCD-1 inhibition, leading to further disruption of lipid rafts and loss of Akt localization and activation.

4.7 Activation of FoxO1 transcriptional activity is essential for autophagy induction in Tsc2$^{-/-}$ MEFs upon SCD-1 inhibition

After establishing the critical role of Akt in autophagy induction in *Tsc2*$^{-/-}$ MEFs following SCD-1 inhibition, we further identified FoxO1 transcriptional factor as a key downstream target in mediating autophagy observed in cells with SCD-1 inhibition (Fig. 27 and 28). At present, the exact mechanisms for FoxO-mediated autophagy are still very debatable. There are studies showing that increased transcriptional activity of nuclear FoxO1 promotes autophagy (Sengupta et al., 2011; Sengupta et al., 2009; Xu et al., 2011). Furthermore, another member of the FoxO family, FoxO3a has been implicated in regulating the expression of pro-autophagy genes for autophagy induction during a variety of stresses, including starvation in haematopoietic stem cells (Warr et al., 2013) and lysosomal proteolysis during muscle atrophy upon denervation (Mammucari et al., 2007; Zhao et al., 2007). On the other hand, there have been studies demonstrating that cytosolic FoxO1 is required for autophagy induction independent of its transcriptional activity (Zhao et al., 2010; Zhou et al., 2012). The cytosolic Foxo1 can bind to Atg7 to modulate the autophagic process that leads to cell death and tumour suppression (Zhao et al., 2010). This is in contrast to our results showing that the transcriptional activity of nuclear translocated FoxO1 is crucial for autophagy induction in *Tsc2*$^{-/-}$ MEFs upon SCD-1 inhibition (Fig. 28). The differences observed between the different studies could be due to the different cell types utilized, as well as the different cellular stimulus for

activation of FoxO1. For example, cytosolic FoxO1 could be important for autophagy induction upon nutrient starvation or oxidative stress as reported (Zhao et al., 2010), while in our study, the nucleus translocated FoxO1 might only particularly important during direct inhibition of Akt activity without affecting MTORC1 signaling pathway.

4.8 *Tsc2*$^{-/-}$ MEFs are 'addicted' to endogenous MUFA production for cell survival and autophagy functions as a cell survival mechanism during SCD-1 inhibition

Previous studies have shown that inhibition of SCD-1 induces apoptosis in a variety of cancer cells (Dobrzyn et al., 2004; Minville-Walz et al., 2010; Scaglia et al., 2009). In this study, we reported that *Tsc2*$^{-/-}$ MEFs are much more susceptible to cell death induced by inhibition of SCD-1 enzymatic activity than the *Tsc2*$^{+/+}$ MEFs while concurrent inhibition of autophagy further increased the cell death in the SCD-1 inhibited *Tsc2*$^{-/-}$ MEFs (Fig. 29). Our results thus imply that the cells with hyperactivated MTORC1 signaling are 'addicted' to the continual production of endogenous MUFAs for cell growth and survival. The dependence of these *Tsc2*$^{-/-}$ MEFs on particular nutrients like glucose and glutamine for cell growth and survival has been reported previously (Choo et al., 2010). In this part of our study, we have gone on to show that survival of these cells is also dependent on MUFAs generation. The difference between the *Tsc2*$^{+/+}$ and *Tsc2*$^{-/-}$ MEFs is presumably due to the differential metabolic demands between the two cell types as a result of the hyperactivated MTORC1 signaling pathway in the *Tsc2*$^{-/-}$ MEFs (Caron et al., 2010; Choo et al., 2010; Duvel et al., 2010). This is consistent with an earlier report that transformed cells are more dependent on *de novo* synthesis of MUFA, rather than the exogenous MUFA (Scaglia and Igal, 2005). In contrast, the *Tsc2*$^{+/+}$ MEFs have a much lower metabolic demand for MUFAs and are more likely to get sufficient exogenous

source from the culture medium and thus are more resistant to inhibition of SCD-1 activity. Induction of autophagy in the *Tsc2*$^{-/-}$ MEFs during SCD-1 inhibition could help to generate free MUFAs from the lipid droplets stored within the cells to support the aberrant cell growth in the *Tsc2*$^{-/-}$ MEFs. Furthermore, we observed that levels of TAG, which are the main components of lipid droplets were significantly decreased in the *Tsc2*$^{-/-}$ MEFs after SCD-1 inhibition (Fig. 21B), suggesting that induction of lipophagy which is the degradation of lipid droplets via autophagic machinery might be occurring (Singh et al., 2009a). Therefore, inhibition of autophagy will further starve the *Tsc2*$^{-/-}$ MEFs from their required MUFA, thus sensitizing cell death mediated by SCD-1 inhibition.

Our findings reported here suggest new therapeutic strategies by targeting lipogenesis for genetic disorders like TSC which are characterized by the deregulation of MTORC1 signaling pathway. Recent clinical trials in the treatment of TSC involved the use of rapamycin analogs to inhibit the MTORC1 signaling pathway and these inhibitors were only able to have cytostatic effects (Bissler et al., 2008). Reappearance of tumour growth was observed in the TSC patients when they were taken off these rapamycin analogs (Bissler et al., 2008). In contrast, our findings here suggest that instead of trying to inhibit the hyperactivated MTORC1 signaling pathway, we can take advantage of this Achilles' heel specifically in the TSC2 deficient cells and target their dependence on endogenous MUFAs as a cytotoxic approach to kill the cells. Furthermore, as we have shown, a combinational therapy together with autophagy inhibition will further increase the efficacy of such treatments, although the efficacy of such combinational approaches needs to be further tested.

Lipids are the major components of cellular membrane structures and play important functions in various cellular signaling pathways. (Vander Heiden et al., 2009; Zoncu et al., 2011b). Autophagy, on the other hand, is known to be involved in lipid metabolism (Singh et al., 2009a). Our study provides novel evidence implicating SCD-1 as a key regulator in lipid metabolism and autophagy, especially in cells with high demand of lipogenesis due to constitutive activation of MTOR. As depicted in Figure 30, we have shown here that under normal conditions, cells deficient of TSC2 protein have constitutively activated MTORC1 activity, leading to increased SCD-1 expression and *de novo* lipogenesis required to maintain the enhanced metabolic demands. In the presence of intact lipid raft structure, Akt is recruited to plasma membrane to be phosphorylated by both PDK1 and MTORC2 to be fully activated. Subsequently, activated Akt phosphorylates its downstream target FoxO1 and prevents its nuclear translocation and gene expression. When SCD-1 is inhibited either via a chemical inhibitor or siRNA knockdown, *de novo* lipogenesis in the cells is suppressed, leading to disruption of lipid raft structures, reduced Akt recruitment to the plasma membrane, and subsequently impaired Akt activation and Akt-mediated FoxO1 phosphorylation. The unphosphorylated FoxO1 then translocates into the nucleus and activates expression of various genes involved in autophagy, a process independent of MTOR regulation. Therefore, it appears that SCD-1 plays a critical role in mediating lipogenesis and autophagy; both are serving as important pro-survival forces in supporting cells with constitutive MTOR activation.

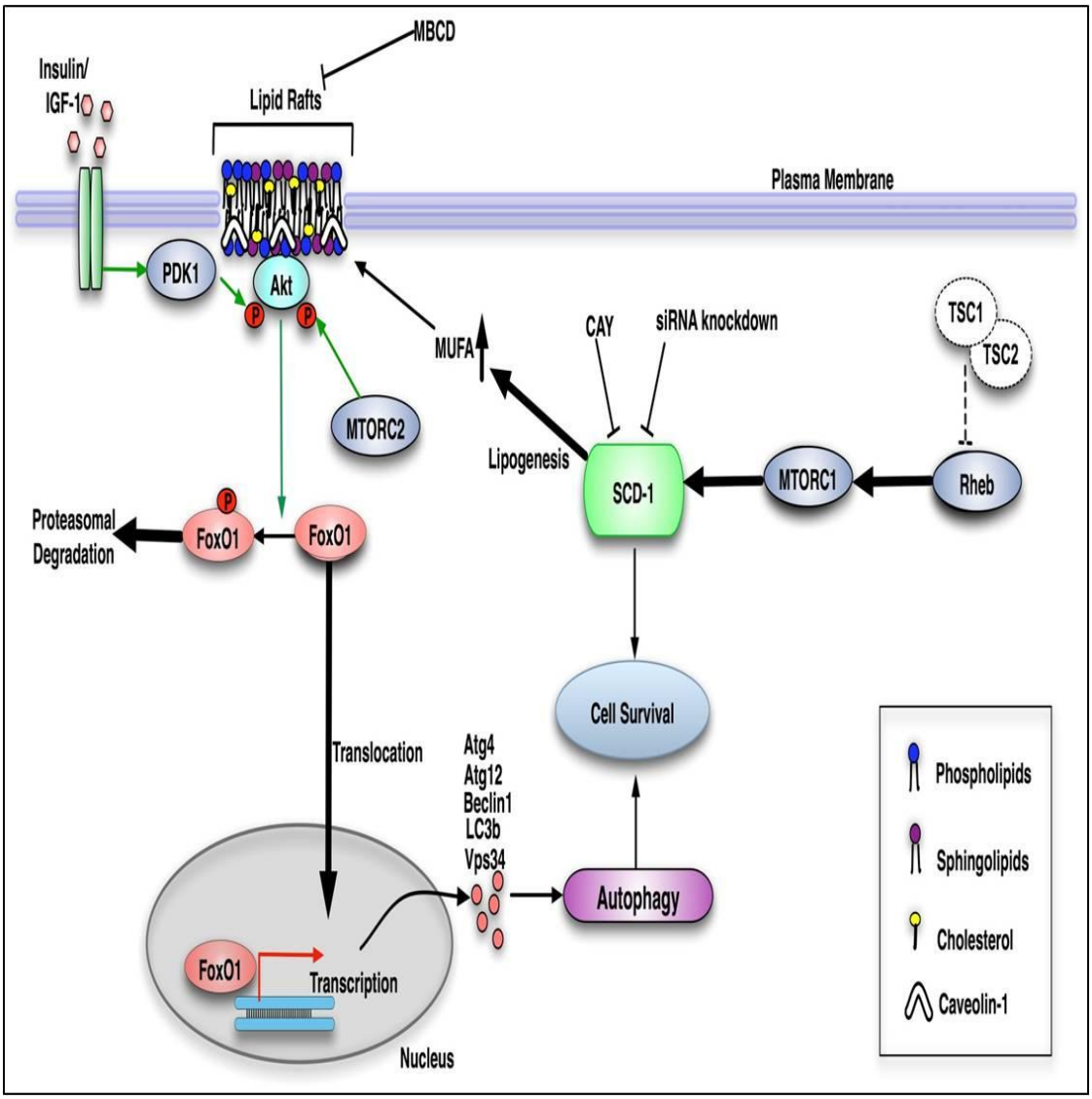


Figure 31: Proposed mechanisms underlying the critical role of SCD-1 in autophagy regulation via lipogenesis and lipid rafts-coupled Akt-FoxO1 signalling pathway.

In summary, data from this part of the study provide novel evidence implicating SCD-1 in autophagy via a signaling pathway involving lipogenesis, lipid rafts, Akt and FoxO1 (Fig. 31). Our study suggests that targeting SCD-1 could be a potential cancer therapeutic strategy in patients with altered metabolic homeostasis caused by constitutive MTOR activation arising from the loss of the functional TSC1-TSC2 complex.

5. Conclusion

In this study, we have investigated how the manipulation of lipids can play a role in the regulation of autophagy using different *in vitro* cell culture models. The whole study consists of 2 major parts. In the first part of the study, we have tried to study the role of exogenous saturated and unsaturated fatty acids in the regulation of autophagy during lipotoxic stresses and the signalling mechanism regulating this fatty acid induced autophagy induction. The major findings from the first part of the study are (i) SFAs like PA but not MUFAs are able to induce autophagy during lipotoxic stress; (ii) induction of autophagy by PA is independent of MTORC1 regulation but is mediated by the DAG-PKCα signalling pathway; (iii) autophagy plays a cell survival role during lipotoxic stress and suppression of autophagy will lead to increased cell death. These major findings have been summarized in Figure 30 above.

In the second part of the study, we have tried to investigate whether modulation of endogenous levels of MUFAs and SFAs through inhibition of SCD-1 can affect autophagy in using the *Tsc2*$^{-/-}$ cell model where MTORC1 is constitutively active leading to high levels of *de novo* lipogenesis. The major findings from the second part of the study are (i) inhibition of SCD-1 activity leads to autophagy induction only in the *Tsc2*$^{-/-}$ MEFs; (ii) induction of autophagy is independent of MTORC1 regulation but mediated by the Akt-FoxO1 signaling pathway due to the disruption of lipid rafts; (iii) autophagy plays a cell survival role in SCD-1 inhibited *Tsc2*$^{-/-}$ MEFs and suppression of SCD-1 and autophagic activity can further sensitize the cells to cell death. These major findings have been summarized in Figure 31 above.

Taken together, data from this study have demonstrated that we can regulate autophagy levels in cells through the modulation of the exogenous or endogenous sources of lipids, specifically free fatty acids under different cellular models. Consistently, we have shown that autophagy can be induced in cells upon excessive SFA treatment in times of lipotoxic stresses. Furthermore, modulation of endogenous MUFA and SFA via suppression of SCD-1 activity in *Tsc2*$^{-/-}$ MEFs can also induce autophagy. In both instances, the induction of autophagy is independent of the classic MTORC1 regulation. More importantly, we have also provided compelling evidence showing that induction of autophagy is an important cell survival mechanism in both parts of the study.

At present, the exact roles that free fatty acids and its secondary lipid metabolites play in regulation of autophagy in various disease states are still not fully understood. Therefore, data from this study has provided novel insight into the intricate relationship between lipid homeostasis and autophagy during situations of lipotoxic stresses or hyperactivated MTORC1 signaling pathway. Such findings also suggest that modulation of autophagic activity can be considered as a therapeutic strategy for therapy in the future. More immediate work involving *in vivo* studies using animal models will be essential to confirm the results we have observed in this study. In future, these studies will potentially lead to the development of therapeutic strategies that is based on the modulation of autophagy either through specific autophagy activators or inhibitors for treatment against diseases like obesity or cancer respectively.

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