INTERPLAY BETWEEN AUTOPHAGY AND LIPID DROPLETS IN STARVING CANCER CELLS

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Doctoral Dissertation
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Doctoral Dissertation

POVEZAVE MED AVTOFAGIJO IN LIPIDNIMI KAPLJICAMI V RAKAVIH CELICAH PRI STRADANJU

Doktorska disertacija

Supervisor: Assoc. Prof. Dr. Toni Petan

Ljubljana, Slovenia, January 2024

To my parents, Šefkija and Melzija Jusović,
and my sister, Minela Aslan

For being reassured that no matter if I fall, you will always be there to lift me up.

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In the end:

Non scholae sed vitae discimus (latin phrase).

– We do not learn for school, but for life.

Abstract

Lipid droplets (LDs) are intracellular organelles that store lipids, mainly triacylglycerols (TAGs) and sterol esters. LDs were initially considered just inert fat reservoirs but have now been identified as critical players in the cellular stress response, especially in cancer cells. In response to various kinds of stress, including nutrient deprivation or excess, LD formation is induced. Recent studies have indicated that there is a complex association between LDs and autophagy. The primary goal of this dissertation was to investigate the crosstalk between autophagy and LD metabolism in promoting cancer cell survival under nutrient stress conditions. Our findings demonstrate that LDs are dynamically formed and broken down in HeLa cervical cancer cells depending on the severity and duration of nutrient stress. Notably, LD biogenesis was upregulated within hours of amino acid starvation, whereas milder starvation in the absence of serum predominantly stimulated LD breakdown pathways. However, during prolonged serum starvation there was a gradual but significant LD accumulation. Inhibition of diacylglycerol acyltransferase (DGAT) 1 and 2 enzymes, which are essential for TAG synthesis and LD formation, decreased the levels of LDs in cells exposed to both conditions of nutrient stress. Autophagy contributed to LD biogenesis during acute starvation, but not during cell growth in nutrient-rich conditions and during serum starvation, when selective autophagic breakdown of LDs through lipophagy was observed. Finally, we demonstrate that joint inhibition of autophagy and DGAT-mediated LD biogenesis, rather than combined suppression of autophagy and lipolysis, is an efficient way to compromise cancer cell survival during both amino acid and serum starvation conditions. In conclusion, the results from this dissertation suggest that autophagy and LDs cooperate in the protection of HeLa cervical cancer cells against nutrient deficiency-induced stress. Our findings provide a better understanding of the interplay between autophagy and LDs in cancer cell survival and could have important implications for the development of novel cancer therapies.

Povzetek

Lipidne kapljice (LK) so celični organeli, ki delujejo kot skladišča za lipide, zlasti triacilglicerole (TAG) in sterolne estre. Sprva so LK obravnavali zgolj kot pasivna skladišča maščob. Nadaljnje raziskave na tem področju so razkrile njihov pomen kot enega od ključnih udeležencev v celičnem odzivu na stres, še posebej v kontekstu rakavih celic. Biosinteza LK se namreč inducira kot odgovor na različne vrste stresa, kot so pomanjkanje ali presežek hranil. Nedavne raziskave so odkrile večplastno povezavo med LK in procesom avtofagije. Glavni cilj te disertacije je bil preučiti povezave med avtofagijo in metabolizmom LK pri omogočanju preživetja rakavih celic v pogojih pomanjkanja hranil. Naše ugotovitve kažejo, da je presnova LK v celicah raka materničnega vratu HeLa dinamična, na njihovo tvorbo in razgradnjo pa vplivata intenzivnost in trajanje pomanjkanja hranil. Ugotovili smo, da se pri pomanjkanju aminokislin v celicah kopičijo LK, pri čemer se je proces biogeneze LK aktiviral že v nekaj urah. Nasprotno pa so se med stradanjem ob odsotnosti seruma spodbujale predvsem poti, odgovorne za razgradnjo LK. Med dolgotrajnim stradanjem v odsotnosti seruma pa je prišlo do postopnega, vendar znatnega kopičenja LK. Ravni LK v celicah, izpostavljenih stresu zaradi pomanjkanja hranil, so se zmanjšale z inhibicijo encimov diacilglicerol aciltransferaz (DGAT) 1 in 2, ki igrajo ključno vlogo pri sintezi TAG in nastajanju LK. Ugotovili smo tudi, da je proces avtofagije pripomogel k nastanku LK pri pomanjkanju aminokislin, vendar pa avtofagija ni prispevala h kopičenju LK med rastjo celic v pogojih, bogatih s hranili, ali med odtegovanjem seruma. Pri slednjih smo ugotovili, da je aktivna posebna oblika avtofagije, znana kot lipofagija, ki selektivno razgrajuje LK. Z meritvami celične smrti pri različnih pogojih stradanja ob inhibiciji avtofagije in presnove LK smo ugotovili, da je, v nasprotju s kombinirano inhibicijo avtofagije in lipolize, hkratna inhibicija avtofagije in z DGAT encimi posredovane biogeneze LK učinkovita strategija za ogrožanje preživetja stradanih rakavih celic. Glavne ugotovitve te raziskave kažejo na sodelovanje med avtofagijo in LK pri varovanju celic raka materničnega vratu HeLa pred stresom, ki ga povzroči pomanjkanje hranil. Rezultati naše raziskave pripomorejo k razumevanju povezav med avtofagijo in LK v kontekstu preživetja rakavih celic in predstavljajo pomemben prispevek k razvoju novih pristopov za zdravljenje raka.

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Abbreviations

(E)FAF	...	(Essentially) fatty acid-free
3MA	...	3-methyladenine
AA	...	Amino acid
ACAT	...	CoA:cholesterol acyltransferase
ADRP	...	Adipose differentiation-related protein
AGPAT	...	Acylglycerol-3-phosphate acyltransferase
AMPK	...	AMP-activated protein kinase
AR	...	Androgen receptor
ATCC	...	American Type Culture Collection
ATGL	...	Adipose triglyceride lipase
ATP	...	Adenosine triphosphate
BafA1	...	Bafilomycin A1
BSA	...	Bovine serum albumin
cAMP	...	Cyclic adenosine monophosphate
ccRCC	...	Clear cell renal cell carcinoma
CE	...	Cholesterol ester
CMA	...	Chaperone-mediated autophagy
CoA	...	Acyl coenzyme A
CQ	...	Chloroquine
DAG	...	Diacylglycerol
DGAT1	...	Diacylglycerol acyltransferase-1
DMEM-GlutaMax	...	Dulbecco’s modified Eagle’s medium with high glucose and GlutaMAX supplement
DPBS	...	Dulbecco’s phosphate buffered saline
EBSS	...	Earle's Balanced Salt Solution
EGFR	...	Epidermal growth factor receptor
ER	...	Endoplasmic reticulum
FA-CoA	...	Fatty-acyl-CoA synthase
FAO	...	Fatty acid oxidation
FASN	...	Fatty acid synthase
FBS	...	Fetal bovine serum
FIT2	...	Fat-inducing transmembrane protein 2
FoxO1	...	Forkhead box O-1
GBM	...	Glioblastoma
GPAT	...	Glycerol-3-phosphate acyltransferase
HBSS	...	Hanks' Balanced Salt Solution
HCV	...	Hepatitis C virus
HER2	...	Human epidermal growth factor receptor 2
HPV	...	Human papillomavirus
HRP	...	Horseradish peroxidase

HSL	...	Hormone-sensitive lipase
KO	...	Knockout
LAMP1	...	Lysosome-associated membrane protein type 1
LAL	...	Lysosomal acid lipase
LD	...	Lipid droplet
LPA	...	Lysophosphatidic acid
MAGL	...	Monoacyl-glycerol lipase
MBOAT	...	Membrane-bound O-acyl transferase
MEF	...	Mouse embryonic fibroblast
mTOR	...	Mammalian target of rapamycin
OGR1	...	G-protein coupled receptor 1
PA	...	Phosphatidic acid
PAT	...	Phosphinothricin acetyltransferase
PE	...	Phosphatidylethanolamine
PI3K	...	Phosphoinositide 3-kinase
PLIN	...	Perilipin
PNPLA	...	Patatin-like phospholipase domain-containing protein
PPAR-γ	...	Peroxisome proliferator-activated receptor gamma
PR	...	Progesterone receptor
PVDF	...	Polyvinylidene difluoride
Rab25	...	Ras-related protein 25
ROS	...	Reactive oxygen species
SDS-PAGE	...	Sodium dodecyl-sulfate polyacrylamide gel electrophoresis
siRNA	...	Small interfering RNA
TAG	...	Triacylglycerol
TBS	...	Tris-buffered saline
TBST	...	Tris-buffered saline with 0.1% Tween
TIP47	...	Tail-interacting protein of 47 kDa
TLR	...	Toll-like receptor
TMRM	...	Tetramethylrhodamine methyl ester
ULK1	...	Unc-51-like kinase 1
WBR	...	Western blocking reagent
WHO	...	World Health Organization

Chapter 1

Introduction

1.1 Work Motivation

Cancer, among all other diseases, is the most significant cause of global mortality. It is classified as one of the diseases that can originate in nearly any tissue or organ of the human body. The development of cancer is not caused by any particular factor or condition; rather, it is believed to arise from the complex interplay of numerous causal factors, including genetic predisposition, environmental influences, and lifestyle choices. According to the World Health Organization (WHO), the disease caused an estimated 10 million deaths worldwide in 2018, making it the second leading cause of death globally (de Martel et al., 2020). Nevertheless, nearly half of all cancer-related deaths could be prevented through effective cancer management, including strategies such as cancer prevention, early detection, and treatment.

According to the WHO (2020), cervical cancer is the fourth most commonly diagnosed cancer in women worldwide (Bray et al., 2013). Around 90% of new cases and deaths globally in 2020 were reported in countries with low or moderate incomes (Sung et al., 2021). The primary cause of cervical cancer cases worldwide is the human papillomavirus (HPV), the most common viral infection of the reproductive system. In 1985, researchers confirmed that HPV can cause various types of cervical cancer using HeLa cells (a widely used human cervical cancer cell line). The lead researcher, Dr. Harald zur Hausen, who was behind this discovery was awarded the Nobel Prize in 2008, which paved the way for the development of an HPV vaccine. Although cervical cancer can be aggressive, invasive, and incurable if detected at a later stage, even in healthy and asymptomatic women, there are still various ways to prevent it, such as screening for HPV infection. Despite extensive research on cervical cancer in recent years, there is still a lack of knowledge about the underlying mechanisms driving the initiation and progression of the disease. This dissertation presents new and original findings that address the poorly investigated role of lipid metabolism in cervical cancer cells that could aid in developing novel treatments for the disease.

In recent decades, considerable efforts have been directed towards the development of efficient cancer treatments. Nonetheless, it has become increasingly evident that the inherent nature of cancer cells poses a significant challenge in our ability to successfully combat the disease. Genetic abnormalities are primarily responsible for alterations in cancer cell growth, proliferation and metabolism. However, an additional and poorly understood factor is that stressful microenvironmental conditions in the tumor compel cancer cells to adapt through specific reprogramming of their metabolism (Beloribi-Djefaflia et al., 2016; Pavlova & Thompson, 2016).

There is a growing amount of evidence that lipid metabolism plays a significant role in various types of cancer. Cancer cells rely on lipids to provide the energy necessary for their growth, proliferation, invasion, and survival (Currie et al., 2013). Consequently, targeting lipid metabolism could be crucial in reducing cancer cell growth. Lipid droplets (LDs), cellular organelles that primarily store fat and are present in almost all cell types, have recently been recognized as important factors in cancer development (Walther & Farese, 2009). Their accumulation is strongly associated with cancer cells' exposure to different stressful stimuli and has been linked to increased tumor aggressiveness and resistance to treatment (Jarc & Petan, 2019; Nieva et al., 2012; Petan et al., 2018). However, there is still a significant gap in our understanding of the molecular mechanisms involved in LD metabolism, which could potentially lead to the development of new strategies to reduce cancer growth.

In addition, the crucial role of autophagy as a mechanism of cancer cells' defense against various stresses has been well documented, as evidenced by the recent research focus (Galluzzi et al., 2015; Lim et al., 2021; Yun & Lee, 2018). The interplay between lipid metabolism, autophagy, and cancer on a molecular level is complex and not well understood. As a result, substantial work is still required to clarify the potential relationships among them.

Resistance to various therapies is a common feature among most cancer types, including those previously mentioned. Therefore, understanding the fundamental processes that cancer cells use to manipulate LDs and autophagy is crucial not only for advancing scientific and clinical research in these areas, but also for developing novel therapeutic and preventative strategies.

This dissertation presents fresh insights and novel findings in the fields of cancer, autophagy, and LD research.

This research investigates the interplay between LD metabolism and autophagy in nutrient-deprived cancer cells, examining the impact of targeting LDs and autophagy on cancer cell survival, and addressing questions about LD turnover under different nutrient stress conditions, the activation requirements of autophagy/lipophagy and lipolysis, and the contributions of LDs and autophagy to the resistance of cancer cells to nutrient deprivation-induced stress.

1.2 LD Metabolism and Basic Morphology

LDs are emerging as key participants in lipid metabolism in a variety of stressed cells. These newly recognized cytosolic organelles have a unique structure among organelles, with a hydrophobic neutral lipid core covered by a phospholipid monolayer embedded with various proteins (Figure 1 A, B). They store mostly triacylglycerols (TAGs) and cholesterol esters (CEs) within their core and have long been regarded only as inert fat storage sites in eukaryotic cells (Walther & Farese Jr., 2012). LDs are heterogeneous in size because they can easily grow, fuse and shrink. The sizes of LDs may range anywhere from 20 to 40 nanometers to 100 micrometers. In adipocytes, these organelles are often much bigger, and in certain cases they may make up the majority of the cell. On the other hand, in other cells, LDs may only be formed under particular circumstances, and if they are present, they are typically much smaller (Farese & Walther, 2009; Walther & Farese Jr., 2012).

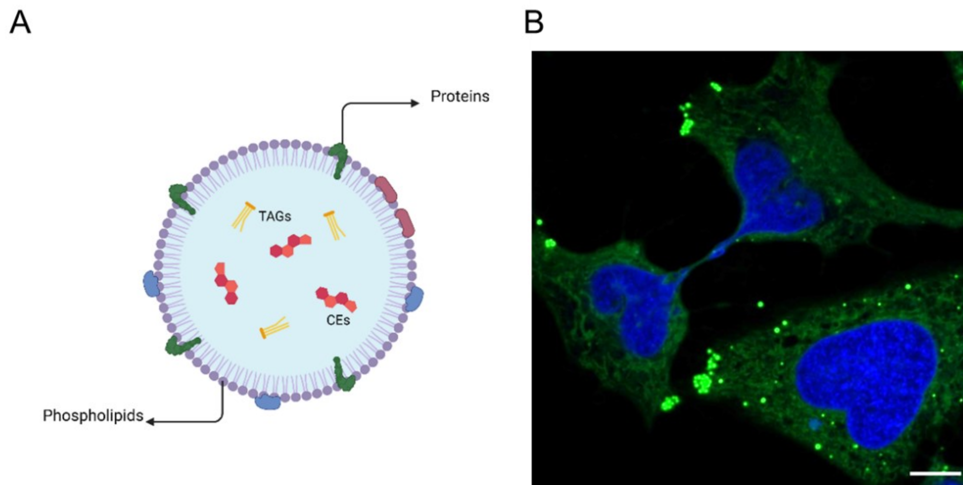


Figure 1: A schematic representation of LD structure. A) The neutral core of LDs is made up mostly of TAGs and CEs, and it is surrounded by a single layer of phospholipids in which numerous proteins may be found. Created in BioRender.com. B) Two HeLa cells undergoing cell division with heart-shaped nuclei (blue) surrounded by LDs (green). Nuclei were stained with Hoechst 33342 nuclear stain, and LDs with Bodipy 493/503 and visualized by live-cell imaging by confocal microscopy.

LDs are typically visible in clusters under the microscope, but they can also be dispersed and mobile. Cancer cells tend to accumulate more LDs compared to normal cells due to their high metabolism and energy demands (Cruz et al., 2020; Tirinato et al., 2020; Wu et al., 2020). Proteomic and other studies have identified hundreds of proteins associated with LDs across various cells and tissues, with perilipins being a common type (Brasaemle et al., 2004; Khaddaj et al., 2022; S. Xu et al., 2018; Yang et al., 2012). Mammalian cells have five perilipin proteins, 1 to 5 (PLIN 1-5), which are the best characterized LD-associated proteins and are discussed in more detail in Section 1.3.3. Briefly, perilipins are important for LD stabilization providing structural integrity and stability of LDs, and are involved in the dynamic regulation of LDs (i.e. their formation, growth, fusion). Perilipins also interact with enzymes involved in lipolysis and lipogenesis, influencing the availability of stored lipids for energy production or other cellular processes (Brasaemle, 2007; Granneman et al., 2011).

LDs are essential for various aspects of cellular metabolism. One of their primary functions is the storage of energy substrates, but they also have other roles including the maintenance of endoplasmic reticulum (ER) homeostasis, protection against lipotoxicity, lipid mediator production, control of fatty acid release to mitochondria necessary for oxidation, storage of vitamins, and may serve as platforms for protein maturation and storage (Olzmann & Carvalho, 2019). These functions make these organelles important for various functions within cells such as cell survival, and understanding their precise role in cell metabolism could lead to the new therapies of many diseases including cancer (Figure 2).

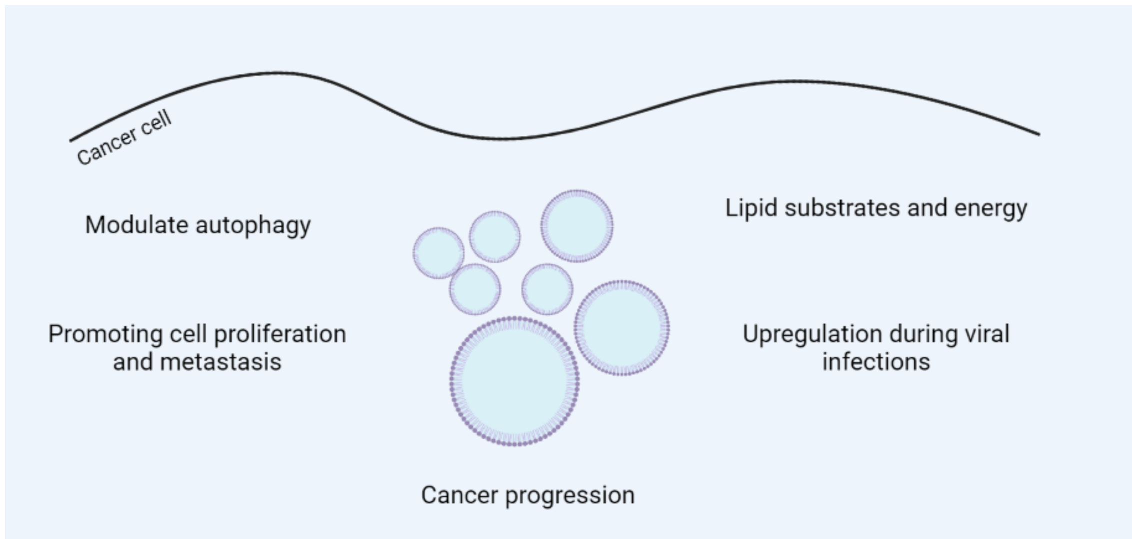


Figure 2: There are several potential roles that LDs may play in cancer. Created in BioRender.com.

1.2.1 LDs in cancer

LDs are dynamic organelles that store and regulate lipids in cells. But, what about their role in one of the most serious diseases worldwide – cancer? Although it is known that cancer cells tend to accumulate more LDs than healthy cells, the exact mechanisms behind this build-up and its correlation with cancer progression, metastasis, and regulation are not yet fully understood. Furthermore, the association between LD function and intracellular processes remains poorly understood. The presence of LDs (at that time called 'vesicles') has been observed in cancer cells since the late 1950s, but at the time, little was known about their composition or functions (Abbas et al., 1982; Aboumrad et al., 1963). It was not known until approximately two decades later when researchers discovered a correlation between the presence of LDs and tumor aggressiveness (Ramos & Taylor, 1974). Today, it is widely recognized that different types of cancer cells have a tendency to accumulate higher levels of lipids and LDs (Z. Li et al., 2020; Lung et al., 2022; W. Luo et al., 2022; X. Luo et al., 2017; Petan, 2023; Petan et al., 2018; Tirinato et al., 2017). Additionally, it is well-established that cancer cells possess unique and fundamental characteristics that distinguish them from normal, healthy tissues (Baghban et al., 2020). These characteristics include uncontrolled growth, abnormal cell proliferation, changes in cellular structure, genomic instability, increased resistance to cell death, and alterations in metabolism, including lipid metabolism (Baghban et al., 2020). When we combine the limited understanding of mechanisms, causes, and regulation of LDs with the complexity of cancer metabolism and the tumor microenvironment, it becomes evident that further investigation of both LDs, lipid metabolism and cancer is necessary as a prerequisite for developing new therapies and treatments. The specific traits mentioned above make tumors particularly challenging to study, comprehend and effectively treat, considering the intricate role of LDs and lipid metabolism in their biology.

Altered lipid metabolism within tumor cells plays a significant role. Tumor cells often exhibit metabolic alterations, such as enhanced glucose uptake or altered nutrient utilization, to meet their high energy demands for rapid growth (Hanahan, 2022). Hence, in order to explore the accumulation and potential functions of LDs in a specific cancer cell line chosen for this study, we conducted experiments on their dynamics and behaviour under varying nutrient conditions. This analysis is crucial as it sheds light on how nutrient

availability can impact the growth and proliferation of cancer cells. Therefore, it is important to emphasize that several studies have highlighted the involvement of LDs or LD-associated enzymes in cancer cells when exposed to fluctuations in nutrient levels. Studies in prostate cancer cells have revealed that inhibiting the enzyme diacylglycerol acyltransferase 1 (DGAT1) induces cell death, while its overexpression promotes the growth of prostate cancer cells. These findings highlighted the significance of DGAT1 in LD synthesis, along with its potential role in prostate cancer development and progression (Mitra et al., 2017). Furthermore, inhibiting DGAT1 has been shown to decrease the aggressiveness of prostate cancer cells and impede tumor growth *in vivo*. This underscores the potential therapeutic value of targeting DGAT1 in mitigating the progression and severity of prostate cancer (Nardi et al., 2019). Moreover, LDs play a crucial role in clear cell renal cell carcinoma (ccRCC). An example of this is a study that showed that interfering with TAG synthesis hampers the growth of ccRCC tumors and ccRCC cells under tumor-like conditions such as hypoxia. In principle, authors observed that simultaneous inhibition of DGAT1 and DGAT2 significantly impaired tumor growth *in vivo* due to an increase in cell death (Ackerman et al., 2018). In one of the recent studies it was showed that in melanoma cells, DGAT1 is up-regulated and acts as an oncoprotein. In addition, it promotes the formation of LDs, serving as a reservoir for excessive fatty acids. Moreover, inhibiting this enzyme leads to an increase in fatty acid oxidation (FAO), generation of reactive oxygen species (ROS) and activation of NF-E2-related factor 2 (NRF2) which is also important for inflammation (Wilcock et al., 2022). Furthermore, high levels of DGAT1 in individuals with glioblastoma (GBM) are linked to poor survival, as DGAT1 protects GBM cells from lipotoxicity by facilitating the storage of fatty acids in LDs. Conversely, in the same study it was shown that inhibiting DGAT1 induces tumor cell death in GBM through oxidative stress (Cheng et al., 2020). In addition, another study showed that DGAT2 decreases liver cancer aggressiveness by suppressing cell cycle-related genes. The same study reported that DGAT2 downregulation is associated with liver carcinoma, while its overexpression inhibits cancer cell proliferation (Y. Li et al., 2019). In autophagy-related studies, it was showed that during starvation-induced autophagy, the formation of LDs through the activity of DGAT1 plays a crucial role in preserving mitochondrial function (Nguyen et al., 2017). In the same study, different cell lines (Hela, Huh7, and U2OS) were employed to observe the behavior of LDs during nutrient deprivation, revealing the consistent accumulation of LDs as a recognized characteristic in cancer cells (Nguyen et al., 2017). Another study demonstrated that in lung cancer cells, LDs promote cancer cell survival during starvation conditions (Lung et al., 2022). Recently it was shown that the activation of phosphoinositide 3-kinase (PI3K)/AKT/mTOR pathway is closely associated with increased LD accumulation (Penrose et al., 2016). De-Gonzalo de Calvo and colleagues showed the accumulation of intratumor cholesterol is linked to increased breast cancer proliferation and aggressive behavior (de Gonzalo-Calvo et al., 2015). Furthermore, additional studies have revealed a positive correlation between CE accumulation, metastasis and poor survival in other tumors as well (Geng et al., 2016; J. Li et al., 2016; Yue et al., 2014).

On the other hand, LDs may be also upregulated during viral infections (Figure 2). To support this, the infection of liver cells by the Hepatitis C virus (HCV) is shown to be linked to the lipid metabolism. The same study demonstrated that DGAT1 enzyme is a crucial host factor for HCV infection (Herker et al., 2010). In addition, an interesting example of the impact of lipid metabolism in cancer cells is the role of FAO-derived adenosine triphosphate (ATP) in driving chemoresistance. Studies have demonstrated that in breast cancer and leukemic stem cells, ATP produced through FAO contributes to the development of resistance against chemotherapy (Farge et al., 2017; Shyu et al., 2018; T. Wang et al., 2018). Interestingly, some of the studies point to the direction of LD

involvement in lipid signalling pathways because they serve as reservoirs for prostaglandins functions. Our group recently summarized their potential roles in mediator production in our review paper (Jarc & Petan, 2020). Briefly, LDs serve as storage compartments for various types of lipids. When these lipids are released from the droplets, they can either directly function as signaling molecules or undergo conversion into other molecules with signaling capabilities. For instance, free fatty acids released from LDs may directly contribute in immune response by binding to cell surface receptors such as Toll-like receptors (TLRs) (Jarc & Petan, 2020; Monson et al., 2021). Altogether, these studies summarize the most important findings on LDs in cancer cell metabolism and this may be summarized as follows: storage of excess lipids (TAGs, CE) and their release when needed, protection against lipotoxicity, cholesterol homeostasis, lipid signalling or chemoresistance.

However, despite this knowledge, we still lack a thorough understanding of the exact mechanisms and regulation of LD accumulation within cancer cells. Given the potential significance of LDs in cancer cells, further research is still needed to elucidate the underlying mechanisms and explore potential therapeutic targets. By doing so, we may be able to develop more effective treatments for cancer and improve outcomes for those affected by this disease.

1.3 LD biogenesis

Over the past decade, there have been many studies dedicated to understanding the cell biology of LDs. However, despite many efforts made in recent years, the precise molecular mechanism responsible for the formation of these organelles is not yet fully described. Studies suggest that in eukaryotic cells, LDs are created through a process of budding from the surface of the ER. This process occurs concurrently with the accumulation of neutral lipids, primarily TAGs and CEs (Wilfling et al., 2014). Thus, the biogenesis of LDs begins with the synthesis of TAGs and CEs within the ER.

1.3.1 Lens formation and synthesis of neutral lipids

TAGs and CEs are formed when an activated fatty acid is esterified to diacylglycerol or a sterol (cholesterol), respectively. Both of these processes within cells are controlled by different enzymes. These lipids are synthesized in the ER from precursor molecules, such as fatty acids and glycerol. The *sn*-glycerol-3-phosphate pathway, also known as the Kennedy pathway, is the major route of TAG biosynthesis. This process was initially characterized in the late 1950s by Professor Eugene Kennedy and his colleagues (Kennedy & Weisst, 1956; Walther et al., 2017). TAGs are produced by the action of DGAT1 and DGAT2 enzymes and other enzymes which will be described below, whereas CEs are primarily formed by the enzymatic action of acyl-CoA:cholesterol O-acyltransferases (ACAT1 and ACAT2) (Wilfling et al., 2014). In the first step of TAG synthesis, fatty acids are sequentially added to glycerol-3-phosphate backbone which results in the production of lysophosphatidic acid (LPA), phosphatidic acid (PA) and diacylglycerol (DAG). Enzymes such as glycerol-3-phosphate acyltransferase (GPAT), acylglycerol-3-phosphate acyltransferase (AGPAT) and lipin are responsible for catalyzing these reactions. DGAT enzymes are responsible for mediating the last stage of the production of TAGs (M. Gao et al., 2019; Walther et al., 2017). Once synthesized, lipids such as TAGs accumulate within the ER membrane allowing for LD formation and their budding from the ER to the cytosol (Figure 3). The nascent LDs then grow in size through the continuous addition of lipids. This growth process involves the recruitment of additional neutral lipids from the ER or the fusion of smaller LDs to form larger LDs. Proteins known as PLINs,

such as PLIN3, and other associated proteins, such as tail-interacting protein of 47 kDa, (TIP47) may be involved in LD stabilization, maturation or anchoring to the ER (M. Gao et al., 2019; Itabe et al., 2017; Skinner et al., 2009).

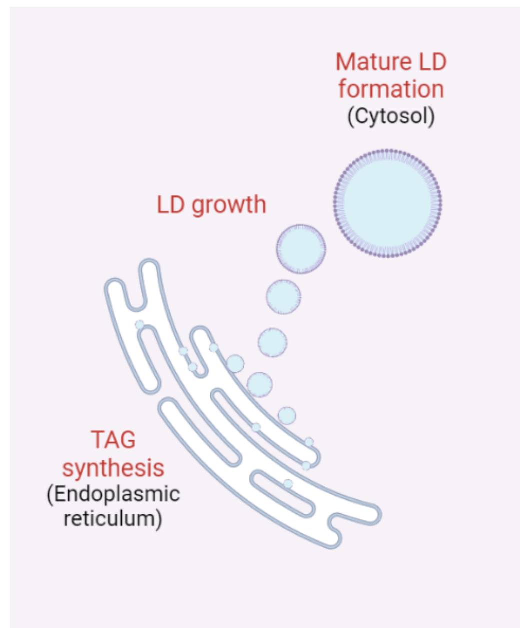


Figure 3: Steps in LD biogenesis. Neutral lipid synthesis, neutral lipid accumulation in ER and LD formation/growth and budding from ER. Created in BioRender.com.

1.3.2 Structure and function of DGAT enzymes

DGAT enzymes play a crucial role in the final step of TAG biosynthesis, which involves converting DAG to TAG by esterifying DAG and acyl coenzyme A (CoA). Two transmembrane proteins, namely DGAT1 and DGAT2, carry out this conversion in the ER. Despite their similar metabolic functions, DGAT1 and DGAT2 have distinct amino acid sequences, which affect their interactions with membranes and organelles. As a result, DGAT enzymes play slightly different but complementary and often overlapping roles at the cellular and organismal levels. This makes them important and essential players in lipid metabolism (Yen et al., 2008).

Similarly as acyl-CoA:cholesterol acyltransferases (ACAT) enzymes, DGAT1 is a member of the MBOAT (membrane bound O-acyl transferase) family, which comprises membrane-bound O-acyl transferases. In the majority of species, DGAT1 encodes a protein of nearly 500 amino acids and has a calculated molecular weight of 55 kDa. One shared characteristic among members of the MBOAT family is a lengthy hydrophobic region that includes asparagine and histidine residues located in the proposed active site. DGAT1 is composed of several transmembrane domains and is predominantly located in the small intestine, whereas also being notably expressed in adipose tissue. DGAT1 is positioned on the ER and plays a significant role in generating LDs while safeguarding ER membranes against the harmful effects of lipotoxicity (Bhatt-Wessel et al., 2018; Nguyen & Olzmann, 2017). In addition, it has the ability to form both di- and tetramers. The C-terminal region of the protein extends into the lumen of the ER (Figure 4), and it plays a crucial role in the enzymatic activity of DGAT1, which is primarily focused on the metabolism of exogenous fatty acids. It appears that DGAT2 is more effective than DGAT1, possessing a greater affinity for its substrates which will be discussed in the next sections.

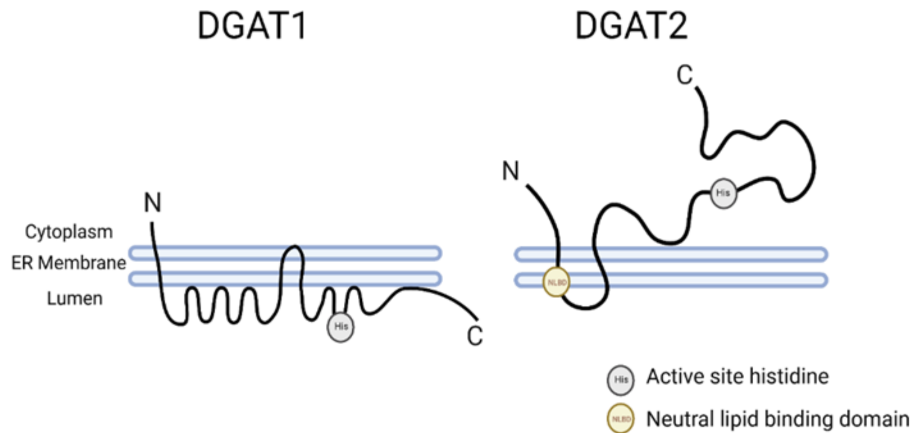


Figure 4: A visual presentation showing the basic structural properties of DGAT enzymes. Created in BioRender.com. Adapted from Stone et al. 2022.

Initially, DGAT2 was isolated from the LD fraction obtained from the fungus Mortierella ramanniana. It contains two transmembrane domains, one of which is mainly responsible for targeting it to ER. In addition, the calculated molecular weight of this protein is around 44 kDa. It is different from DGAT1 because it only has a hydrophobic region (Figure 4). DGAT2 is primarily found to be present on ER, but also a small fraction of it was shown to be associated with LDs (McFie et al., 2018). While DGAT1 has been found to possess acyltransferase activities beyond the esterification of DAG *in vitro*, DGAT2 does not exhibit such activities. Recently, it was shown that the DGAT2 enzyme may also compensate for the deficiency of DGAT1 when it comes to LD formation in intestinal stem cells (Van Rijn et al., 2019). More recently, the impact of PF-06424439, a selective DGAT2 inhibitor, on MCF7 breast cancer cells that were exposed to X-rays was analyzed. Results indicate that treatment with the inhibitor for 72 hours decreased LD content, reduced cell migration, and had no effect on cell proliferation. Furthermore, pre-treatment with PF-06424439 followed by radiation exposure increased the radiosensitivity of MCF7 cells (Nisticò et al., 2021). Additionally, the physiological functions of DGAT enzymes have been studied using knockout (KO) animals, revealing significant differences between these two enzymes. DGAT1-deficient mice have been found to be viable and possess body fat but are comparatively lean and exhibit resistance to high-fat diet-induced obesity (H. C. Chen et al., 2002; Smith et al., 2000). These findings have raised interest in the physiological functions of DGAT1, as it has been suggested that it may play a crucial role in lipid metabolism and adipose tissue biology. Additionally, recent studies have shown that DGAT1-mediated TAG synthesis not only functions in energy storage but also serves to protect adipocytes from lipotoxicity during lipolysis, indicating that DGAT1 has multiple roles in regulating lipid homeostasis (Chitraju et al., 2017). Such discoveries have implications for potential therapeutic strategies targeting DGAT1 in the treatment of obesity, diabetes, and other metabolic disorders (Smith et al., 2000). Unlike DGAT1, DGAT2-deficient mice died soon after birth due to impairment of skin permeability, which may lead to death (Chitraju et al., 2017). Recently it was shown that overexpression of DGAT2 stimulates LD biogenesis in bovine satellite. Researchers also found that both DGAT1 and DGAT2 enzymes exhibit similar specificity for fatty-acyl-CoA synthase (FA-

CoA) substrates (Cases et al., 2001). Most importantly, inhibition of these two enzymes has been considered as a therapeutic target. Other researchers conducted experiments to evaluate the impact of T863 (DGAT1 inhibitor) both *in vitro* and *in vivo*. They showed that oral administration of T863 in mice resulted in postponed fat absorption, as well as weight reduction, enhanced insulin sensitivity and reduced hepatic steatosis in a mouse model with diet-induced obesity (Cao et al., 2011). Most recently, the effects of PF-06424439 were reported on the levels of ceramide and O-acylceramide in the liver and LDs from 8-week old mice fed a high-fat diet enriched with oleate. Mice loaded with oleate had increased LDs and steatosis as compared to the control group (Tomimoto et al., 2015). Adding this inhibitor to drinking water did not alter the levels of total ceramides and O-acylceramides in the control group, but it led to a reduction in the group of mice that were fed with a high-fat diet. These findings confirmed that the synthesis of O-acylceramide in mice is primarily governed by DGAT2 (Senkal et al., 2017).

In conclusion, although biochemically different, both DGAT1 and DGAT2 enzymes are important for lipid metabolism and LD biogenesis.

1.3.3 LD-associated proteins

Protein composition of LDs received a significant amount of attention over the last two decades of LD research. In order for LDs to perform all of the many biological roles that have been ascribed to them, a large number of proteins have been suggested as candidates for the roles on the surface of LDs. LDs are only cellular organelles that are surrounded by a phospholipid monolayer rather than a bilayer (Vanni, 2017). As such, LD binding proteins may have distinct and unique structural characteristics compared to the other membrane proteins. Different studies have revealed the presence of proteins that have more than one transmembrane domain (Brasaemle et al., 2004). However, it is difficult to determine if the proteins are directly related to the surface of the LDs or whether they are localized to the membranes of other organelles. Two most important LD resident or structural proteins found on the surface of LDs are PLIN1 and PLIN2. PLINs are considered as main markers of LDs, since they have been discovered on the surface of these molecules (Bickel et al., 2009; S. Xu et al., 2018). PLIN1 was shown to be a prominent phosphorylated protein in white adipose tissue when it was first identified (Blanchette-Mackie et al., 1995). In addition, this protein is highly expressed in mature adipocytes, whereas PLIN2 protein expression is mostly present in immature preadipocyte cells. PLIN2 is also known as adipose differentiation-related protein (ADRP), and is shown to be highly expressed in the liver and may strongly contribute to the development of fatty liver disease. In mouse embryonic fibroblasts (MEFs), PLIN2 has shown to stimulate the process of degradation of lipids by lipolysis, and following the stimulation of lipolysis is heavily marked near LDs (Takahashi et al., 2016). Additionally, it has been shown that the stability of this protein is increased when it is situated on the surface of LDs. Structurally, all PLIN proteins share a similar domain, called Phosphinothricin acetyltransferase (PAT) domain and 11-mer motif repeat, with the exception of the PLIN4 where this domain is not found. What is known about this domain is that usually it is associated with the surface of LDs. Structure of these proteins is very important for various studies because it was shown that several point mutations within the 11-mer repeats of PLIN1–3 altered the amphipathic amino acid alignments, which consequently led to eliminated connection to LDs (Itabe et al., 2017). All these proteins share structural similarities and as such they are performing similar functional roles. Their binding ability to LDs is shown to be essential especially in response to metabolic stimuli and they may contribute to the lipid flow into and out of LDs. PLIN5 is a more ER-associated protein and is important for lipid trafficking, storage and mobilization (Bartholomew et al., 2012). Additionally, these

proteins may interact with other cellular organelles that are essential for the formation of LDs because they may govern the access of other proteins (Blanchette-Mackie et al., 1995). Another protein found to be associated with LDs is TIP47 (tail-interacting protein) which is responsible for maintaining trans-Golgi network and it has apolipoprotein-like characteristics (Bulankina et al., 2009). Beside this, a more recent protein found to be connected to LDs is comparative gene identification-58 (CGI-58). CGI-58 is involved in the regulation of triglyceride metabolism and lipolysis. It binds to LDs and activates the enzyme adipose triglyceride lipase (ATGL), promoting lipolysis and release of fatty acids (Gruber et al., 2010). Additionally, fat-inducing transmembrane protein 2 (FIT2) is showed to be involved in LD formation and size regulation. It interacts with the LD membrane and promotes lipid incorporation into the droplet. Research suggests that in addition to the proteins mentioned earlier, other organelle-related proteins, such as septins (a type of cytoskeletal protein), together with FIT2, help to organize or assist in LD biogenesis. If the function of these proteins is impaired, it was shown that it can significantly delay the process of nascent LD formation (F. Chen et al., 2021). Adipose triglyceride lipase (ATGL) is also found to be recruited on LD surface where it can facilitate TAG hydrolysis in LDs (Lass et al., 2011). Additionally, some proteomic studies also showed that ribosome proteins may be found on LDs in different organisms (S. Xu et al., 2018).

While our understanding of LD proteins continues to advance, it is crucial to explore their potential roles in various diseases, particularly cancer. One such example is the investigation of PLIN proteins, where it has been observed that their expression can differ across different types of cancer cells. Specifically, research has demonstrated that the expression of PLIN3 is higher in breast cancer tissue samples compared to the healthy, normal tissue samples. In contrast, the expression of other proteins belonging to the PLIN family was found to be lower in cancerous tissues compared to normal tissues (X. Zhang et al., 2021). These findings highlight the importance of studying LD proteins and their implications in diseases, as they can provide valuable insights into the molecular mechanisms underlying cancer development and progression.

Despite the fact that these proteins are important for managing LD dynamics, movement or transport of lipids in and out of LDs, there is still a lack of information about all of the roles that these proteins may play in controlling the dynamics of LDs and in their participation in other processes such as lipolysis, autophagy/lipophagy, lipogenesis and other similar processes.

1.3.4 Molecular mechanism of autophagy

Autophagy is a fundamental process that is crucial for maintaining cellular homeostasis, as it enables the degradation of unnecessary or damaged components within cells (Dikic & Elazar, 2018; Glick et al., 2010). The process is highly conserved, and it occurs in all eukaryotic cells. Autophagy is activated in response to various stresses, including nutrient deprivation, hypoxia, oxidative stress, and pathogen infection (Gatica et al., 2018; Klionsky et al., 2016; Koizume & Miyagi, 2016; Parzych & Klionsky, 2014). When a cell undergoes autophagy, it sequesters cytoplasmic material, including proteins, organelles, and lipids, in a double-membrane vesicle called an autophagosome. The autophagosome then fuses with a lysosome, a specialized organelle that contains hydrolytic enzymes, leading to the degradation of the sequestered material. The most known type of autophagy is macroautophagy, which is commonly referred to as autophagy. This type of autophagy is initiated by the formation of a phagophore, a crescent-shaped membrane structure that encloses cytoplasmic material. The phagophore then expands to form an autophagosome, which eventually fuses with a lysosome (Parzych & Klionsky, 2014). The sequestered material is then degraded and recycled. Another type of autophagy is chaperone-mediated

autophagy (CMA), which involves the direct transport of specific proteins to lysosomes for degradation. CMA is mediated by heat shock proteins, which recognize specific protein sequences and deliver them to lysosomes for degradation (Parzych & Klionsky, 2014). Microautophagy is another type of autophagy, which allows for the direct engulfment of cytoplasmic material by lysosomes. In this process, the lysosomal membrane invaginates and engulfs the cytoplasmic material directly, leading to its degradation (Parzych & Klionsky, 2014). Furthermore, autophagy can also occur in a cargo-selective manner, where specific components are targeted for degradation (Vargas et al., 2023). For instance, mitophagy is a type of autophagy that involves the degradation and removal of damaged mitochondria, while lipophagy is a type of autophagy that selectively degrades LDs (Shin, 2020; Singh & Cuervo, 2012).

Autophagy is a complex process that plays a critical role in maintaining cellular homeostasis. Its ability to selectively degrade and recycle cellular components makes it an essential process in many physiological and pathological conditions. The different types of autophagy have specific functions, and their dysregulation can lead to various diseases, including cancer, neurodegeneration, and metabolic disorders. Thus, understanding the mechanisms of autophagy is crucial for developing new therapeutic strategies for treating these diseases.

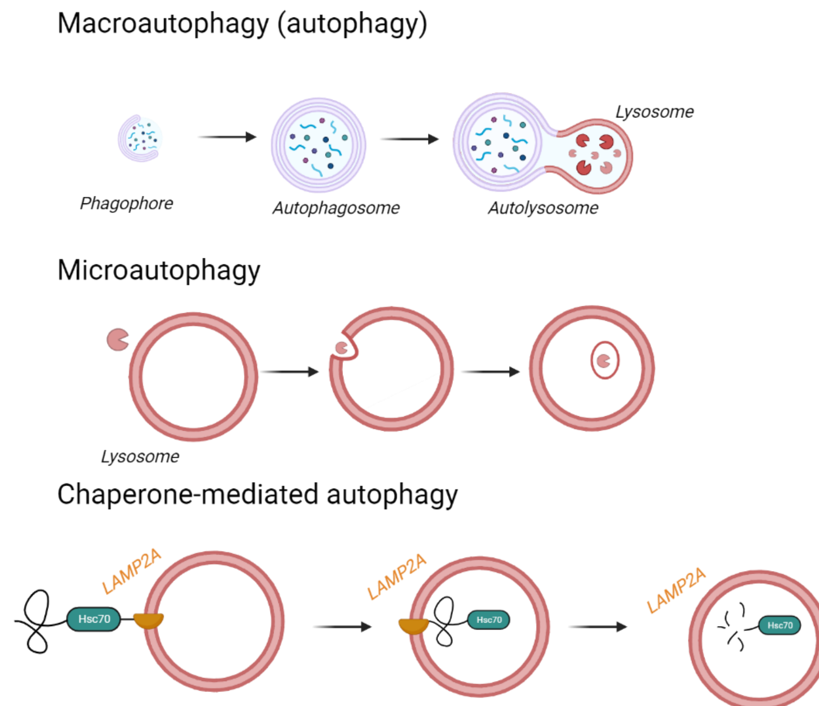


Figure 5: Three different types of autophagy: macroautophagy, microautophagy and chaperone-mediated autophagy. Adapted from Aburto et al., 2012 (Aburto et al., 2012). Created using BioRender.com.

Two important conjugation systems involved in autophagy are: ubiquitin-like conjugation system, which involves the covalent attachment of ubiquitin-like proteins to substrates, leading to their targeting for autophagic degradation. The key components include ATG7, an E1-like enzyme, and ATG10, an E2-like enzyme, which facilitate the conjugation of Atg12 to Atg5. This ATG12-ATG5 complex then interacts with ATG16L1 to form the autophagy-specific E3-like ligase complex, which is essential for autophagosome

formation (Mizushima, 2020). The second one is LC3-phosphatidylethanolamine (PE) conjugation system: LC3, a ubiquitin-like protein which is conjugated to PE to form LC3-II, the lipidated form that is tightly associated with autophagosomal membranes. This conjugation process involves the sequential action of several enzymes, including ATG7 and ATG3 (Figure 5). ATG7 activates LC3 and transfers it to ATG3, which facilitates the conjugation of LC3 to PE, leading to its incorporation into autophagosomal membranes (Tanida et al., 2004).

These conjugation systems are critical for the proper progression of autophagy, cargo recognition, and autophagosome formation. Dysregulation of these systems can impair autophagy and contribute to the development of various diseases, including cancer, neurodegenerative disorders, and metabolic disorders.

1.3.5 LDs and autophagy/lipophagy

The interplay between LDs and autophagy is a complex relationship marked by intricate regulatory mechanisms and functional crosstalk. As mentioned earlier, LDs serve as organelles specifically designed for lipid storage and undergo dynamic alterations in response to cellular signals and metabolic requirements (Jarc & Petan, 2019; Petan, 2023; Zadoorian et al., 2023). Interestingly, these conditions that trigger changes in LDs and their turnover often coincide with the activation of autophagy. Autophagy, a highly regulated cellular process, plays a critical role in maintaining cellular homeostasis by degrading and recycling cellular components, including also LDs (Galluzzi et al., 2014; Galluzzi, Baehrecke, et al., 2017; Galluzzi, Bravo-San Pedro, et al., 2017). Autophagic activation can be initiated by a diverse range of stimuli, such as nutrient stress, energy stress, oxidative stress, ER stress, infections, cellular and DNA damage, as well as the accumulation of misfolded proteins and protein aggregates and many others (Galluzzi et al., 2014). Therefore, this complexity arises from the bidirectional interactions between LDs and autophagy. On the one hand, LDs play a significant role in regulating autophagy, while on the other hand, autophagy is emerging as a crucial cellular process that can influence both the biogenesis or degradation of LDs. To understand this work effectively, it is necessary to analyze its components from different perspectives. Nevertheless, it is crucial not to overlook the fact that this initial separation between perspectives or stories can also serve as a crucial connection between them. Taking that into consideration, the next sections aim to provide insights into the potential influence of LDs on autophagy and its components and vice versa, the influence of autophagy/lipophagy on LD turnover within different types of cells or tissues.

One well-established association is the ability of autophagosomes to engulf LDs through a process known as lipophagy. A more detailed discussion on this topic can be found in Chapter 1.4.2. Briefly, the majority of research investigating lipophagy has focused only on hepatocytes. A recent study conducted on primary rat hepatocytes and cells subjected to acute nutrient deprivation revealed a close association between LDs and lysosomes. This proximity enables direct lipid transfer between these cellular compartments (Schulze et al., 2020). In addition, it was observed that LDs can supply lipids essential for the formation of autophagosomes, which are crucial for the process of autophagic degradation (Drizyte-Miller et al., 2020). Furthermore, in addition to the aforementioned research conducted on mammalian cells, it has been demonstrated that the initiation of autophagy is closely linked to LDs. These LDs act as reservoirs for lipids, facilitating the formation of autophagosomes. Notably, this initiation process heavily relies on the presence of the PNPLA5 protein, which is located on LDs. Furthermore, it was observed that *de novo* phospholipid synthesis can take place on LDs, thereby facilitating the formation of autophagosomes (Ogasawara et al., 2020; Shpilka et al., 2015). Overall, there is a direct correlation between the levels of

LD content and the autophagic activity within cells (Dupont et al., 2014). Additionally, it was shown that lipophagy plays a role in the release of fatty acids during nutrient starvation (W. Cui et al., 2021). Recently, a captivating study shed light on the accumulation of LDs during nutrient deprivation via autophagy. This investigation highlighted the crucial involvement of a small GTPase known as RalA. RalA assumes a pivotal role by recruiting phospholipase D to lysosomes, initiating the localized generation of phosphatidic acid, and ultimately promoting the growth of LDs (Hussain et al., 2021). These are only several examples that are covered in more detail in Chapter 1.4.2. In summary, the relationship between LDs or LD-derived fatty acids and autophagy-related compartments such as autophagosomes or lysosomes has received significant attention. However, the detailed mechanisms underlying these connections are not yet fully understood, including the exact process by which LDs are degraded through lipophagy.

On the other hand, in yeast cells, it was shown that upon nitrogen starvation which is a general stimulus for activating autophagy, storage of lipids is necessary for autophagy induction (D. Li et al., 2015). However, in mammalian cells (i.e. mice), it was shown that autophagy is highly contributing to TAG generation and LD biogenesis during differentiation of neutral lipid-storing cells (Singh, Xiang, et al., 2009; Y. Zhang et al., 2009). This was also shown more recently in endothelial vascular cells (Ren et al., 2021). Most importantly and for our investigations, it was shown that the autophagy may provide lipids for the biogenesis of LDs under certain starvation conditions in MEFs (Nguyen et al., 2017; Rambold et al., 2015). The intricate interplay between lipid metabolism and autophagy plays a significant role in governing cell homeostasis, cell survival, and programmed cell death. Specifically, already mentioned studies with MEFs have shown that when MEFs experience acute amino acid starvation, autophagy drives LD biogenesis (Nguyen et al., 2017). For this instance, LD biogenesis occurs in order to protect cells from possible mitochondrial damage that may be caused by overload of free fatty acids (Nguyen et al., 2017). Moreover, LD biogenesis during acute stress is shown to be highly dependent on DGAT1 enzyme (Nguyen et al., 2017). Additionally, a separate study showed that excess fatty acids that have been temporarily stored within LDs are then released via lipolysis, allowing for a more controlled transfer of autophagy-derived fatty acids into mitochondria (Rambold et al., 2015). In addition, studies have shown that during periods of starvation, LDs can transport fatty acids to various locations or compartments within cells, such as mitochondria, where they are subsequently utilized for FAO. The same study also revealed a close association between LDs and mitochondria, which is further enhanced during nutrient deprivation. Moreover, the activation of AMP-activated protein kinase (AMPK) has been found to strongly influence LD mobility (Herms et al., 2015). Nevertheless, in hepatocytes, LC3 lipidation system which is necessary for autophagosome formation is found to be involved in LD formation and this formation happened to occur in ATG7-dependent manner under starvation conditions (Shibata et al., 2009). Contrary, in a study done on mice and liver, it was shown that LD-associated protein PLIN2 can influence autophagy. More precisely, authors demonstrated that PLIN2 KO mice had increased levels of autophagy in liver (Tsai et al., 2017). Interestingly, it was shown that during starvation, LDs may help maintain ER homeostasis serving as a buffering system for fatty acids that ER needs, and therefore regulate autophagy and that the possible LD deficiency may lead to conditional inhibition of autophagy (Velázquez et al., 2016). In hepatic stellate cells, it has been demonstrated that autophagy plays a role in regulating the turnover of LDs and that this process is mediated by ROS-dependent activation of Ras-related protein 25 (Rab25) (Z. Zhang et al., 2017). However, although there is some understanding of LDs in MEFs or hepatocytes, there is still a significant lack of knowledge regarding the role of LDs and autophagy-dependent formation of them in other cell types, including cancer cells. In recent studies, it has been demonstrated that lipogenesis,

facilitated by ovarian cancer G-protein coupled receptor 1 (OGR1), plays a vital role in enabling metabolic adaptation to acid stress through autophagy in various cancer cell lines, such as MCF7, MDA-MB-231, MDA-MB-468, and others (Pillai et al., 2022). The formation of LDs under these circumstances is attributed to the autophagic degradation of ketogenic amino acids (Pillai et al., 2022).

In conclusion, our understanding of the connection between LDs and autophagy in cancer cells is still in its early stages. However, studies on various cell types offer promising insights for cancer cell lipid metabolism. Further research is needed to reveal the specific mechanisms of LD-autophagy interaction and the role of altered lipid metabolism in cancer development. Additionally, considering the heterogeneity of cancer types and their unique metabolic characteristics is crucial for tailored therapeutic interventions. Exploring the crosstalk between LDs, autophagy, and other cellular processes can enhance our understanding of cancer biology.

1.4 LD Breakdown

LDs can be broken down by two distinct pathways: lipolysis and lipophagy (Zechner et al., 2012). Lipolysis is mediated by cytosolic neutral lipases, enabling a gradual release of lipids, whereas in lipophagy, LDs are broken down by acid lipases in the lysosome along with proteins and other material.

1.4.1 Lipolysis

TAGs are well known due to their ability to offer a source of fatty acids that may be used to generate energy and provide various functions within the cell. Lipolysis is the cellular process in which TAGs are broken down by hydrolysis into their component molecules, which are glycerol and free fatty acids (Lass et al., 2011; Zechner et al., 2012). The most important enzymes catalyzing this process are lipases, including hormone-sensitive lipase (HSL), ATGL and monoacylglycerol lipase (MAGL) (Zechner et al., 2012). The order of enzymatic reactions of LD lipolysis is the following: ATGL catalyzes first step in TAG degradation in order to produce DAG, which is further degraded by HSL to MAG, and lastly, MAG is degraded by MAGL enzyme. Fatty acids that are released by this process may serve different functions in the cell. For example, during the process of mitochondrial FAO, they can be oxidized within the mitochondria, they can be used again for the production of TAGs, help maintain cellular and ER homeostasis, they can serve as intermediates for the generation of various membranes or different signaling molecules, or they can even be released outside the cell (Walther & Farese Jr., 2012) (Figure 6).

Belonging to the group of lipolytic enzymes, for a long time HSL has been considered as primary enzyme in the process of lipolysis. It is a multifunctional enzyme that can hydrolyze a range of TAGs but, most importantly, this enzyme catalyzes the enzymatic reaction of DAG breakdown (Haemmerle et al., 2006). Stimulated activity of HSL can be caused by increased levels of cyclic adenosine monophosphate (cAMP)-dependent protein kinase. On the other hand, it is shown that insulin may lower cystolic cAMP levels, resulting in less HSL assembling (Holm, 2003). Found mostly in adipocytes, it is a multidomain protein where its alpha/beta hydrolase domain constitutes a central region and is primarily involved in the process of hydrolysis. In humans, when the body wants to access its stored reserves of energy, the activity of HSL is triggered.

Structurally, MAGL also contains an alpha/beta hydrolase fold domain, which is an important characteristic of several lipases. This enzyme may be found inside the cytoplasm as well as the plasma membrane and is highly connected to LD metabolism. The white

adipose tissue, liver, kidneys and heart are only few of the organs in which it is abundantly expressed (Karlsson et al., 1997). ATGL is one of the most studied enzymes in the field of lipolysis and belongs to the patatin-like phospholipase domain-containing protein (PNPLA) family of proteins (Lass et al., 2011). ATGL has a strong affinity for the *sn*-2 and *sn*-1 locations of TAGs when operating *in vitro*, which results in the production of *sn*-1,3- and *sn*-2,3-DAG. This enzyme may be found mainly in white and brown adipose tissues, but also in some others. In addition, it is found also in adipocytes, and it is considered the major mammalian lipase for delivering fatty acids from adipose tissue to the working muscle. ATGL is expressed in different cells and tissues (Peyot et al., 2009; Schlager et al., 2015). Both transcriptional and post-transcriptional processes are involved in the regulation of ATGL expression and activity. As an example for this, during transcription, peroxisome proliferator-activated receptor gamma (PPAR-γ) is a transcription factor that interacts with the ATGL promoter region and increases the production of ATGL mRNA in adipocytes (Ji et al., 2006). In hepatocytes, another transcription factor known for stimulating ATGL expression is forkhead box O-1 (FoxO1). According to the findings of a research on palmitate's effect on lipid formation, ATGL-dependent lipolysis may function as a defense mechanism inside a cell (Zhao et al., 2021). Interestingly, ATGL levels may also increase upon fasting (Nielsen et al., 2011). The mechanisms that regulate ATGL-mediated lipolytic breakdown of LDs are complex and not completely understood. ATGL interaction with PLIN5 has been shown to result in decreased lipolysis in studies done by Wang and his colleagues (H. Wang et al., 2011).

ATGL has been shown to play an essential role in the process of mammalian TAG catabolism. This conclusion has been reached after investigations with animals with a deficiency in ATGL as well as people whose genes had certain anomalies or mutations of this gene (Schreiber et al., 2019). Since 2004 (Zimmermann et al., 2004), when ATGL was discovered, several genetic KO models of research have provided crucial insights into the novel functions played by ATGL. In humans, genetic mutations in ATGL have been linked to the development of various disorders, including myopathy (Fischer et al., 2007; Kaneko et al., 2014; Missaglia et al., 2015).

The majority of investigations point to a function for ATGL that is protumorigenic in cancer. To be more specific, a number of studies have indicated that ATGL depletion results in a suppression of proliferation in a variety of cancer cell lines (Zagani et al., 2015). In another study, ATGL overexpression led to a decrease in cell proliferation and an increase in TAG hydrolase activity in a number of cancer cell lines; however, limiting ATGL activity by its chemical inhibition has been shown not to have any effect on hydrolyse activity (Xie et al., 2020). In addition, ATGL expression is enhanced by obesity and is seen at higher levels in human colon cancers. Moreover, the same study reported that ATGL mRNA levels were also elevated in humans with the same colon cancer (Iftikhar et al., 2021). In contrast, depletion of ATGL in colorectal cancer cells and non-small-cell lung carcinoma cell line was shown to consistently decrease the tumor development, multiplication, and tumor invasion (Ou et al., 2014; Zagani et al., 2015). Additionally, it has been shown that the levels of ATGL are relatively high in breast cancer cells compared to normal breast epithelium, pointing to the potential of targeting this enzyme in order to inhibit the development and invasiveness of cancer cells (Y. Y. Wang et al., 2017).

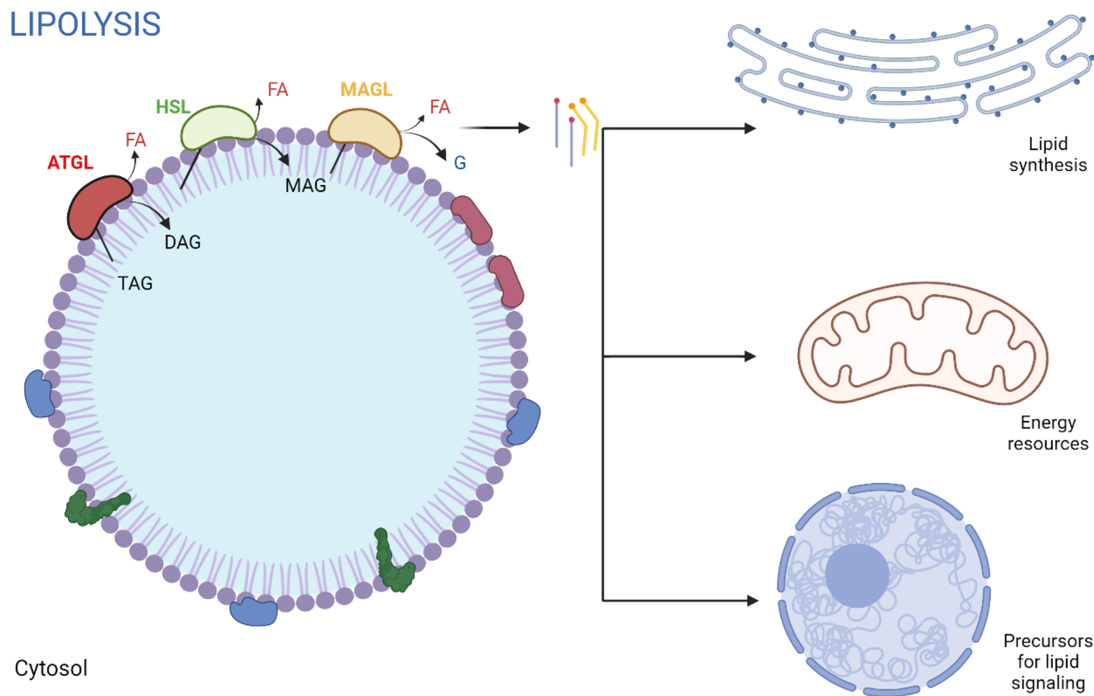


Figure 6: A schematic view of how intracellular lipolysis works. Adapted from Hofer et al. 2020 (Hofer et al., 2020). Created in BioRender.com.

1.4.2 Lipophagy

Beside the role of autophagy in LD formation, autophagy can also play a role in LD breakdown. This process is called lipophagy. Lipophagy, like autophagy and lipolysis, is triggered by nutrient stress. Even though the precise molecular pathway of lipophagy is not known yet, lipophagy is believed to occur along the same molecular mechanisms as bulk autophagy, with the only difference being that only lipids/LDs or parts of LDs are being trapped/engulfed within autophagosomal structures. These structures further fuse with lysosomes enabling the degradation of fat. Lipophagy has been observed in a variety of cell types since its discovery in 2009 in the liver of fasted mice (Hubbard et al., 2010; Singh et al., 2009). It is still exceedingly difficult for scientists to detect and define this process despite the fact that they put a lot of effort into it. This is primarily due to the fact that relatively little is known about the receptors, proteins and all of the signaling pathways that are involved in this process. Instruments, methods and tools that are almost always used to verify the presence of bulk autophagy are also employed in the majority of the research for the aim of detecting lipophagy inside cells of diverse origins. This is because these tools have already been proven to be effective in determining the presence of autophagy (Klionsky et al., 2016).

Hepatocytes have been the subject of the great majority of research investigations that have been conducted on lipophagy. In the study that was conducted not too long ago on primary rat hepatocytes that were subjected to acute nutrient stress, it was shown that LDs and lysosomes are found in close connection to each other, which allows for a direct lipid transfer between these two cellular compartments (Schulze et al., 2020). Another study has shown that lipophagy contributes to nutrient stress-induced fatty acid release (W. Cui et al., 2021). In addition, the results of a more recent investigation on hepatocytes

demonstrated that the exogenous addition of fatty acids, such as oleic acid, induced lipophagy. This result was confirmed by the detection of autophagosomal and lysosome-associated proteins on LDs (Garcia-Macia et al., 2021). It has also been suggested that the size of the LD determines whether lipophagy or lipolysis is the metabolic pathway of choice during breakdown process (Schott et al., 2019). Beside hepatocytes, lipophagy has been shown to be induced in enterocytes, glial cells, T cells and neurons (Hubbard et al., 2010; Kaushik et al., 2011; Khaldoun et al., 2014; Klionsky et al., 2016; Martinez-Vicente et al., 2010). Much less is known about lipophagy in cancer. The breakdown of lipids that is reliant on lipophagy has the ability to provide rapidly proliferating cancer cells with the energy substrates and intermediates that are essential for biosynthesis and the proliferation of the cells. Docosahexaenoic acid treatment is one of the ways when lipophagy may be induced in breast cancer cell lines (Pizato et al., 2019). In addition, lipophagy may rapidly fulfill the need for (phospho)lipids, required for phagophore elongation, which is a necessary step for initiating autophagy (Drizyte-Miller et al., 2020).

In conclusion, although the exact mechanism of lipophagy is not known yet, this field is emerging very fast. Therefore, this study has the potential to highlight the significance of lipophagy within cancer cells.

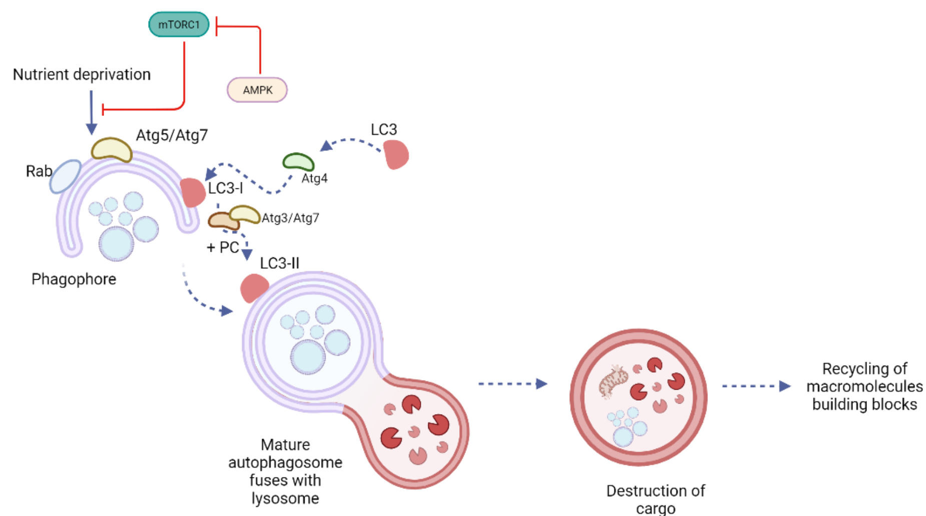


Figure 7: A schematic illustration of autophagy/lipophagy. Created in BioRender.com.

1.4.3 Role of autophagy/lipophagy-related proteins in regulating lipid transfer and LD metabolism

Inhibition of autophagy may lead to increased LD accumulation (Jarc & Petan, 2019). It was shown that the absence of essential autophagy-related proteins such as ATG5 may lead to increased TAG accumulation in hepatocytes (Singh et al., 2009). Besides the proteins discussed earlier, it is crucial to acknowledge that proteins involved in autophagy can also have various impacts on LD metabolism. As an example for this, the colocalization

between LDs and lysosome-associated membrane protein type 1 (LAMP1) has been demonstrated, which is one of the known glycoproteins found on the lysosomal membrane. There is evidence to suggest that autophagy plays a significant role in transporting the contents of LDs to lysosomes. In support of this, it was observed that when inhibitors of autophagosome formation (i.e. 3-methyladenine (3-MA)) or autophagosome-lysosome fusion (vinblastine) were used, the co-localization of LDs with LAMP1 was significantly reduced. Similarly, LD/LAMP1 co-localization was lower in cells where ATG5 was knocked down using siRNA. Moreover, this co-localization did not increase even when lysosomal proteolysis was inhibited. These findings suggest that autophagy is involved in the delivery of LD content to lysosomes for degradation (Singh et al., 2009). LDs were found to directly associate with autophagosomes, as evidenced by their co-localization with the autophagosome marker LC3. It was also demonstrated that when there is no trigger for the accumulation of lipids, there is a higher degree of co-localization between LDs and microtubule-associated protein 1A/1B-light chain 3 (LC3) in the presence of lysosomal inhibition. This suggests that autophagy has a role in regulating LDs (Singh et al., 2009).

ATG9A is a protein that acts as a scramblase during autophagy by moving phospholipids between membrane leaflets (Mailler et al., 2021; Noda, 2021). Recent studies have revealed that depletion of ATG9A leads to an increase in LD size and number in human cells and in C. elegans, and blocks the transfer of fatty acids from LDs to mitochondria for energy production (Mailler et al., 2021). ATG9A is found in vesicular-tubular clusters near the ER and other membrane scramblases and in close proximity to autophagosomes, LDs, and mitochondria. The findings of this study indicated that ATG9A is crucial for lipid mobilization and utilization in both autophagic and non-autophagic processes (Mailler et al., 2021). Compelling evidence has also demonstrated that both yeast and human ATG9 function as lipid scramblases, proficiently translocating phospholipids between the outer and inner leaflets of liposomes *in vitro* (Matoba et al., 2020). Interestingly, it has also been demonstrated that ATG9 serves as a receptor, facilitating the transfer of lipids from the ER to the phagophore through Atg2. Moreover, it is crucial for these lipids to be evenly distributed across both leaflets of the phagophore membrane. In addition, it was also shown that specific transmembrane regions of ATG9 have a specific role in phagophore expansion and that specific mutations such as those found in transmembrane domains may completely eliminate this activity (Chumpen Ramirez et al., 2023). Furthermore, a separate study has elucidated the remarkable capabilities of ATG9 as a highly efficient transport protein, successfully facilitating the transbilayer movement of lipids. Notably, this transport process predominantly takes place within the two leaflets of autophagosomes or autophagic bodies, underscoring the pivotal role played by ATG9 in these cellular structures (Orii et al., 2020). Although the exact formation of *de novo* autophagosomes is still not clear, recently it was shown that, by using recombinant proteins from yeast, ATG9 recruits different complexes such as ATG2-ATG18 that may facilitate ATG8 lipidation process. Additionally, ATG2 may support this lipidation by lipid transfer (Sawa-Makarska et al., 2020).

Recent studies have demonstrated that ATG2 plays a crucial role in facilitating lipid transfer between membranes during the process of autophagosome formation (Osawa et al., 2019). In addition, the characteristics of ATG2A and ATG2B, which are similar to the mammalian ATG2 protein, were investigated. The simultaneous inhibition of ATG2A and ATG2B led to a disruption in the process of autophagic flux, resulting in the build-up of autophagic structures that contain Atg proteins but remain unclosed. ATG2A was observed to localize on the surface of both, the autophagic membrane and LDs (Velikkakath et al., 2012). The depletion of ATG2A and ATG2B proteins leads to the formation of enlarged LDs that aggregate together, without involving autophagy. This study suggested that these proteins play a role not only in autophagosome formation but also in regulating

the morphology and dispersion of LDs in the cell. The clustering of enlarged LDs may also have implications for lipid metabolism and cellular homeostasis, highlighting the importance of ATG2A and ATG2B in maintaining proper cellular function. Nevertheless, further research is needed to fully understand the mechanisms by which ATG2 and ATG9 proteins regulate LD dynamics and their impact on cellular physiology.

1.5 The Interaction Between Cancer Lipid Metabolism, Autophagy and Cancer Cell Survival

Lipids play an essential role in the storage of fat and the generation of energy in cells. Lipids are not only vital for the storage of fat and the generation of energy in cells but also play a significant role in the efficient transportation of fatty acids. These fatty acids serve a crucial purpose in the management of energy metabolism within the cell, further emphasizing the importance of lipids in cellular processes. As a result, ensuring that a cell has access to an adequate supply of nutrients for development, growth and proliferation is of the utmost significance. When discussing nutrient stress, it mainly refers to the perspective of how cells detect and react to an inadequate supply of nutrients in order to satisfy their energy demands (Wellen & Thompson, 2010). A cell that is subjected to the toughest circumstances may have fatal consequences due to nutrient stress, which may ultimately result in the death of the cell. These conditions may be either an excess of nutrients (such as an excess of lipids or fatty acids), which can lead to lipotoxicity, or a deficiency of nutrients, which can also contribute to the cell death (lack of glucose, amino acids, lipids, etc.). Highly conserved signaling networks and pathways lie at the heart of the cellular metabolism and cell proliferation. These pathways are mainly mediated by mammalian target of rapamycin (mTOR) and AMP-activated protein kinase (AMPK) (Hardie, 2014; Ling et al., 2020). When cells are exposed to nutrient stress, AMPK is activated. However, upon inhibition of mTOR, autophagy is induced (Hardie, 2014; Jung et al., 2010; Wellen & Thompson, 2010; Zoncu et al., 2011). The availability of nutrients has a significant amount of influence on each of these pathways. Something that should not be left out and is already mentioned earlier is the fact that LD biogenesis is primarily induced when cells are subjected to a variety of stressful conditions (Jarc & Petan, 2019; Petan et al., 2018). This is true regardless of whether the cells are being stressed by an abundance of nutrients or a lack of nutrients (Jarc & Petan, 2019). It is well known that cancer cells have the capacity to rewrite/reprogram the instructions for their metabolism (Pavlova & Thompson, 2016). This indicates that they are able to survive and adapt even in the most aggressive microenvironment. Surprisingly, the fact that cancer cells are able to live in any environment is one of the most critical factors that contributes to the difficulty of treating and curing this illness. Beside nutrient stress, some of the main characteristics or conditions of tumor microenvironment are oxidative stress, acidic pH, mitochondrial damage, high autophagic flux, etc. This could be one of the reasons why LD biogenesis happens more often and in greater quantity in cancer cells as opposed to typical healthy tissues. Due to their rapid proliferation, cancer cells rely on high levels of lipids. One of the most fundamental characteristics of cancer is altered lipid metabolism. The illness manifests itself in a number of different stages, all of which are influenced by the metabolism of lipids. In healthy tissues, cell development and proliferation are sustained by sufficient energy, such as ATP energy, which is created mostly by glycolysis, an aerobic process. Nevertheless, aberrant and uncontrolled development and multiplication of cancer cells is maintained by many energy sources. This indicates that cancer cells are able to survive even at the lowest amount of nutrients and oxygen in their microenvironment. There are several sources of lipids that are accessible to cancer cells, and the following are

just a few examples: lipids obtained extracellularly (cancer cells can acquire lipids from the extracellular environment. This can occur through the uptake of lipids present in the surrounding microenvironment or through the uptake of lipoproteins); intracellular utilization via lipolysis/lipophagy (cancer cells can utilize intracellular lipid stores through processes such as lipolysis and lipophagy); or *de novo* fatty acid synthesis (cancer cells also have the capability to synthesize fatty acids *de novo*. This involves the synthesis of fatty acids from non-lipid precursors within the cell, such as glucose and amino acids). This pathway allows cancer cells to produce lipids for their specific needs (Petan et al., 2018). So, how do cancer cells survive under altered lipid metabolism characteristics? Elevated levels of *de novo* fatty acid synthesis are characteristic of various cancers. Therefore, the increased expression of the enzymes involved in *de novo* fatty acid synthesis is one of the reasons leading to this consequence. Several growth factors, including the epidermal growth factor receptor (EGFR) and human epidermal growth factor receptor 2 (HER2), as well as steroid hormones and steroid hormone receptors, such as the estrogen receptor (ER), androgen receptor (AR), and progesterone receptor (PR), exert a significant amount of control on the expression of fatty acid synthase (FASN). However, although it sounds very simple, the connection between elevated expression of FASN enzyme and cancer is complex. For instance, blocking the activity of this enzyme in the cells of hepatocellular carcinoma is shown to be a potential new strategy to the therapy of this illness (Hao et al., 2014). In colorectal cancer cells, overexpression of FASN leads to the promotion of cancer cell survival during stressful conditions (Zaytseva et al., 2015). On the other hand, the integrity of the cell's structure is essential for the functioning of any cell, including cancer cells. As being part of cellular membranes, sphingolipids and important enzymes in their metabolism have also shown to have some prosurvival effects for cancer (Ogretmen & Avenue, 2018).

To summarize, lipid metabolism in cancer cells is complex and not yet fully understood. Therefore, better understanding of the crosstalk between lipid metabolism and other cellular processes including autophagy is important for monitoring cancer cell death and this is essentially important in cancer research.

Chapter 2

Aims and Hypothesis

This research work intends to investigate the interplay between LD metabolism and autophagy in nutrient-deprived cancer cells and examine the possibility that targeting LDs and autophagy impairs cancer cell survival. In this work, we wanted to answer the following questions: 1) Is LD turnover affected by the type and length of nutrient stress; 2) What are specific conditions of nutrient stress that are required to activate autophagy/lipophagy and lipolysis; 3) Are LD turnover and autophagy/lipophagy dynamically coupled in stressed cells; 4) Whether both LDs and autophagy/lipophagy contribute to the resistance of cancer cells to nutrient deprivation-induced stress?

The first objective of our study was to examine the impact of various nutrient stress conditions on the formation and degradation of LDs in HeLa cancer cell line under diverse stressful conditions. To accomplish this, we formulated a hypothesis that the duration and intensity of nutrient stress could significantly influence LD behavior. Subsequently, we exposed cells to different types of nutrient stress and tracked the changes in LD dynamics over a specific time frame.

Our second objective aimed to identify the conditions that trigger the activation of autophagy/lipophagy. To accomplish this, we formulated a hypothesis suggesting that the extent of autophagy within cancer cells might differ depending on the culturing conditions and could also vary among different cell types. Consequently, we investigated various stressful conditions in a cervical cancer cell line and selected the most suitable ones for our research based on the observation of the highest/lowest levels of activation of this process.

Our third objective was to establish a connection between LD metabolism/dynamics and autophagy/lipophagy. To accomplish this, we formulated a hypothesis suggesting that by simultaneously inhibiting key components involved in LD biogenesis and autophagy, we could gain a deeper understanding of the relationship between these two processes. This approach aimed to provide valuable insights into how LDs and autophagy/lipophagy are interconnected and influence each other.

Lastly, and of utmost importance, our fourth objective was to investigate the interplay between autophagy/lipophagy, lipolysis and LD metabolism at the molecular level, and examine its contribution to the survival of cancer cells. We hypothesized that disturbing the intricate interconnection between these cellular processes may potentially lead to cancer cell death, either directly or indirectly. To reach this goal, we examined cancer cell death under various conditions involving the inhibition of LD biogenesis, autophagy, or both simultaneously, across diverse experimental settings.

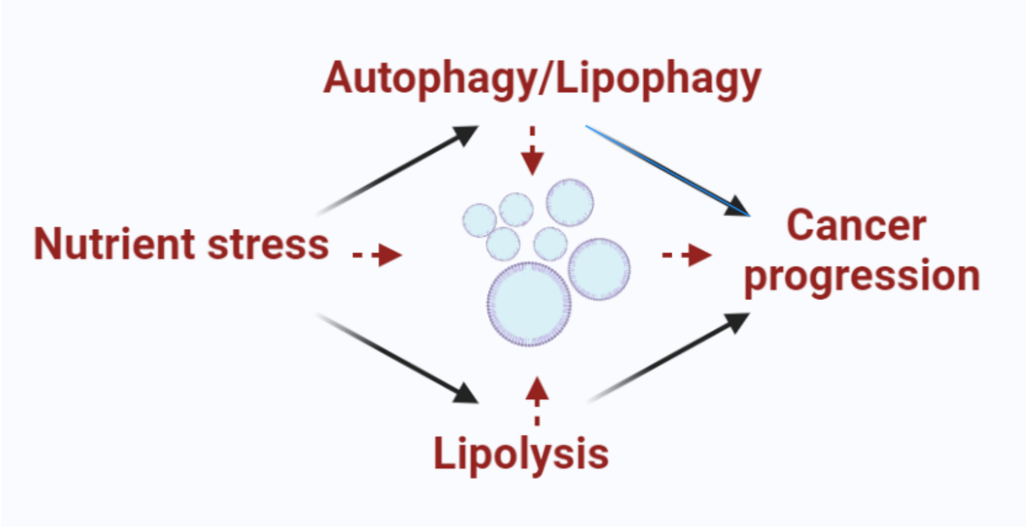


Figure 8: An illustration depicting the primary hypotheses and objectives of this dissertation. Created in BioRender.com.

Chapter 3

Materials and Methods

3.1 Materials

HeLa human cervical adenocarcinoma cells were obtained from ATCC (USA). Dulbecco's modified Eagle's medium with high glucose and GlutaMAX supplement (DMEM-GlutaMax) (cat. no. 61965-059), heat inactivated fetal bovine serum (FBS) (cat. no. 10500-064), Dulbecco's phosphate buffered saline (DPBS) (cat. no. 14190-169), TrypLE Select (cat. no. 12563-029) were from Gibco (USA), and Opti-MEM from Life Technologies (USA). Lipi-Deep Red (cat. no. LD04-10) was from Dojindo (Dojindo, Kumamoto, Japan). BODIPY 493/503 (cat. no. D3922), BODIPY FL C12 (cat. no. D3822), BODIPY 558/568 C12 (cat. no. D3835), Lipofectamine RNAiMAX, dithiothreitol (DTT) (cat. no R0862), Halt™ Protease Inhibitor Cocktail, EDTA-free (100X) (cat.no. 78425), Pierce 660 nm Protein Assay Reagent (cat. no. 22660) were from Thermo Fisher Scientific (USA) and Hoechst 33342 nuclear stain, Cyto-ID Autophagy detection kit 2.0 and chloroquine (CQ) from Enzo Life Sciences (USA). Tris-Glycine SDS Sample Buffer (2X), (cat. no. LC2676) was from Novex by Life Technologies (USA). Human ATGL- and ATG5-targeting siRNAs and AllStars Negative Control siRNA were from Qiagen (Germany). ProLong™ Diamond Antifade Mountant with DAPI was from ThermoFisher (cat. no. P36962, USA). Autophagy inhibitor bafilomycin A1 (BafA1) was from Cayman (USA), T863 (DGAT1 inhibitor), PF-06424439 (DGAT2 inhibitor), essentially fatty acid-free bovine serum albumin (BSA) (cat. no. A7511), fatty acid-free BSA (cat. no. A8806), Western Blot-grade BSA (cat.no. A7030) and Nile Red were from Sigma-Aldrich (USA). β-actin antibodies (cat. no. NB600-532) were purchased from Novus Biologicals (UK), LC3 (cat. no. 2775), ATG5 (cat. no. 12994) and ATGL (cat. no. 2138) antibodies from Cell Signaling (USA) and horseradish-peroxidase-labelled secondary antibodies from Jackson ImmunoResearch Laboratories (USA). Invitrogen Anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody/Alexa Fluor 488 conjugate (cat. no. A11070) was used. Lumi-Light Western Blotting Substrate (cat. no. 12015196001) and Western Blocking Reagent (WBR) (cat. no. 11921673001) were from Roche Applied Science (Germany). All other chemicals were of at least analytical grade and purchased from Sigma-Aldrich (USA) or Serva (Germany).

3.2 Cell Culture and Treatments

HeLa cells obtained from ATCC were cultured in DMEM-GlutaMAX supplemented with 10% FBS. The cells were seeded in 24-well plates at a concentration of 1.5×10^4 cells/well, unless otherwise stated. Cells were left for 24 h to attach in complete medium. After 24 h, depending on the experimental objective, two methods of cell starvation were conducted, including a) serum-free mild starvation in DMEM-GlutaMax or RPMI-1640 and b) severe starvation in HBSS medium, both supplemented with 0.02% EFAF-BSA for different time

intervals. For prolonged mild starvation experiments, the cells were serum-starved for additional 96 h. For short, acute nutrient starvation experiments, a 16-hour time point was typically selected. In case of low glucose starvation, cells were starved in glucose- and glutamine-depleted RPMI-1640 medium supplemented with 1 g/L glucose and 2 mM L-glutamine. In experiments with pre-starvation, cells were pre-starved in RPMI-1640 medium containing 0.02% BSA for 24 h. The pre-starvation medium was then replaced with either complete medium, serum-free medium, HBSS, or low-glucose medium for the time period indicated. In experiments with only nutrient-rich conditions, complete medium was replaced with fresh complete medium for next 24 hand LD dynamics over time indicated was studied.

3.2.1 Cell culture and treatments-pulse chase assay

HeLa cells were cultured in DMEM-GlutaMAX supplemented with 10% FBS, unless otherwise indicated, HeLa cells were seeded in 24-well plates at a concentration of 3×10^4 cells/well. Depending on the experimental goal, two main procedures of acute cell starvation were performed following cell seeding and attachment for 24 h in complete medium, including a) serum-free mild starvation in DMEM-GlutaMax/RPMI-1640 and b) severe starvation in HBSS medium, both supplemented with 0.02% EFAF-BSA. DGAT and autophagy inhibitors were added to culture media during acute starvation phase.

LD dynamics in HeLa cells was also followed by pulse chase assay and using exogenously added fatty acid analogues BODIPY FL C12 and BODIPY 558/568. The FA analogues were complexed to BSA by incubating each fluorophore (at a concentration of 1 µM) in the presence of 0.02% EFAF-BSA in serum-depleted culture medium (for mild starvation) or HBSS (for severe starvation) for 1 h at room temperature before its addition to the cell culture. Cells were first reverse transfected with ATG5-coding siRNA or SCR control and left in complete medium for 24 h. After the feeding, the cells were washed and medium replaced with serum-free medium containing 1 µM BODIPY 558/568 C12 and a mixture of DGAT inhibitors (pre-starvation phase). After 24 h, images of serum-free starved cells were captured using confocal microscopy at zero timepoint (0 h), while remaining cells were washed twice with DPBS and starved for additional 6 h in HBSS before images were taken. Finally, 1 µM of BODIPY FL C12 was added to the cells 15 min prior microcopy.

3.3 Silencing of ATGL, ATG5 and ULK1 Proteins Using Small Interfering RNA (siRNA)

In a 24-well plate, reverse transfection was performed on HeLa cells at cell density of 3×10^4 cells/well, whereas for 6-well plates (primarily used for seeding cells for Western Blot analysis) cell density was 1.5×10^5 cells/well. Transfection was done with 20 nM (mixture of two siRNAs for single gene silencing) or 40 nM (mixture of four siRNAs for double gene silencing) total siRNA targeted at ATGL, ATG5 and/or ULK1, or with 20 nM or 40 nM AllStars Negative Control siRNA, used as negative control (SCR) in all experiments. Transfection complexes were generated using 1 µl/well in 24-well plates and 7.5 µl/well in 6-well plates of lipofectamine RNAiMAX in Opti-MEM medium in accordance with the guidelines of the manufacturer.

3.4 Flow Cytometry Analysis of Cellular Neutral Lipid Content

Neutral lipid analysis was performed as described before (Pucer et al., 2013). Briefly, cells were seeded in complete medium in 24-well culture plates at a concentration of 1.5 x 10^4 cell/well (HeLa) and left to adhere for 24 h, and then treated with either the DGAT1 inhibitor, TB63 (DGAT1i), the DGAT2 inhibitor, PF-06424439 (DGAT2i) or with a mixture of both DGAT inhibitors (DGATi) for blocking LD formation, or with 5 nM BafA1 or 100 µM CQ for inhibition of autophagy for 6 or 16 h in serum-free or -free (HBSS) media. During experiments on cells cultured in a complete medium, the cells were subjected to treatment with a mixture comprising 10 µM each of lysosomal acid lipase (LAL) inhibitors lalistat-1 and lalistat-2, denoted as LALi, with a combined concentration of 20 µM. After the cells were collected, the pellet was resuspended in 500 µl of 1 µg/ml Nile Red solution in DPBS and incubated in the dark for 10 mins before flow cytometry measurements using a BD FACSCalibur system (BD Biosciences, USA) equipped with a 488-nm Ar-ion laser, and CellQuest software (Becton 877 Dickinson, USA). The FL1 filter was used to capture fluorescence signals from at least 2 x 10^4 events per sample.

3.5 Live-Cell Confocal Microscopy Imaging

3.5.1 Staining of LDs with Bodipy 493/503

The BODIPY 493/503 neutral lipid staining was used to visualize LDs by live-cell confocal imaging. For this purpose, cells were first washed twice with DPBS and then stained with staining mixture containing 1 µg/ml BODIPY 493/503 and 1 µg/ml Hoechst dye in DMEM or RPMI-1640 medium (medium without FBS/BSA) for 15 mins in a CO_2 incubator. After staining, the cells were washed twice with DPBS and fresh culture medium (either serum-free RPMI-1640 or HBSS) was added to the cells. Live cell imaging was performed using a confocal laser scanning microscope (LSM 710; Carl Zeiss, Germany) depending on the selected time points in starvation conditions. Images were processed using the Zen software (Carl Zeiss, Germany).

3.5.2 LC3 immunofluorescence assay

Hela cells were seeded on glass coverslips in complete medium at a density 3 x 10^4 cell/coverslip. After 24 h, the cells were washed and treated with 100 µM CQ in serum-free DMEM Glutamax or HBSS for 16 h. HeLa cells reverse transfected with ATG5 siRNA or control siRNA were subjected to nutrient deprivation in HBSS without additional treatment with autophagy inhibitors. After 16 h starvation, the cells were washed with DPBS, fixed in 4% paraformaldehyde in DPBS for 15 mins at room temperature and permeabilized with 0.1% Triton X-100 in DPBS (DPBST) for 5 mins. Fixed cells were blocked with 3% BSA in DPBST for 1 h at room temperature and incubated overnight at 4 °C with an anti-LC3 primary antibody diluted at a ratio of 1:200. After washing with DPBS, a secondary antibody conjugated with Alexa Fluor 488 (1:1000) was added to the cells for 1 h at room temperature in the dark. LDs were stained with the Lipi-Deep Red dye diluted at 1:1000 in DPBS for 30 mins at room temperature in the dark. Cells were then washed with DPBS and mounted on slides using mounting medium with DAPI.

Images were captured using a confocal laser scanning microscope (LSM 710; Carl Zeiss, Germany) and processed using the Zen software (Carl Zeiss, Germany).

3.5.3 LD counting and diameter analysis

Live-cell confocal microscopy images of BODIPY 493/503 stained LDs were used for computer image analysis using ImageJ software (National Institutes of Health, USA) and the LipidDroplet Counter Plugin (https://doi.org/10.5281/zenodo.2581434). The analysis was carried out on 32-bit two-dimensional pictures, where the quantities of LDs as well as their sizes were calculated in accordance with the instructions provided by the plugin. The surface areas of the LDs were used to calculate the diameters of LDs. Each sample was subjected to approximately 30 cells for each analysis.

3.6 Western Blot Analysis

Following the steps of serum-free or AA-free starvation outlined above, Hela cells were first seeded in complete media in 6-well plates at a density of 1.5 x 10^5 cells/well and, if necessary, reverse transfected with siRNA. Cell lysates were prepared by washing adherent cells in ice-cold DPBS and scraping the cells in Tris-Glycine SDS Sample Buffer (2X) with 800 mM DTT and by adding Halt Protease Inhibitor Cocktail (Thermo Scientific, USA) to the mixture. After a ten-minute incubation at 95 °C, the lysates were kept on ice or frozen at –80 ºC until western blot analysis. The Pierce 660 nm Protein Assay was used to determine the total protein concentration. 5 μg of total protein was separated on 12.5% SDS-PAGE gel for LC3 and 10 μg of total protein on 10% SDS-PAGE gel for ATG5 or ATGL protein detection, and then transferred to PVDF (Merck Millipore Ltd., Germany) or nitrocellulose membrane, respectively (Serva, Germany). Following the transfer, membranes were blocked for each protein separately, including 1 h in 5% non-fat dry milk in TBS/0.1% Tween-20 (TBST) (ATGL), 3% BSA (#A7030) in TBST for LC3 and 5% BSA (#A7030) in TBST for ATG5, or in 1% WBR in TBS for β-actin. After blocking, membranes were incubated overnight at 4 ºC in the presence of anti-rabbit primary antibodies at 1:1000 (ATGL, ATG5 and LC3) or 1:5000 (β-actin) dilution. The primary antibodies were diluted as follows: ATGL in 5% non-fat dry milk in TBST, ATG5 and LC3 in 5% BSA in TBST, and β-actin in 0.5% WBR in TBS. Following multiple washing steps in TBST, the membranes were subjected to one hour incubation with HRP-conjugated secondary antibodies (1:10000) diluted in the primary antibody solution. Lumi-Light Western Blotting Substrate was used for visualization and imaging done on Gel Doc XR system (Bio-Rad, USA).

3.7 TMRM/YO-PRO-1 Cell Death Assay

Cell death was determined using TMRM/YO-PRO-1 assay and flow cytometry as described previously. Briefly, cells were seeded in complete medium in 24-well plates at cell density of 1.5 x 10^4 cells/well and allowed to adhere for 24 hours. After attachment, cells were treated with different inhibitors, blocking LD formation (40 μM mixture of DGATi) or autophagy (5 nM BafA1) for the time indicated in experimental settings. For analysis, cells were harvested, and the pellet re-suspended in 100 μl of 25 nM TMRM in DPBS and incubated in the dark for 15 minutes. To detect dead cells, YO-PRO-1 was added to a final concentration of 50 nM and cells incubated for additional 10 minutes. The cell suspension was diluted with 200 μl (D) PBS containing fatty acid-free BSA (0.1% m/v) from Sigma (USA). Flow cytometry using the FL1 and FL3 filters was used to analyze the TMRM and

YO-PRO-1 signals from at least 20,000 cells in each sample, respectively. TMRM negative and YO-PRO-1 positive cells were considered apoptotic.

3.8 Statistical Analyses

All statistical analyses were carried out using Prism 9.4.1 (GraphPad Software, USA). Data are presented as means ± SEM. Statistical significance was determined by one-way or two-way ANOVA, followed by Bonferroni or Tukey's post-hoc tests. P values lower than 0.05 were considered statistically significant.

Chapter 4

Results

4.1 Hypothesis I: LD Turnover is Affected by the Type and Length of Nutrient Stress

Limited nutrient availability in the tumor microenvironment has previously been shown to drive LD formation (Bozza & Viola, 2010). In this study, we focused on the HeLa cervical cancer cell line. To set the basis for our further studies, we first examined changes in the abundance of LDs in HeLa cervical cancer cells in response to different starvation conditions that are defined in Materials and Methods section of this thesis (Figure 9). It is important to note that it was already reported that elevated levels of LD accumulation were observed in various human cell lines when cultivated in amino acid (AA)-deficient conditions (Nguyen et al., 2017; Rambold et al., 2015). This chapter describes our latest work on the role of autophagy/lipophagy in driving LD formation/breakdown when cells are exposed to different types of nutrient (metabolic) stress.

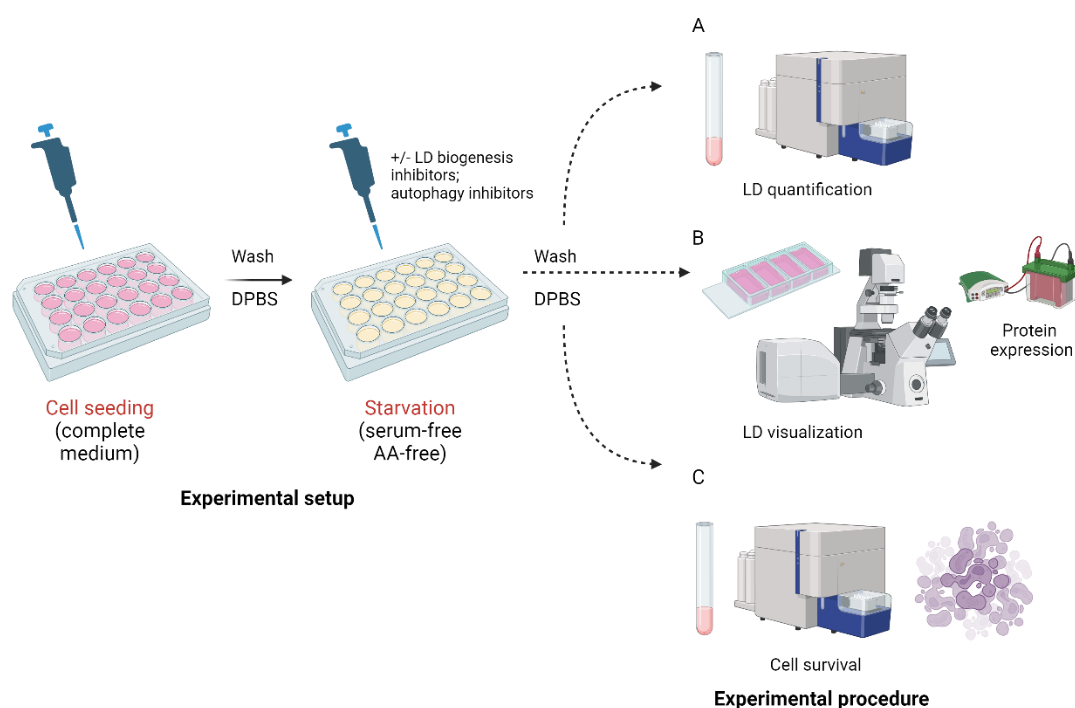


Figure 9: Schematic presentation of experimental setup and main procedure. Created in Biorender.com.

To gain deeper insights into the regulation of LD metabolism in HeLa cervical cancer cells under acute nutrient stress, our initial focus was on assessing alterations in LD abundance concerning variations in feeding and various starvation conditions (Figure 10 A). To reduce the initial LD levels, HeLa cells were subjected to a 24-hour pre-starvation phase in a serum-free medium to induce a near-complete depletion of LDs. There were no subsequent fluctuations in LD abundance in serum-free media over the next 16 hours of the experiment (Figure 10 Figure 10B, C). However, upon transitioning cells from a serum-free medium to a complete medium or subjecting them to total amino acid deprivation in HBSS, a notable rise in LD abundance was observed (Figure 10 B, C). Importantly, in amino acid-starved cells, LD accumulation reached levels comparable to those in fed cells after 6 hours of starvation (Figure 10 C). However, LD numbers and LD diameters were higher in amino acid-starved cells in comparison to those in fed cells after 16 h of starvation (Figure 10 C, D). Cells cultured in a low-glucose-containing medium accumulated LDs as well, although to a significantly lower degree than those cultured in HBSS or complete media (Figure 10 B, C).

4.1.1 LDs are formed in DGAT1-dependent manner during severe nutrient stress

Next, to determine whether the observed increase in LD content under amino acid-depleted conditions results from the initiation of new TAG synthesis and resulting LD formation, we treated cells with T863 and PF-06424439, which are inhibitors of the DGAT1 and DGAT2 enzymes, respectively (Cao et al., 2011; Futatsugi et al., 2015). Our results revealed that when we inhibited DGAT1 or performed a dual inhibition of both DGAT1 and DGAT2 enzymes, it effectively blocked the accumulation of LDs induced by amino acid deprivation. On the contrary, the single inhibition of DGAT2 did not exhibit any significant impact on either the LD number or the levels of neutral lipids (Figure 10 E, F, G) (Jusović et al., 2023). These findings strongly indicate that the process of LD formation in response to amino acid starvation in HeLa cells is predominantly dependent on the activity of DGAT1. Moreover, when the pre-starvation phase was excluded, and HeLa cells were directly shifted from the complete medium to either a fresh complete medium or HBSS (Figure 10 H, I), a marked elevation in LD accumulation was noted, relative to the initial LD levels after 16 hours of amino acid starvation. On the contrary, LD abundance in fed cells decreased over time relative to initial levels. Therefore, whether or not HeLa cells experience pre-starvation-induced LD depletion, they still have a higher quantity of LDs when subjected to starvation conditions in HBSS compared to their growth in full nutrient-rich media. These findings imply that different conditions of sudden starvation can lead to diverse impacts on LD metabolism, such as the breakdown of LDs caused by serum depletion and the significant accumulation of LDs triggered by amino acid depletion.

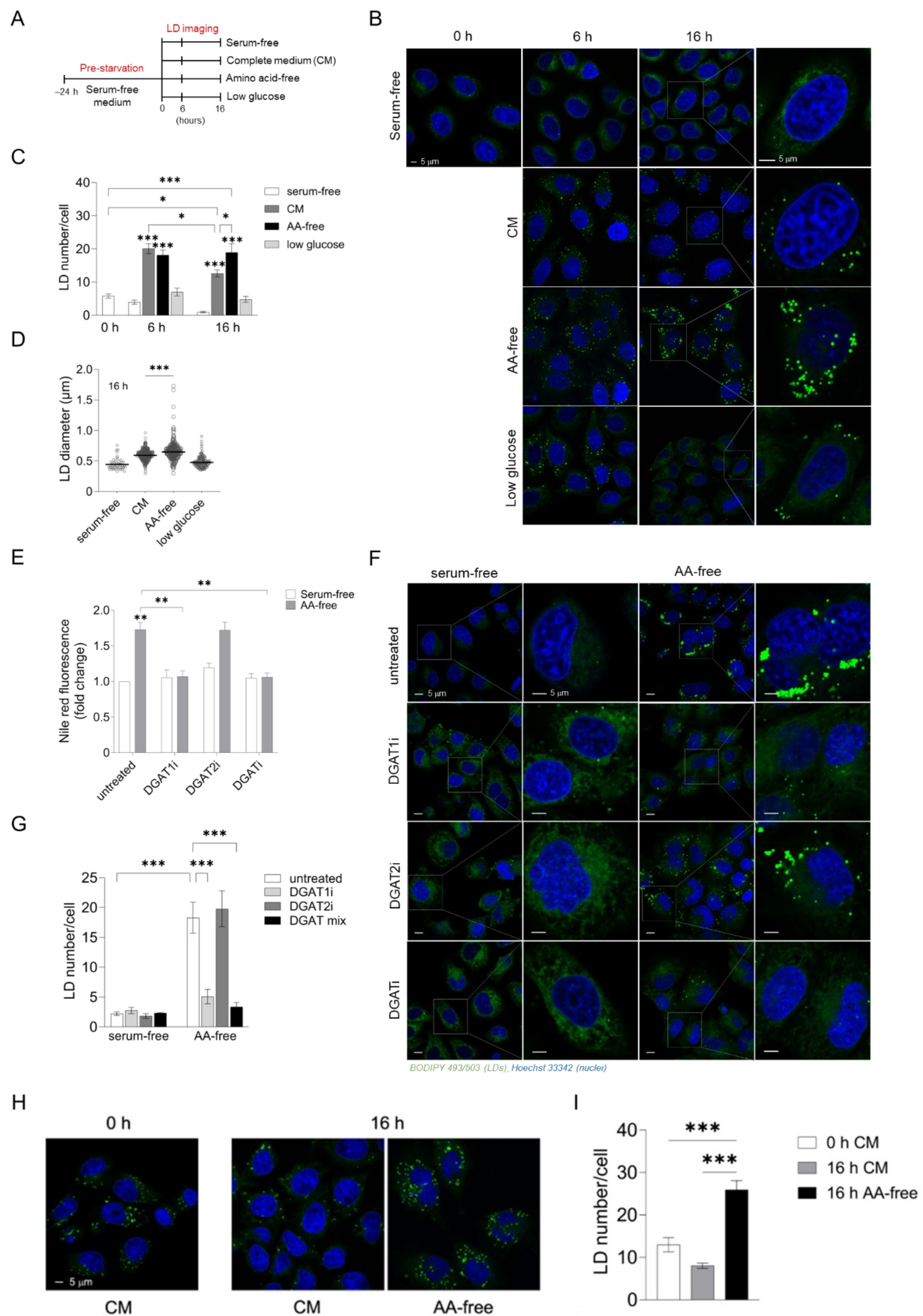


Figure 10: DGAT1 stimulates the formation of LDs in HeLa cells when exposed to amino acid deprivation. B, C) LD levels in HeLa cells pre-starved in serum-free medium (0 h) followed by 6–16 h of growth in different media (complete, serum-free, low glucose, or HBSS media), as shown in the A) experimental setup scheme. D) Alterations in LD diameters in HeLa cells grown under the same experiment design. E, F) Total neutral lipid

content and LD abundance in amino acid-starved HeLa cells treated with DGAT inhibitors. Starved HeLa cells were treated with 20 µM T863 (DGAT1i), 20 µM PF-06424439 (DGAT2i), or both (DGATi). After 16 h of starvation, neutral lipid content and LD abundance levels were determined. (G) Alterations in LD abundance in serum-starved and AA-starved HeLa cells treated with DGAT inhibitors. B, F) Cells were stained with 1 µg/mL BODIPY 493/503 to visualize LDs (green) and Hoechst stain solution to visualize nuclei (blue), and images (C, D, G) were analyzed using ImageJ. E) Neutral lipid content was quantified using Nile red staining and measured by flow cytometry. H, I) Microscopic images and a quantitative assessment of LD abundance in fed and starved HeLa cell. HeLa cells were initially cultured in complete medium (CM) for 24 hours (0 h), after which the medium was substituted with either fresh CM or amino acid-depleted medium for the next 16 hours. Data presented means SEM (C, E, G) or geometric means SEM (D) of at least two independent experiments. Quantification of LD number/cell (C, G) and size (D) was determined from more than 50 (C, D) or 30 cells/sample (G). Results that are statistically significant are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$ (one-way ANOVA with Šidak adjustment (C, D), two-way ANOVA with Tukey adjustment (E, G))).

Next, we checked for the effect of cultivating HeLa cells in serum-free medium for a prolonged period of time (4-5 days). Surprisingly, when HeLa cells were starved under these conditions, we saw that LDs also tend to accumulate (11 A, B). Furthermore, we noted a similar effect with DGATi treatment even under prolonged serum starvation conditions (Figure 11 A, B). This suggests that DGAT enzymes play a role in promoting LD biogenesis even in the absence of serum for an extended period. Bringing together these findings, we have provided support for our initial hypothesis in this study, which suggests that nutrient stress, in terms of both its type and duration, impacts the dynamics of LDs in HeLa cancer cells.

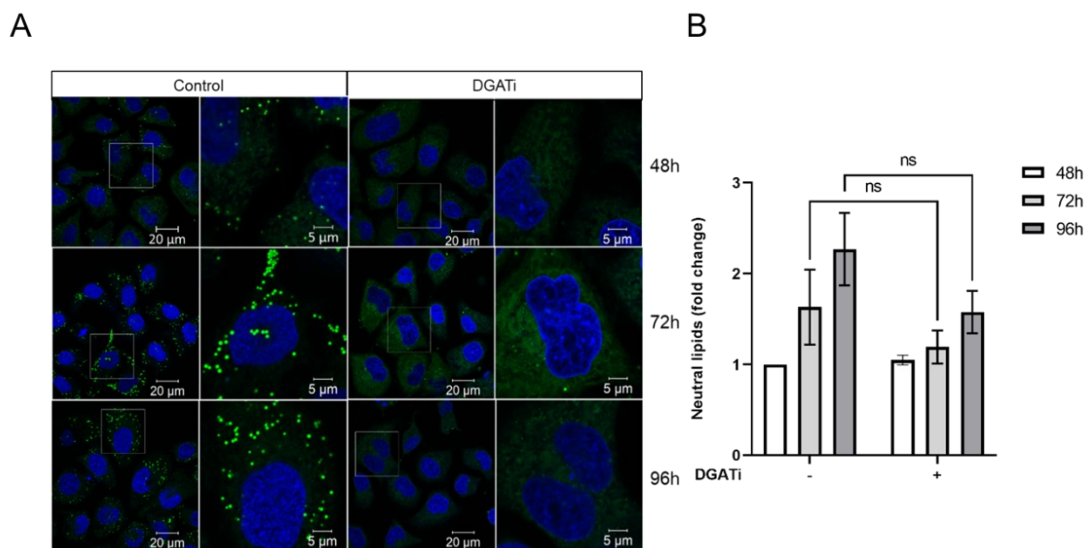


Figure 11: Prolonged serum deprivation induces LD formation in DGAT-dependent manner. A) Representative microscopic images of HeLa cells treated with DGATi and starved with serum-free medium for 48-96 h. Cells were stained with 1 µg/mL BODIPY 493/503 to visualize LDs (green) and Hoechst stain solution to visualize nuclei (blue). B) Total neutral lipid content in serum-free starved HeLa cells for 48-96 h. Neutral lipid content was quantified using Nile red staining and measured by flow cytometry. Data presented are means SEM of at least two independent experiments. Results that are

statistically significant are indicated (*, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$, (two-way ANOVA with Bonferroni's adjustment).

4.2 Hypothesis II: LD Turnover and Autophagy/Lipophagy are Dynamically Coupled in Stressed Cells

4.2.1 Autophagy contributes to LD biogenesis during severe nutrient stress

The interaction between autophagy and lipid metabolism has seen a significant increase in research in recent years. Based on this, we investigated how inhibiting autophagy using a range of approaches affects the accumulation of LDs within the predetermined experimental conditions. Autophagy was inhibited using late-stage autophagy inhibitors, BafA1 or CQ, which impede lysosomal acidification. We assessed their influence on LD accumulation during amino acid starvation. Our results for a 16-hour starvation showed that treatment of HeLa cells with the autophagy inhibitor BafA1 resulted in suppression of starvation-induced accumulation of neutral lipids, depleting the cells of LDs (Figure 12 A, B) (Jusović et al., 2023).

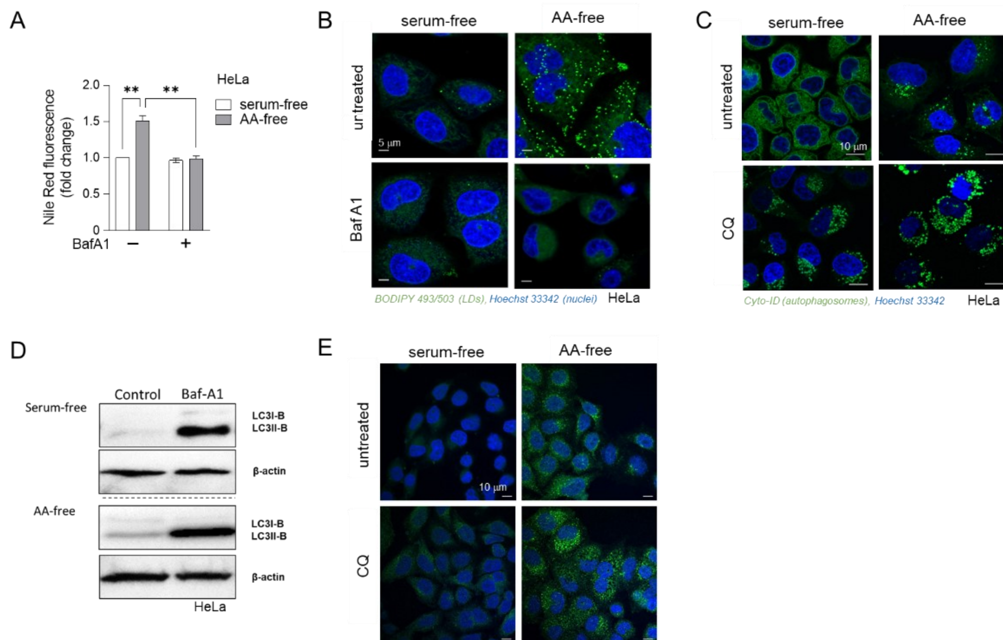


Figure 12: Inhibition of autophagy reduces LD accumulation in amino acid-starved HeLa cells. A, B) BafA1 (5 nM) treatments resulted in suppression of HBSS starvation-induced accumulation of LDs in HeLa cells as observed by flow cytometry (A) and by live-cell confocal microscopy (B). C) Cyto-ID-stained autophagosomes in amino acid-starved HeLa cells treated with CQ. D) Autophagic flux in starving HeLa cells estimated by LC3-II turnover in the presence and absence of BafA1. E) LC3 puncta in starving HeLa cells treated with CQ. Scale bars: 10 µm. Values on the graphs are means ± SEM of at least two experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Tukey's adjustment.

In addition, to confirm the involvement of autophagy in this context, we evaluated changes in autophagosome abundance and autophagic flux. The findings indicate that amino acid starvation led to a significant rise in Cyto-ID autophagosome staining (Figure 12 C) and that CQ treatment led to even higher autophagosome accumulation. Additionally, starvation triggered the turnover of LC3-II (Figure 12 D) and resulted in an increase in the number of LC3 puncta (Figure 12 E), strongly indicating a robust activation of autophagy in these circumstances.

Next, to verify that autophagy promotes the biogenesis of LDs, we employed siRNA-based technology to knock down autophagy-related protein 5 (ATG5), a crucial autophagic gene needed for phagophore elongation, and followed LD dynamics over time. Our time-lapse microscopic examinations demonstrated that many small LDs progressively accumulate in HeLa cells after 3 h of amino acid starvation (Figure 13 A). In comparison with control cells, the average number of small LDs observed per cell was significantly reduced in ATG5-silenced cells, most evidently at the 3 h time point (Figure 13 A). As the duration of amino acid deprivation extended, the count of small LDs notably diminished, while the reduction in the number of large LDs was relatively minor (Figure 13 B). Consequently, ATG5-deficient cells exhibited a corresponding rise in the average diameter of LDs during the period of starvation (Figure 13 C). During HBSS starvation, silencing of ATG5 suppressed autophagic flux (Figure 13 D, E, F) but did not significantly reduce total neutral lipid content measured by flow cytometry (Figure 13 G). However, when viewed under the fluorescent microscope, we were able to detect the effect of knockdown of ATG5 on reducing LD content in HBSS-starved HeLa cells (Figure 13 H). Moreover, depletion of ATG5 resulted in lower abundance of LC3 puncta (Figure 13 I). Thus, the reduced presence of both small and large LDs in ATG5-silenced cells may be linked to impaired LD formation due to autophagy suppression. Nevertheless, the notable increase in LD diameters in ATG5-depleted cells hints at the potential involvement of autophagy also in LD breakdown in these circumstances. In summary, these experiments reveal that depleting HeLa cells of ATG5 primarily suppresses amino acid starvation-induced LD accumulation.

Subsequently, we postulated that the decrease in LD number, along with the enlargement of LDs in ATG5-deficient cells during HBSS starvation, could be attributed to the influence of ATG5 depletion on pre-existing LDs that had formed during cell growth in complete media before the initiation of starvation. Specifically, we noticed that the depletion of ATG5 results in an increased accumulation of LDs in cancer cells when they are cultured in both nutrient-rich media with serum (complete medium) – (Figure 14 A) and in serum-free media (Figure 13 I). When combined with the reduced turnover of LC3-II observed in ATG5-deficient HeLa cells cultured in complete media (Figure 14 B), these findings indicate that ATG5-mediated lipophagy plays a role in LD breakdown not only during nutrient abundance but also during serum deprivation.

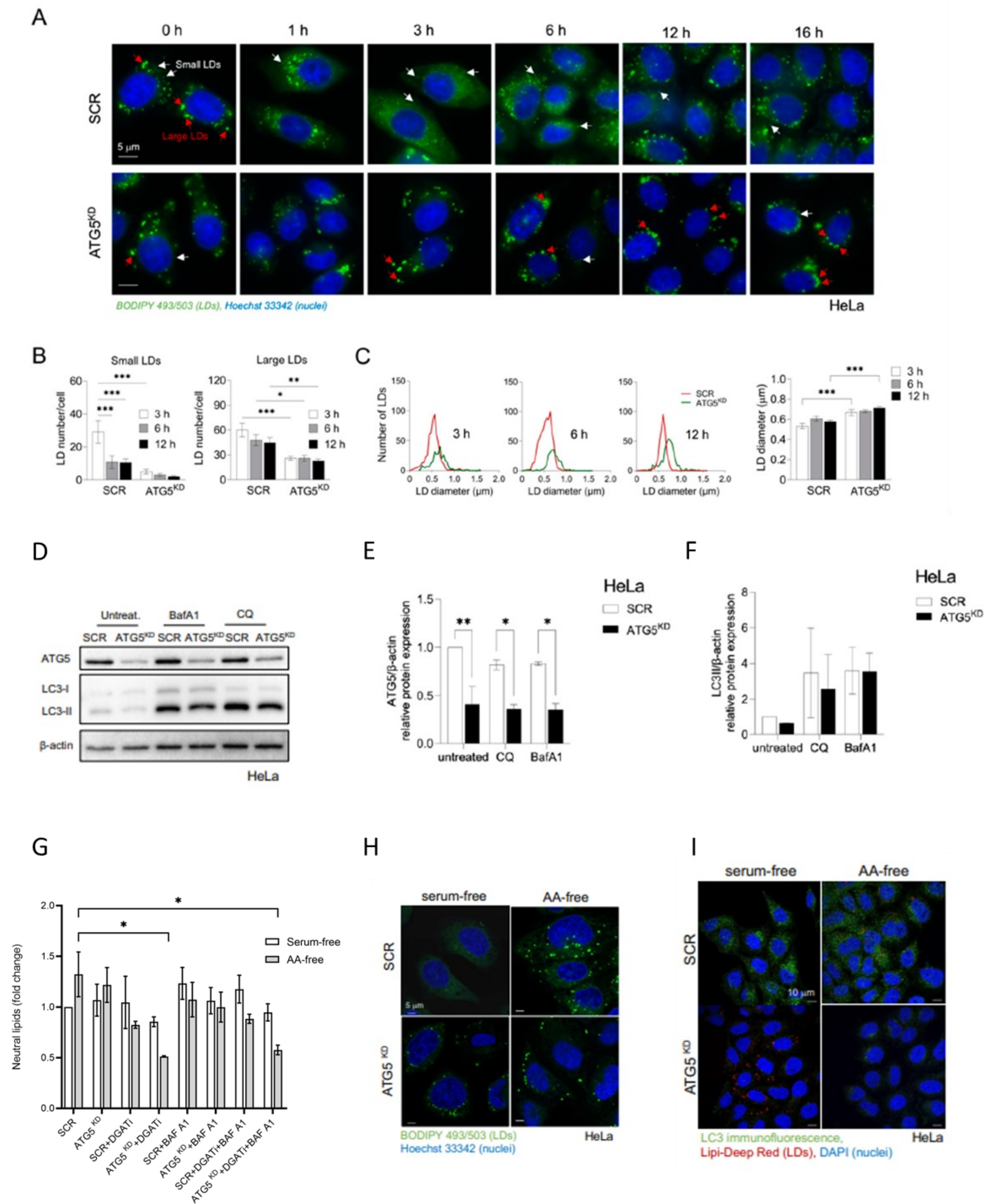


Figure 13: The depletion of ATG5, leading to the inhibition of autophagy, impairs the accumulation of LDs induced by starvation. A) Changes in LD abundance in control and ATG5-deficient HeLa cells during starvation in HBSS. B, C) Changes in LD number and diameter in control and ATG5-deficient HeLa cells during starvation in HBSS. D) Cell lysates were subjected to Western blot analysis to assess the presence of ATG5 protein, levels of the basal form of LC3 (LC3-I), levels of the lipidated form (LC3-II), and were normalized using β-actin as a control. E, F) Densitometric analysis of protein levels of ATG5 and LC3-II corresponding to experiment shown under experimental conditions shown in D. The blots are representative of two independent experiments. G) Cellular neutral lipid content and LD abundance in amino acid and serum-starved control, ATG5-deficient HeLa cells treated with DGATi and BafA1. H) LD abundance in control and ATG5-deficient HeLa cells during starvation in amino acid-free and in serum-free medium. I) LC3 puncta and LD abundance in control and ATG5-deficient HeLa cells during starvation in amino acid-free and in serum-free medium. Scale bars: 10 µm, unless otherwise

indicated. LDs and nuclei were visualized using 1 µg/mL BODIPY 493/503 or Lipi-Deep Red dye and Hoechst stain solution, respectively. Values on the graphs are means ± SEM of at least two experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Tukey's adjustment or Šidak adjustment; unpaired t-test.

To confirm that LDs are formed during starvation and assess the role of ATG5 in the process, we inhibited LD biogenesis during growth in serum-rich conditions by treatment with DGATi and used fluorescent BODIPY-labelled fatty acid analogues to distinguish between preexisting and newly formed LDs. Under these conditions, we investigated the effects of ATG5 silencing on the abundance of LDs specifically during HBSS starvation (H. Wang et al., 2010). In Figure 14 C, LDs labeled with green BODIPY FL C12 dye and indicated by white arrowheads were seen in live-cell confocal microscopy images at the end of the growth phase in complete media (0 h). Notably, these LDs were not present in cells without ATG5. Our results showed that following a 6-hour starvation in HBSS, the labeled LDs decreased, attributed to ATG5 depletion (Figure 14 C). The overlapping red and green signals observed in ATG5-deficient cells suggest that these cells mainly contain pre-existing LDs formed in complete medium but lack the LDs that were induced by acute nutrient stress. This indicates that under acute starvation conditions, ATG5-deficient HeLa cells probably lost their ability to generate new LDs (Figure 14 C).

Collectively, these experiments demonstrate that severe amino acid starvation in HeLa cancer cells triggers the activation of autophagy, and this activation, in turn, promotes the biogenesis of LDs.

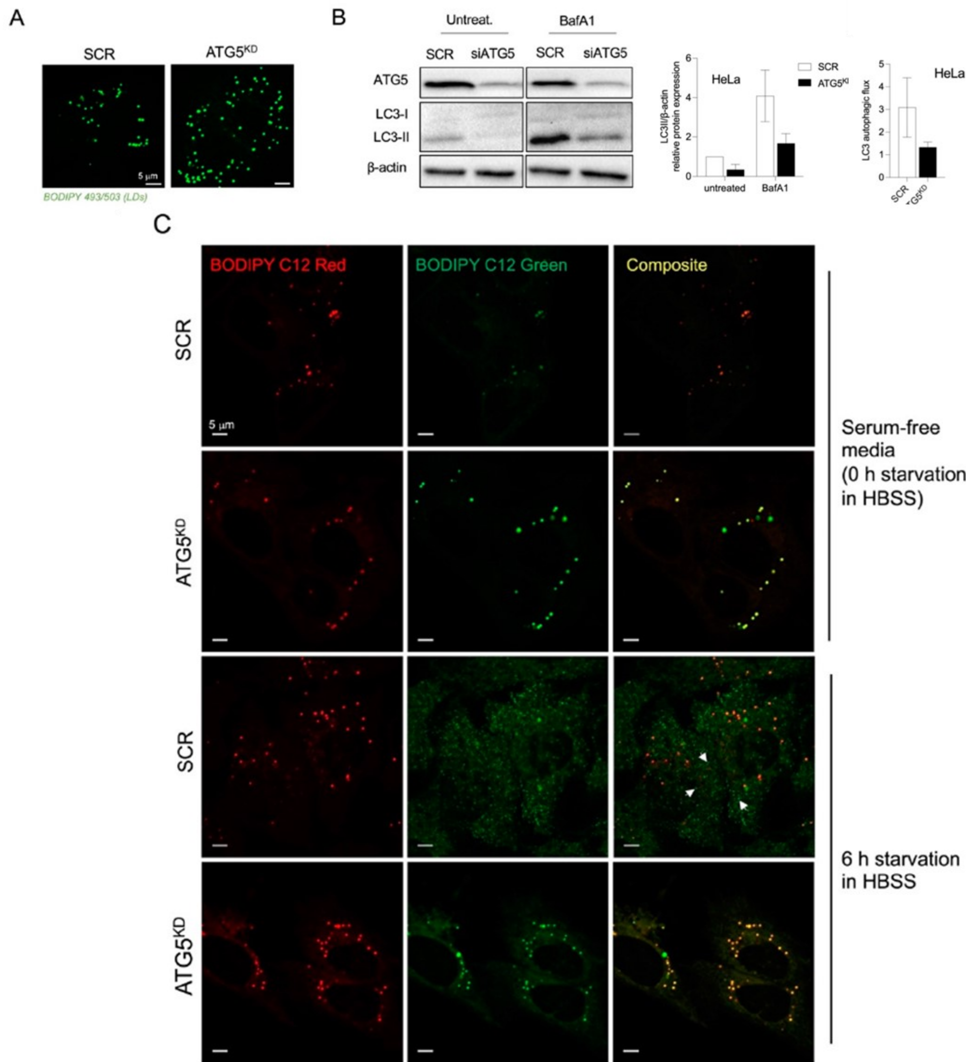


Figure 14: ATG5 participates in stimulating LD degradation under conditions of nutrient abundance, while it supports LD formation during amino acid deficiency. A) LD abundance and B) LC3-II turnover that was assessed to evaluate autophagic flux in control and ATG5-deficient HeLa cells cultured in complete media. Cells were treated with BafA1 to inhibit lysosomal degradation, followed by Western blot analysis of LC3-II levels. LC3-II turnover was determined by comparing LC3-II levels in the presence and absence of BafA1. Additionally, to quantitate LC3-II levels, densitometric analysis of Western blot bands was performed using ImageJ software. The intensity of LC3-II bands was normalized to a loading control (e.g., β-actin) to account for variations in protein loading. The fold change in LC3-II levels was calculated relative to control cells. C) Live cell imaging of pre-existing (BODIPY C12 Red) and newly formed LDs (BODIPY C12 Green) during HBSS starvation of control and ATG5-deficient HeLa cells. Serum-depleted and DGATi-treated HeLa cells were labelled with red Bodipy FA analogue (Bodipy 558/568) for 24 hours before severe nutrient deprivation and with green C12 during severe stress (for the next 6 hours). Green FL C12 was shown to be incorporated into newly synthesized LDs, whereas red C12 remained accumulated in LDs already formed before starvation. Representative confocal microscopy results are from at least three independent experiments. Scale bars: 10 µm. Values on the graphs are means ± SEM of at least two independent experiments. A) LDs were visualized using BODIPY 493/503. B) Cell lysates were analyzed via Western blotting

for the presence of ATG5 protein, LC3 basal (LC3-I), and lipidated forms of (LC3-II) and β-actin. The images and blots shown are representative of at least two independent experiments.

As ATG5 has been shown to have autophagy-independent effects, we next aimed to corroborate our findings by targeting autophagy initiation. To address this question, we utilized siRNA-based technology to genetically silence an essential gene involved in the initiation of the autophagic process, the Unc-51-like kinase 1 (ULK1). Our findings revealed a similar effect of ULK1 depletion as with ATG5, where there were no apparent changes in the total amount of neutral lipids after 6 hours of amino acid starvation (Figure 15 A, D). However, the diameter of LDs in ULK1-depleted cells was notably larger compared to control cells (Figure 15 C) and the LD number per cell exhibited a slight decrease upon ULK1 depletion (Figure 15 A), similar to the results observed in Figure 13 B and C.

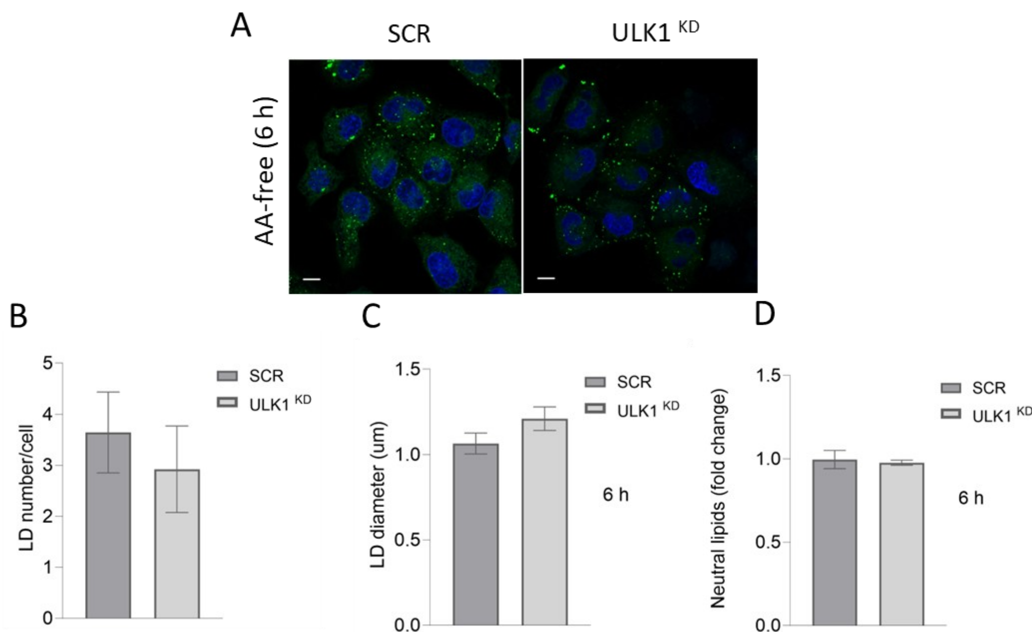


Figure 15: Effect of ULK1-depletion on LD abundance and morphology during severe nutrient stress. A) Representative microscopic images of control and ULK1-depleted HeLa cells during amino acid deprivation for 6 h. B, C) Changes in LD number and diameter in control and ULK1-deficient HeLa cells during starvation in HBSS. D) Total neutral lipid accumulation in control and ULK1-deficient HeLa cells during starvation in HBSS. Scale bars: 10 µm, unless otherwise indicated. LDs and nuclei were visualized using 1 µg/mL BODIPY 493/503 and Hoechst stain solution, respectively. The absence of statistical error bars indicates the absence of significant changes.

4.2.2 Interplay between lipolysis and autophagy/lipophagy in LD metabolism during nutrient stress

Past research involving amino acid-starved MEFs has demonstrated that the degradation of autophagy-derived LDs takes place through ATGL-mediated lipolysis (Rambold et al., 2015). Additionally and more recently, it was demonstrated that a combination of lipolysis and a specific form of autophagic lipid breakdown known as lipophagy promoted LD

catabolism in hepatocytes (Schott et al., 2019). Based on this idea, in this work, we investigated what would happen to LD dynamics if both processes were genetically inhibited simultaneously. We reached several findings using various techniques including live cell confocal microscopy and flow cytometry that are explained below.

To find out if our cell model, HeLa, employs ATGL for TAG lipolysis, we knocked down ATGL using siRNA and followed LD content and morphology within cells. Our results demonstrate that in the initial stages of severe nutrient stress, there was an increase in total LD content in HeLa cancer cells due to ATGL depletion (Figure 16 A, B). Next, we performed parallel inhibition of autophagy by silencing ATG5. Co-inhibition of lipolysis (ATGL ^{KD}) and autophagy (ATG5 ^{KD}), compared with single depletion of ATGL, resulted in a significant reduction in neutral lipid levels and a decreased LD accumulation in amino acid-starved cells (Figure 16 A, B). This indicates that when amino acid starvation conditions are present, the primary mechanism for the breakdown of LDs formed through autophagy is ATGL-mediated lipolysis, rather than lipophagy. Therefore, ATGL plays a role in the degradation of autophagy-derived LDs in amino acid-starved HeLa cells.

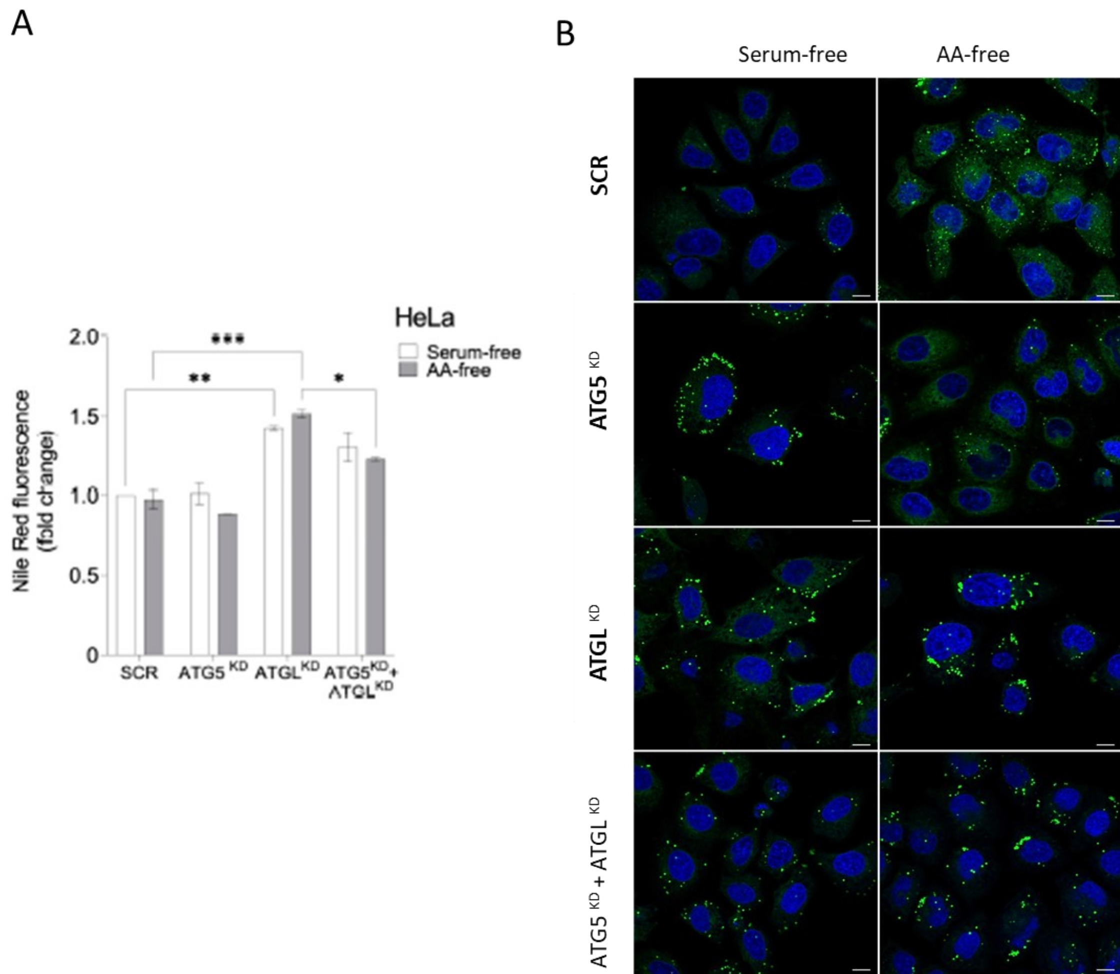


Figure 16: ATGL plays a role in the degradation of autophagy-derived LDs in amino acid-starved HeLa cells. A) Neutral lipid levels in control, ATG5-deficient, and ATGL deficient serum- and amino acid-starved HeLa cells. B) Representative confocal microscopy results of LD abundance in control, ATG5-deficient, and ATGL-deficient serum- and amino acid-starved HeLa cells. Cellular LDs in starved cells were visualized using 1 µg/mL BODIPY 493/503 and nuclei using Hoechst stain solution. Scale bars: 10 µm. Images are from at

least two independent experiments. Values on the graphs are means ± SEM of at least two experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Tukey's adjustment.

4.2.3 Autophagy and LD formation in nutrient-rich media

Considering that basal levels of autophagy and lipophagy can occur in nutrient-rich conditions, we next examined the interplay between autophagy/lipophagy and lipolysis in LD metabolism under nutrient-rich conditions. After incubating cells in complete medium for 24 hours following reverse transfection, we assessed their total neutral lipid content. Our findings revealed that ATG5 ^{KD} led to the formation of slightly larger LDs (Figure 17 A) while the overall levels of neutral lipids measured by flow cytometry showed a slight decrease compared to the control group (Figure 17 C). Interestingly, inhibiting LD biogenesis using a DGAT1 inhibitor resulted in reduced total neutral lipid content across all groups, even when lipolysis was blocked (Figure 17 C). However, the DGAT2 inhibitor alone did not have any significant effect. These observations led us to infer that ATG5 ^{KD} caused the accumulation of larger LDs (Figure 17 A), suggesting the presence of active lipophagy under nutrient-rich conditions which explained our results in HBSS-starved cells (see Figure 13 B), where we also saw larger LDs.

To gain further insights, when the samples were exposed to various autophagy inhibitors, the treatment with BafA1 resulted in a slight increase of LD content in the control cells but had no discernible impact on ATG5 ^{KD} cells (Figure 17 D). However, in cells where lipolysis was blocked (ATGL ^{KD}), BafA1 alone and together with LALi inhibitor significantly affected the neutral lipid content, indicating that the interplay between autophagy and lipolysis may be influenced by ATGL (Figure 17 D). Finally, the experiments on HeLa cancer cells under nutrient-rich conditions revealed that inhibiting autophagy through ATG5 depletion led to an increased expression of the lipolysis-related protein ATGL (Figure 17 B). Additionally, cells in which both autophagy and lipolysis were genetically inhibited showed a reduced autophagic flux compared to control cells.

In summary, these findings indicate that there is a complex interplay between these processes, even in the context of cells grown under nutrient-rich conditions.

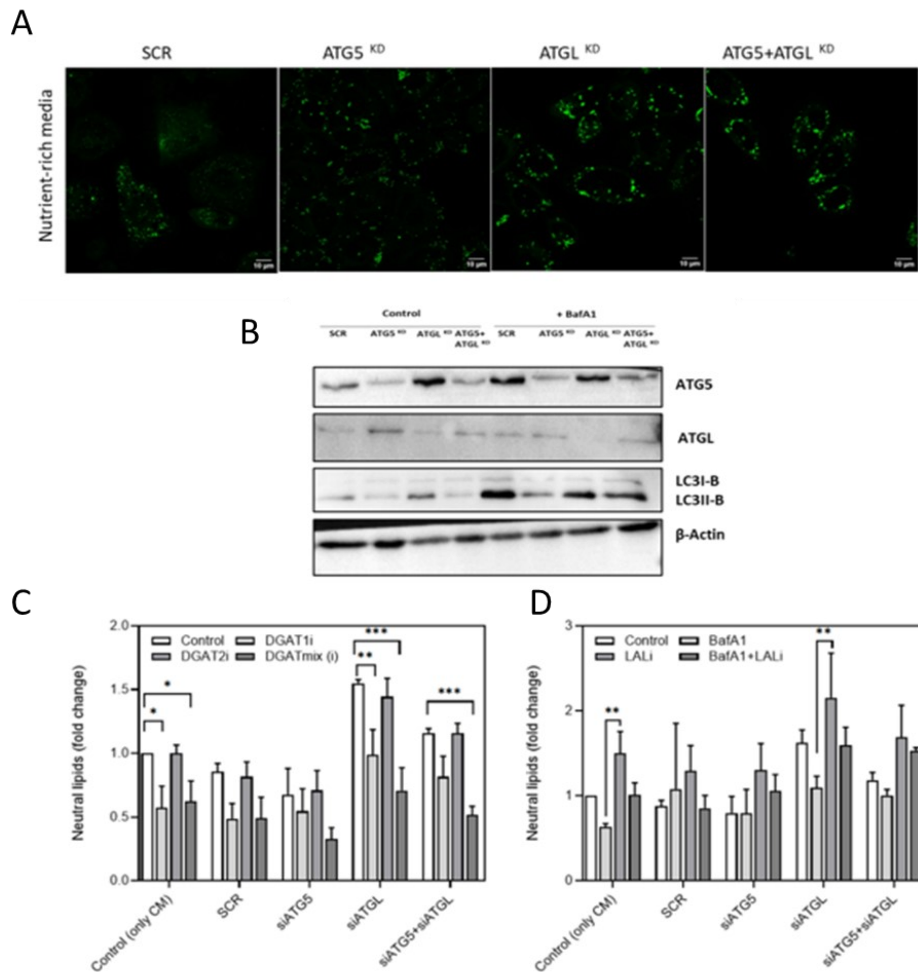


Figure 17: Lipolysis and lipophagy contribute to LD breakdown in nutrient-rich conditions. A) Representative microscopy images of LDs in cells depleted of ATGL, ATG5 or both. B) Cell lysates were analyzed via Western blotting for the presence of ATG5, ATGL, LC3 basal (LC3-I), and lipidated forms of (LC3-II) andβ-actin. C) Neutral lipids in cells depleted of ATGL, ATG5 or both, and treated with DGAT1 and DGAT2 inhibitors or both (DGATi). D) Neutral lipids in cells depleted of ATGL, ATG5 or both, and treated with LAL and BafA1 inhibitors or both (BafA1+LALi). Scale bar: 10 µm. The images and blots shown are representative of at least two independent experiments. Values on the graphs are means ± SEM of at least two experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Tukey's adjustment).

4.3 LD Dynamics in HeLa Cells During Prolonged Serum Deprivation

4.3.1 Crosstalk between ATGL-mediated LD breakdown and autophagy/lipophagy during prolonged starvation

Having established that LDs do indeed form in response to severe nutrient deprivation, we wondered whether we could elicit a similar effect by starving cells in serum-free media for several days (4-5 days). Our main question was: what is the effect of prolonged starvation

on LD abundance and how is it affected by inhibition of autophagy and lipolysis? Surprisingly, our results showed an increase in LD abundance over time (Figure 18 A, B). Next, when autophagy was inhibited by depletion of ATG5, we observed a decrease in LD numbers (Figure 18 A). This suggested that LD biogenesis might be driven by autophagy under these conditions, similar to what we already saw during severe nutrient stress.

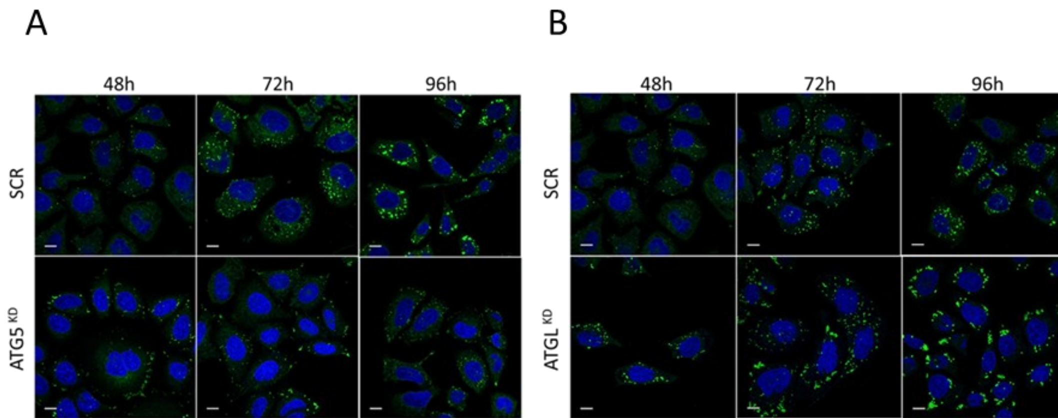


Figure 18: Changes in LD abundance during prolonged serum starvation are controlled by autophagy and lipolysis. A, B) Representative confocal microscopy images of live cells under serum-free conditions and genetic knockdown of ATG5 and ATGL. Silencing of essential autophagic gene ATG5 (20 nM siRNA) reduced total LD content during prolonged, mild nutrient stress in HeLa cells, whereas depletion of ATGL led to increased accumulation over time. ATGL-mediated LD breakdown may thus compensate for the reduced autophagy/lipophagy caused by ATG5 ^{KD}, leading to unexpectedly low levels of LDs during prolonged serum deprivation. Scale bar: 10 µm.

In addition, our results demonstrated that the inhibition of ATGL consistently led to the sustained build-up of LDs, which suggested that lipolysis is active throughout the prolonged starvation phase (Figure 18 B).

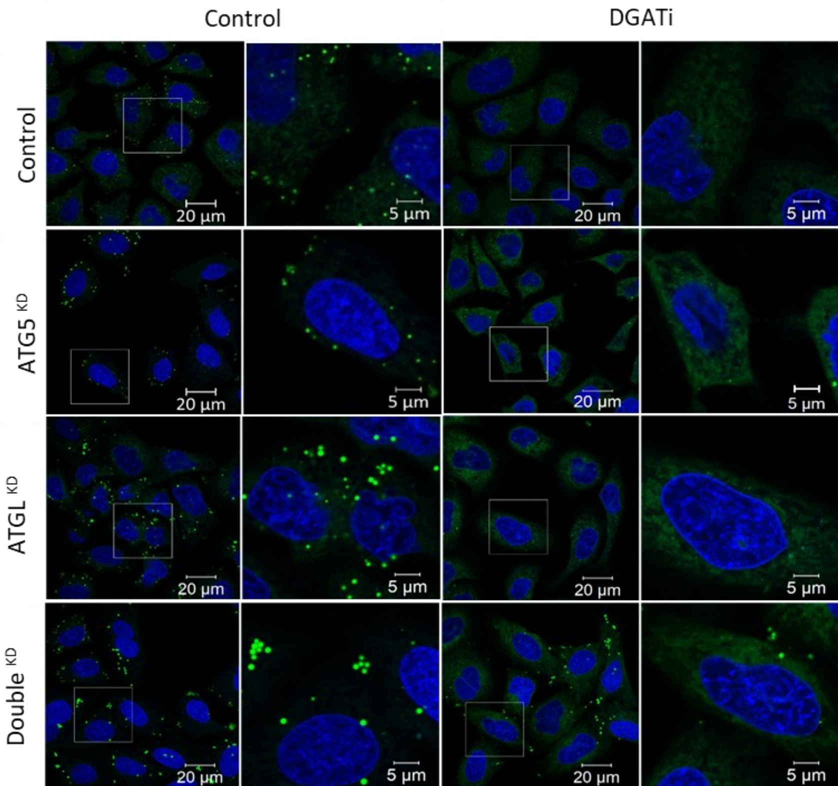
Next, we asked whether LD biogenesis indeed occurs during prolonged starvation and how LD dynamics is affected when both autophagy/lipophagy and lipolysis were simultaneously blocked.

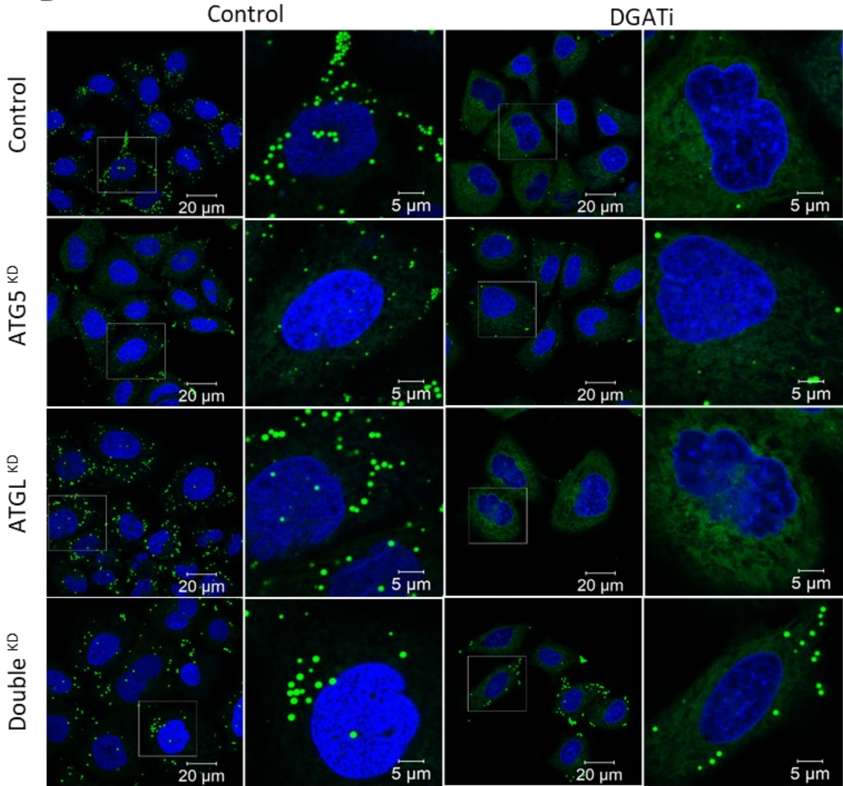
4.3.2 LD biogenesis and lipolysis occur during prolonged serum deprivation

During first 48 h of serum removal, cells slightly accumulated LDs in DGAT-dependent manner (Figure 19 A). In later time points, trend of DGAT-dependent LD synthesis is more profoundly present compared to 48 h, and the effect of DGAT inhibitors was more evident (Figure 19 B, C).

When ATGL-deficient cells were examined over several days of serum starvation (48–96 h), we consistently found elevated LD abundance suggesting that ATGL-mediated TAG lipolysis is continuously active under these conditions (Figure 19 A, B, C). Accordingly, ATGL-deficient cells had more LD number/cell only after 48 h. In addition, DGATi treatment in ATGL-depleted cells reduced LD accumulation (Figure 19 A, B, C). Surprisingly, when autophagy/lipophagy was inhibited by depletion of ATG5, there was a consistent decrease in LD numbers (Figure 19 A, B, C). However, a combined silencing of ATGL and ATG5 led to LD accumulation, indicating that a compensatory elevation of

ATGL-mediated LD breakdown might be responsible for the low LD levels observed after autophagy/lipophagy was blocked with ATG5 depletion. Additionally, the reduction in LD numbers was evident in cells lacking ATG5 and undergoing DGATi treatment, reflecting a similar outcome seen in cells depleted of both genes (Figure 19 A, B, C).


A
Control
DGATi
Control
ATG5 KD
ATGL KD
Double KD
20 µm
5 µm


B
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DGATi
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ATGL KD
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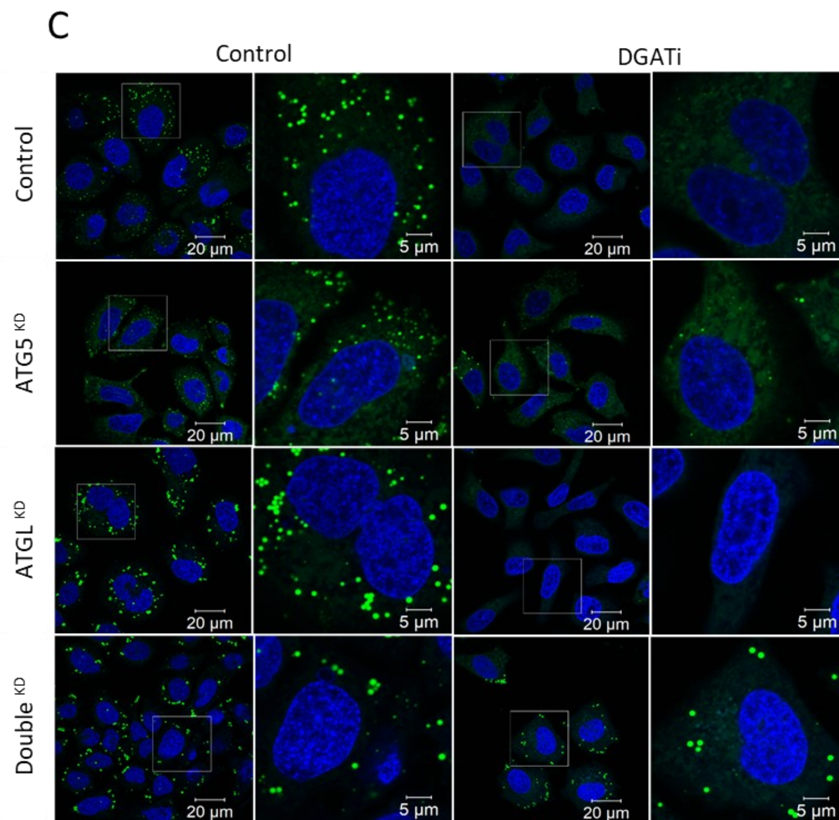

Figure 19: LD biogenesis and lipolysis occur during prolonged serum deprivation. A, B, C) Representative confocal microscopy images of LDs in HeLa cancer cells depleted of ATGL, ATG5 and both after 48 h (A), 72 h (B) and 96 h (C) of serum starvation with and without a mixture of DGAT1 and DGAT2 inhibitors (DGATi; 20 uM each). Images were taken from three independent experiments. Scale bars: 20 and 5 µm, respectively.

Next, we decided to verify this effect by measuring the total LD content (Figure 20 A, B, C) and quantify LD number/cell under these experimental settings (Figure 20 D, E, F). We confirmed our results with respect to DGATi treatment for separate genetic knockdowns and the resulting reduction in the amount of LDs also by flow cytometry and by quantification of LDs from microscopic images.

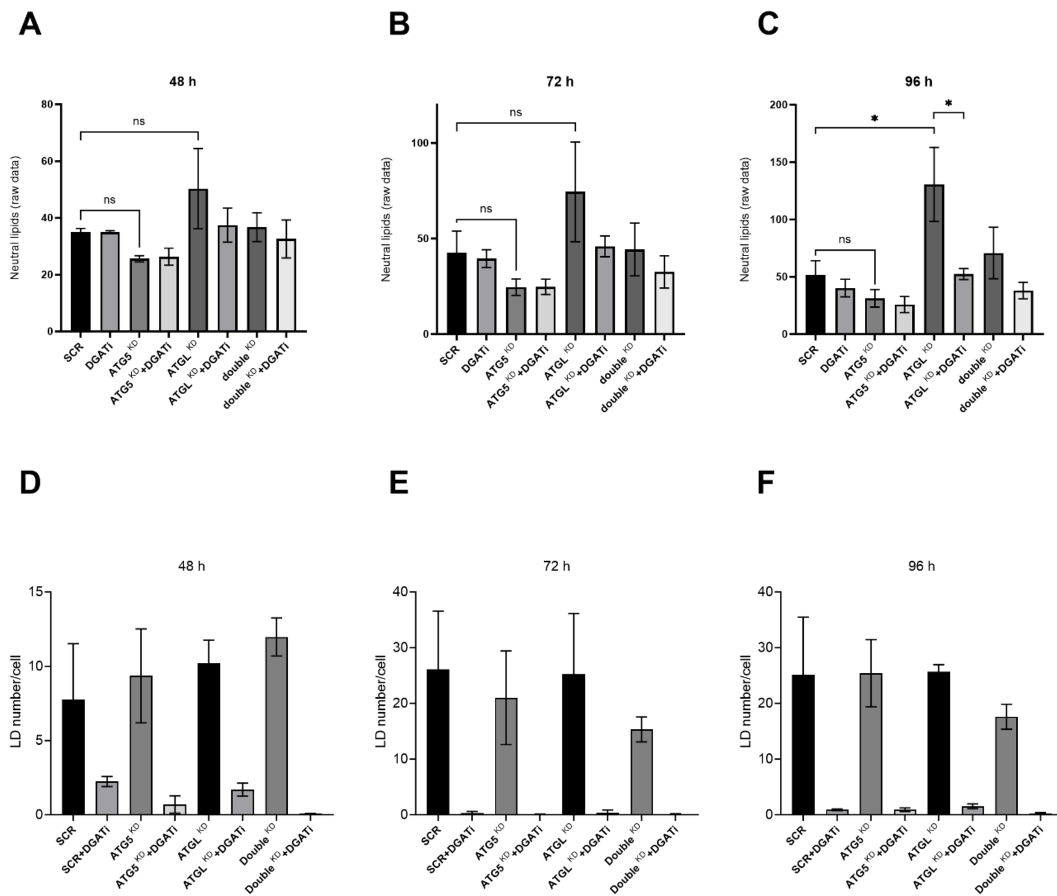

Figure 20: HeLa cells progressively accumulate LDs during prolonged serum deprivation in DGAT1/2-dependent manner. (A–C) Total neutral lipids were quantified upon genetic suppression of autophagy and lipolysis-related genes. (D–F) LD number/cell for at least 30 cells/sample and per one biological replicate were quantified using ImageJ analysis from three different experiments. Values on the graphs are means ± SEM of at least three independent experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA with Šidák's or Tukey's adjustment).

Next, for the purpose of better understanding the effects we observed during prolonged serum deprivation, we silenced another gene involved in autophagic process: ULK1. We wondered whether ULK1 depletion will result in the same effect as ATG5 depletion which was observed in Figure 19. In the initial 48 h of serum deprivation, the depletion of ULK1 did not lead to a noticeable reduction in the number of LDs, as observed through fluorescent microscopy. However, with an extended period of starvation, this observation became consistent with what was seen in the case of ATG5 depletion (Figure 19). Therefore, we confirmed our hypothesis that also in this case both autophagy/lipophagy and lipolysis occur during this time period and under these stressful conditions (Figure 21).

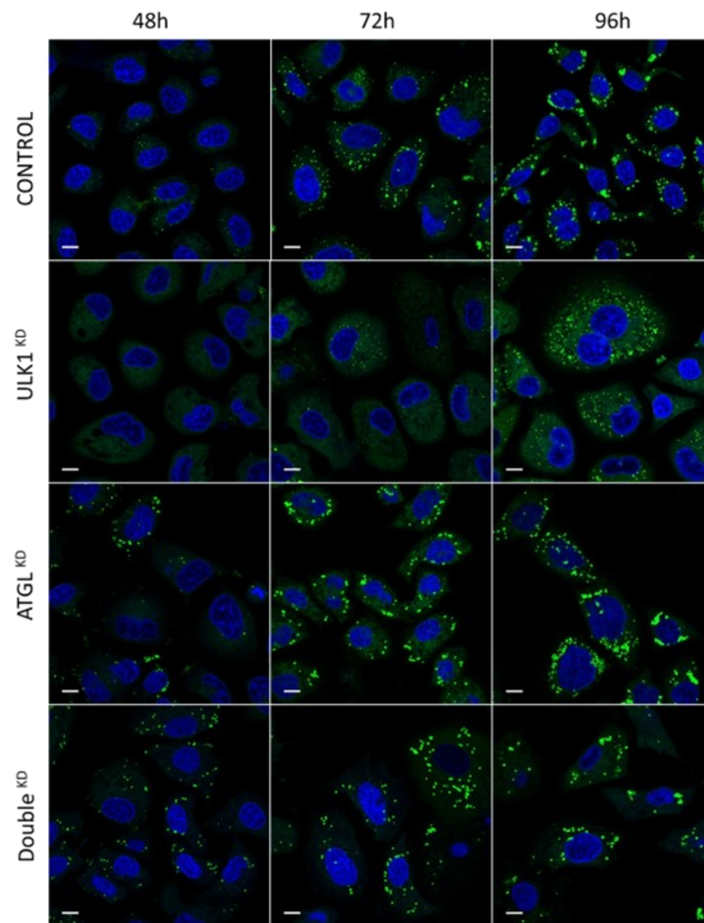

Figure 21: Autophagy and lipolysis occur during prolonged serum deprivation. Representative confocal microscopy images of HeLa cancer cells showing the impacts of individual, as well as combined, genetic depletion (ATGL and ULK1 ^{KD}). Scale bars: 10 µm. Images are taken from at least two independent experiments.

4.4 Hypothesis III: Both LDs and Autophagy/Lipophagy Contribute to the Resistance of Cancer Cells to Nutrient Deprivation-Induced Stress

4.4.1 Autophagy and LDs collaborate for cell survival, while lipolysis has no impact

Autophagy-driven LD biogenesis has been shown to prevent mitochondrial damage in starving MEFs by sequestering autophagy-derived FAs into LDs (Nguyen et al., 2017). Here, we investigated whether interfering with LD turnover (biogenesis or breakdown), combined with inhibition of autophagy or lipolysis, alters the capacity of HeLa cancer cells to survive different forms of nutrient stress. First, inhibition of LD biogenesis with DGATi treatment increased cell death during amino acid starvation, but not during serum depletion (Figure 22). Moreover, inhibiting autophagy through ATG5 ^{KD} intensified cell death (Figure 22). Intriguingly, the concurrent application of both treatments, ATG5 silencing and DGATi treatment, led to an unexpectedly high percentage of apoptotic cells,

underscoring the crucial roles of both autophagy and LDs in promoting cancer cell survival (Figure 22).

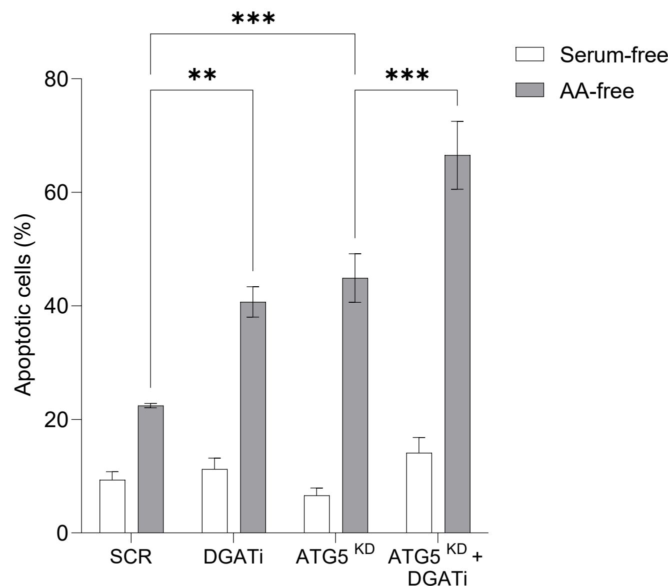

Figure 22: Inhibition of autophagy and DGAT-mediated LD biogenesis increase HeLa cell death during amino acid starvation. Cell death rates in control and ATG5-deficient serum- or amino acid-starved HeLa cells treated with an equimolar mixture of DGAT1 and DGAT2 inhibitors. Values on graphs are means ± SEM of at least two independent experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Tukey's adjustment).

Next, inhibition of autophagy by the late-stage autophagy inhibitor BafA1 significantly compromised HeLa cell survival during severe starvation, but not during mild starvation in serum-free media (Figure 23 A). In addition, to investigate the significance of lipolysis for cell survival under these conditions, HeLa cells were depleted of the rate-limiting neutral lipase ATGL using siRNA (Figure 23 B, C). We found that ATGL depletion alone or in combination with ATG5 ^{KD} does not affect cell survival in serum-depleted cells for early hours (Figure 23 B). Surprisingly, we found that ATGL depletion did not influence cell survival in severely starved cells (Figure 23 C) as it was not the case with ATG5 depletion (Figure 22) and combination of ATG5 and ATGL depletion (Figure 23 C). Additionally, we also found that DGAT co-inhibition led to a further increase in cell death in severely starved HeLa cells (Figure 23 C), whereas we failed to observed the effect of DGAT co-inhibition during mild serum deprivation (Figure 23 B). Altogether, our results show that whereas inhibition of DGAT enzymes and autophagy are effective in killing starving HeLa cancer cells, targeting ATGL-mediated lipolysis does compromise HeLa cell survival during severe nutrient stress.

We next asked how autophagy, LD biogenesis and lipolysis affect cell survival during prolonged serum starvation (Figure 23 D, E, F). We found that interfering with LD biogenesis by DGAT inhibition or lipolysis by depletion of ATGL did not affect cell survival even after 96 hours of serum depletion (Figure 23 F). However, inhibition of autophagy by depletion of ATG5 led to an increase in cell death, which was particularly evident at later stages of mild nutrient starvation (Figure 23 E, F) and was further augmented by inhibition of both LD biogenesis and autophagy (Figure 23 E, F). Surprisingly, ATGL depletion had no effect on cell death during mild stress, even when both lipolysis and autophagy were

inhibited (Figure 23 D, E, F). Altogether, these results suggest that ATG5-driven autophagy and DGAT-mediated LD biogenesis, but not ATGL-mediated LD lipolysis, work together to ensure cell survival during both severe and mild nutrient stress.

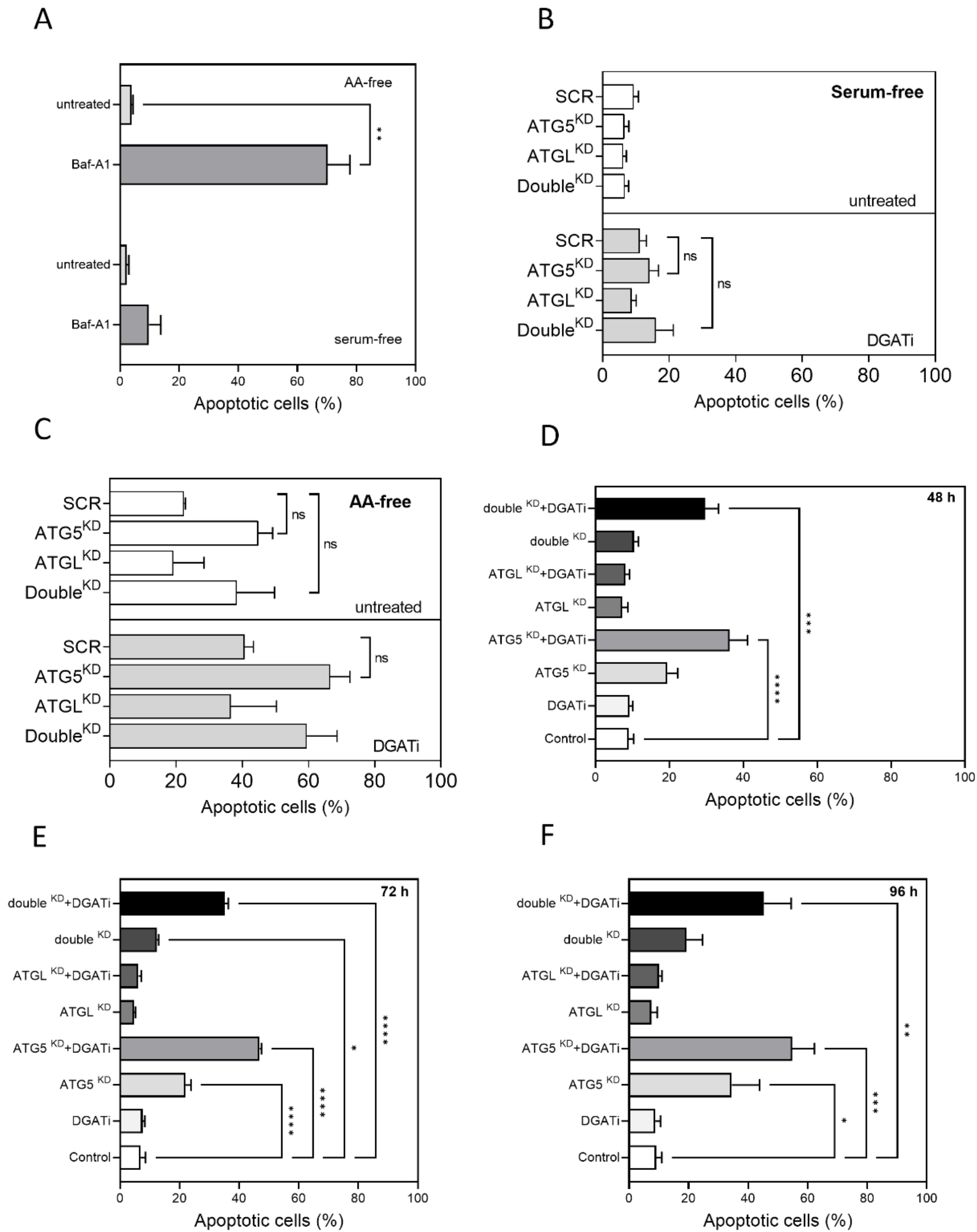

Figure 23: Cell survival during different stages of nutrient stress exposure and under different conditions. A) Cell death rates in serum or amino acid-starved HeLa cells treated with autophagy inhibitor BafA1. B) Cell death rates in control, ATG5-deficient, and ATGL-deficient serum-starved HeLa cells treated with an equimolar mixture of DGAT1 and DGAT2 inhibitors. C) Cell death rates in control, ATG5-deficient, and ATGL-deficient severely-starved HeLa cells treated with an equimolar mixture of DGAT1 and DGAT2 inhibitors. D, E, F) Cell death rates (48 h, 72 h, 96 h) in control, ATG5-deficient, and ATGL-deficient serum-starved HeLa cells treated with an equimolar mixture of DGAT1

and DGAT2 inhibitors. Values on graphs are means ± SEM of at least two (A) or three (B-F) independent experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Tukey's adjustment).

Chapter 5

Discussion

Cancer cells face significant challenges due to the limited availability of nutrients and oxygen, which can hinder their growth and development. However, they have developed distinct mechanisms that enable their uncontrolled proliferation. This metabolic reprogramming is crucial for the growth, metastasis, and resistance of tumors to various treatments. The survival of cancer cells during periods of stress heavily relies on the availability of different nutrients, including lipids (Koundouros & Poulogiannis, 2020; Pavlova & Thompson, 2016). Lipids can either be synthesized *de novo* within cells, recycled from other cellular compartments, or taken up from the extracellular environment (Petan et al., 2018). Autophagy is a biological process used by cells to recycle cellular material, including lipids (Dikic & Elazar, 2018; Parzych & Klionsky, 2014). The intricate interplay between lipid metabolism, cancer metabolism and autophagy coordinates multifaceted cellular mechanisms essential for cellular and energy homeostasis. Therefore, this study along with other studies that investigate this crucial relationship between lipid metabolism and autophagy/lipophagy and other cellular processes including lipolysis in cancer cells is gaining significant interest nowadays. Therefore, this dissertation has the potential to shed light on the molecular mechanisms that enable cancer cells to thrive under unfavorable conditions such as nutrient stress and provide new avenues for developing effective cancer therapies (Fu et al., 2021; Santos & Schulze, 2012; Walther & Farese Jr., 2012). In the following paragraphs, we will delve into the significance of this research in relation to the other important studies within the field. This comparative analysis aims to provide a deeper understanding and greater appreciation for the contributions of this work.

5.1 LDs and Autophagy/Lipophagy Have Complex Relationship

This work provides compelling evidence that LDs are key players in the lipid metabolism of HeLa cancer cells subjected to nutrient stress. They are no longer considered mere inert storage depots for many reasons and some of them are covered in next paragraphs. These organelles' pivotal role in cancer cell stress resistance and, potentially, tumor growth stems from their close association with other cellular processes critical to overall cellular metabolism such as ER, mitochondria, autophagosomes, lysosomes, etc. One of the most significant connections is observed between LDs and the ER (Q. Gao & Goodman, 2015). The ER serves as a primary site for lipid synthesis, and LDs emerge as distinct structures from the ER membrane (Walther & Farese Jr., 2012; Walther & Farese, 2009). LD formation is intricately linked to ER lipid metabolism, with lipids being synthesized on the ER and then channeled to LDs for storage. Moreover, proteins involved in lipid metabolism, such as enzymes and lipid transporters, often localize to both LDs and the ER, further highlighting their interconnected functions (Q. Gao & Goodman, 2015). In addition, it is

shown that LDs interact with mitochondria by the 'kiss-and-run' model of organelle connection (L. Cui & Liu, 2020). Moreover, it was shown that the formation of LDs relies on a specific enzyme which is involved in LD biogenesis and TAG synthesis, DGAT1, and serves as a protective mechanism for cells against mitochondrial damage induced by an excess of fatty acids derived from autophagy, which may be converted into acylcarnitines (Nguyen et al., 2017). The interesting link between LDs and autophagy-related compartments, like autophagosomes or lysosomes, introduces complexity to the field. This intricate interplay between these distinct organelles is evident in processes such as lipophagy or LD breakdown, specifically in their connection to lysosomes, and autophagy, or in their association with autophagosomal structures known as autophagosomes. This research delves into this intricate interplay between LDs and autophagy/lipophagy primarily through a range of approaches starting from the hypothesis that LDs are being formed and/or broken down in response to the different nutrient stress conditions to which cancer cells are usually exposed.

First, by exposing cancer cells to various stressful factors such as severe or mild nutrient stress for different time periods, we were able to follow LD dynamics. In this study, we showed that DGAT-dependent LD formation is more pronouncedly observed in cells exposed to severe nutrient stress compared to milder, serum-free conditions or low glucose conditions (Figure 10 E–G). Interestingly, following LD dynamics for longer periods of mild nutrient starvation, we were able to see that LDs also start to accumulate after prolonged periods of serum-free starvation in DGAT-dependent manner (Figure 11 A, B). However, this study demonstrates that mild starvation during initial hours of serum depletion promotes LD breakdown (Figure 10 A–D). While previous studies have demonstrated LD accumulation in HeLa cell line subjected to intense 16 h nutrient stress (Nguyen et al., 2017), the formation of LDs under milder nutrient conditions over extended durations has not been observed until now. Under the conditions described in previously mentioned study, DGAT1 enzyme mediates FA channeling into LDs downstream of mTOR1-regulated bulk autophagy and protects against lipotoxic disruption of mitochondrial function while promoting cancer cell survival (Nguyen et al., 2017). Although they can have distinct functions in various conditions that promote fat storage, DGAT1 and DGAT2 can easily compensate for each other as recently reported (Chitraju et al., 2019). Consequently, these findings underscore the significance of this dissertation and make the basis for our other experiments.

LDs' close and complex connection to autophagy made this work challenging and complex (C. Xu & Fan, 2022). Autophagic process is mediated by the group of the proteins named autophagy-related genes (ATGs). More than 35 known genes are found in yeast with most of them also being conserved in mammals and plants. ATG12–ATG14, ATG16 and others are important for autophagosomal formation and their fusion with lysosomes. This study shows that HeLa cervical cancer cells under HBSS starvation conditions accumulate LDs, whose synthesis is governed by upregulation of autophagy (Figure 12 A, B and Figure 13 D–F). In exploring the role of DGAT enzymes which are important for LD biogenesis, we demonstrated that inhibition of DGAT1, but not DGAT2, prevents starvation-induced LD accumulation in HeLa cells, suggesting its prerequisite role in the process (Figure 10 E–G). Using live cell imaging, we found that ATG5 ^{KD} HeLa cells lose the capacity for new LD formation during HBSS starvation, but contain less LDs with bigger diameter that remain present in the cells from the cultivation in complete medium (Figure 13 A, C). In addition, this study showed that ATG5 depletion resulted in reduced levels of LD content (Figure 13 H, I; C; Figure 18 A) under both types of stressful conditions used throughout the study (short, acute and prolonged, mild). Previous studies showed that complete nutrient deprivation induces LD biogenesis in various cells including HeLa and human LN18 gluoblastoma cells (Cabodevilla et al., 2013). Additionally, the

same study reported that cell death was associated with depletion of LDs (Cabodevilla et al., 2013) which will be discussed also in some of the next paragraphs of this chapter. Our study adds up to the growing evidence that LDs are induced primarily during the exposure of cells to severe nutrient deprivation (Nguyen et al., 2017; Rambold et al., 2015), while pointing to the complexity of this interplay when it comes to the prolonged serum deprivation.

For HeLa, despite being among the most significant and often employed cell lines in studies on metabolism and cancer, little is known about their metabolism from the perspective of LDs, and it is still unknown how they are formed or broken down and whether autophagy/lipophagy has an impact on this. As previously mentioned, we checked for the possible role of ATG5 protein in reducing the content of newly synthesized LDs and/or managing FA analogues transfer to LDs (Figure 13 A and C). We put an emphasis on newly formed LDs, because we found out that LDs are also present in nutrient-rich conditions, and it was hard to distinguish between those whose synthesis was induced by stress and those that were already formed. In a previous study done on hepatocytes, the FA analogues were introduced as tracers for TAG synthesis (and therefore LDs) to investigate the triacylglycerol hydrolase-mediated changes in cytosolic LD dynamics (H. Wang et al., 2010). Therefore, in our study, we used the same technique to further examine the potential role of ATG5 protein in FA analogues transfer to LDs and thus, LD formation. Study on hepatocytes was examining how inadequate autophagy influences LD biogenesis and showed that autophagy-deficient (L-Atg5) knockout mice (thus with decreased autophagy) had difficulty responding to fasting-induced hepatic production of LDs (Y. Li et al., 2018). Our findings demonstrate that HeLa cells behave differently when cultured in complete medium due to genetic knockdown of ATG5. In addition, we show that ATG5 may be of major relevance for LDs produced in such conditions also prior to starvation as well as during the starvation. All of this points to the direction that lipophagy may be occurring even during growth in complete medium as we were able to observe larger LDs before even starving cancer cells. So, for our understanding of what is actually going on regarding LD dynamics during various cell growth conditions, this was one of the most crucial findings that we obtained. However, when we started severe nutrient starvation, LDs started to accumulate very fast and even during early hours of severe nutrient stress (Figure 13 A). This was in line with the data obtained on different lung cancer cells as it was shown that lipids are an important nutrient source for lung cancer cells under starvation (Lung et al., 2022). Moreover, ATG5 silencing resulted in reduced autophagic flux as we expected and was observed also in other papers (cell lines/animals) (Figure 13 D) (Xi et al., 2016). In microglia cells, it was shown that the genetic removal of ATG7 induces LD formation *in vitro* (Y. Xu et al., 2021). Additionally, in MEFs, it was demonstrated that the deletion of ATG5 leads to impairment of adipogenesis in cellular model and in mice (Baerga et al., 2009). In lymphatic endothelial cells, the authors reported that autophagy regulates LD homeostasis. More precisely, they demonstrated that within these cells, the deletion of ATG5 or ULK1, along with the inhibition of autophagy through CQ treatment, resulted in a noteworthy increase in LD content. This elevation was quantified using fluorescent staining for neutral lipids and observed through microscopic imaging (Meçe et al., 2020). Nonetheless, there remains an existing knowledge gap concerning the involvement of autophagy in the formation of LDs in various cancer cell types, as well as the precise mechanisms that govern this formation, despite recent coverage of this topic in a review paper (Fader Kaiser et al., 2022). For this reason, this thesis offers a fresh perspective on the dynamics of LDs in response to various types of stresses commonly encountered by cancer cells.

Beside using siRNA-based technology to block and follow autophagy levels within cells, we also employed other techniques in order to prove high autophagy levels during severe

nutrient stress that are influencing LD biogenesis. By CYTO-ID staining, we were able to observe a high amount of autophagosomal structures in CQ-treated samples compared to respective control cells during severe nutrient stress (Figure 12 C). Furthermore, by measuring the levels of endogenous LC3 protein within cells, we also showed that amino acid starvation led to higher levels of endogenous LC3 protein which was further potentiated by CQ treatment (Figure 12 E). It was already shown that in HeLa cells Earle's Balanced Salt Solution (EBSS)-treatment resulted in an increase in LC3 puncta compared to control cells (Ni et al., 2011). In summary, our findings underscore the importance of autophagy in promoting the formation of LDs in cancer cells experiencing severe nutrient starvation. Throughout this dissertation, we have demonstrated the occurrence of high levels of autophagy under such challenging and stressful conditions.

5.2 ATGL-Mediated TAG Lipolysis is Continuously Active During Various Starvation Conditions

The hydrolytic cleavage of stored TAGs, the production of FAs and glycerol, and their release are essential for fat catabolism and the availability of energy substrates during periods of nutrient restriction or increased energy demand. ATGL is the primary enzyme responsible for TAG hydrolysis (lipolysis) in adipocytes as well as in nonadipocyte cells (Vegliante et al., 2018). In our study, we looked at the involvement of this enzyme by genetically inhibiting its activity and tracking the dynamics of LD degradation during a period of different nutrient starvation conditions for a certain amount of time. When ATGL-deficient cells were examined either during short, severe (6 h) or mild, prolonged nutrient stress (48–96 h), we consistently found elevated accumulation of LDs, suggesting that ATGL-mediated TAG lipolysis is continuously active under these conditions (Figure 16 A, B; Figure 18 B; Figure 19 A–C). Accordingly, ATGL-deficient cells had more LDs/cell during mild stress (Figure 20 D–F). ATGL has been showed to enhance autophagy and lipophagy (Sathyanarayan, Mashek, & Mashek, 2017), however its specific involvement or possible crosstalk between both processes and LD metabolism remains unclear. It is shown that LD size directs lipolysis and lipophagy catabolism in hepatocytes (Schott et al., 2019). The same study showed that inhibiting lipolysis, lipophagy, or both resulted in similar LD amount, but different LD morphology. In addition, ATGL inhibition caused large LD accumulation, whereas lysosomal inhibition produced small LDs, and combined inhibition of ATGL and LAL led to large LDs (Schott et al., 2019). ATGL was found in larger LDs, while lipophagic vesicles were limited to small LDs. Thus, researchers of this study concluded that lipolysis targets larger LDs to generate both small LDs for lipophagic internalization and newly synthesized LDs (Schott et al., 2019).

Compared to the previously mentioned study, our results show that ATGL depletion leads to an increase in total neutral lipid content in 6 h severely starved cells (Figure 16 A, B) and also during prolonged serum deprivation. However, the decrease in LD accumulation upon ATGL and ATG5 co-inhibition as compared to just ATGL inhibition was significant (Figure 14 A, B) and proves that LDs formed due to autophagy are then broken down by ATGL under severe starvation conditions (Figure 16 A, B). Also, by employing siRNA-based technology by employing ULK1 ^{KD}, we were able to see the similar effect on LD dynamics compared to ATG5 ^{KD} and also in combination with ATGL ^{KD} (Figure 15 A–D). This data presents a distinctive observation that has not been demonstrated in starved HeLa cells before. It is important to emphasize that LD dynamics upon simultaneous inhibition of autophagy/lipophagy and lipolysis was covered only by few papers (Getiye et al., 2022; Kloska et al., 2020; Schott et al., 2019). Considering that the exact mechanism of lipophagy is not known yet, these results arise to be a necessity

for further exploring the connection between lipolysis, autophagylipophagy and LD metabolism.

5.3 Autophagy and LD Biogenesis, but not Lipolysis, Are Important for the Survival of Starving HeLa Cancer Cells

The role of DGAT-mediated LD synthesis in mitigating FA lipotoxicity has been recognized in cells exposed to different metabolic stresses, including increased autophagic flux in severely starved cells (Bailey et al., 2015; Jarc et al., 2018; Listenberger et al., 2003; Nguyen et al., 2017). In severely starved MEFs and cancer cell lines, the synthesis of TAGs is a major source of FAs for mitochondrial energy production (Cabodevilla et al., 2013) but TAG synthesis also prevents FA-induced mitochondrial damage (Nguyen et al., 2017; Rambold et al., 2015). Our results suggest that severely starved HeLa cancer cells have the capacity to mitigate the lipotoxicity of free FAs released upon inhibition of TAG synthesis, which is important for their survival during starvation (Figure 24). Re-esterification of FAs into TAGs not only protects from lipotoxicity, but it also regulates the oxidation of FAs and controls the cellular energy state (Sharma et al., 2023). In MEFs, the transfer of FAs from autophagy-derived LDs to mitochondria is regulated by the main cytosolic lipase ATGL (Nguyen et al., 2017; Rambold et al., 2015). We have shown previously that ATGL-mediated TAG lipolysis is not necessary for MDA-MB-231 cell survival during prolonged serum deprivation (Jarc et al., 2018). The results of this study suggest that silencing of ATGL contributes to increased LD accumulation, but it does not affect HeLa cell survival during acute nutrient stress (Figure 23 C). However, here we show that the inhibition of autophagy leads to the induction of cancer cell death, further suggesting the importance of autophagy for HeLa cell survival (Figure 22 and Figure 23 A, C) during severe nutrient stress and also during prolonged mild starvation (Figure 23 D E, F). It would be interesting to determine whether other lipases may compensate for the inhibition of ATGL-mediated lipolysis for FA transfer and contribute to cell survival under these conditions.

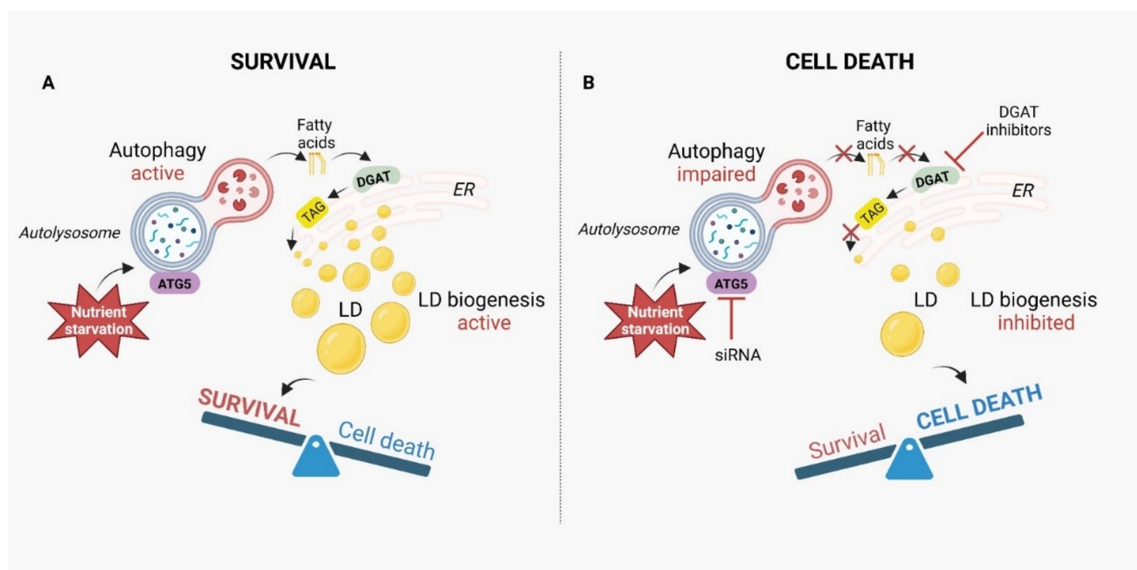

Figure 24: Autophagy-regulated LD formation through DGAT1 protects HeLa cells against intense nutrient stress. A) Our findings propose a conceptual framework wherein autophagy

induced by HBSS starvation triggers LD formation in cancer cells. This process relies on the functioning of DGAT1 enzyme and serves as a protective mechanism against cell damage induced by starvation. (B) Blocking autophagy by silencing ATG5 or LD biogenesis by inhibition of DGAT1 activity with the T836 inhibitor increases HeLa cancer cell death during severe nutrient stress. Created in BioRender.com.

In conclusion, focusing on LDs and autophagy may represent a viable approach in fighting the resistance of certain cancer cell types to nutrient stress. In this context, additional research is essential to understand the extent to which cancer cells with varying genetic characteristics depend on the complex relationship involving LDs, autophagy, lipophagy, and lipolysis.

Chapter 6

Conclusions

LDs are emerging as central regulators of lipid metabolism, indicating their importance in cellular processes such as energy storage and utilization. Understanding how cancer cells adjust to stressful stimuli and their interaction with LD metabolism and autophagy/lipophagy is crucial for compromising the development and aggressiveness of cancer. Our study explored the probable interaction between LD metabolism and autophagy/lipophagy, and lipolysis in terms of LD dynamics and cancer cell survival during two types of stressful conditions: short, acute stress and long, mild nutrient stress.
Following our three main hypotheses, we reached the several conclusions and answers to the hypotheses and/or questions we made. Therefore, the answer to our first hypothesis/question: Is LD turnover affected by the type and length of nutrient stress is:

- YES, LD turnover is affected by the type and length of nutrient stress.
- Moreover, DGAT1 stimulates the formation of LDs in HeLa cells when exposed to amino acid starvation highlighting the importance of DGAT enzymes in driving LD biogenesis.

Our second hypothesis was to answer what are the specific conditions of nutrient stress that are required to activate autophagy/lipophagy and lipolysis.
We reached several main conclusions following this hypothesis and these are:

- Acute nutrient starvation promptly triggered autophagy activation, while serum depletion effectively induced lipophagy/lipolysis. Nonetheless, given the intricate interconnection between these processes, we investigated lipophagy even under conditions of complete medium, where we observed its occurrence. Overall, following this hypothesis we reached the conclusion that LD turnover and autophagy/lipophagy are dynamically coupled in stressed cells. Specifically:
- Inhibition of autophagy, whether through ATG5 depletion or inhibition with BafA1/CQ highly impacts LD accumulation in severely starved HeLa cells.
- ATG5 participates in stimulating LD degradation under conditions of nutrient abundance.
- ATGL plays a role in the degradation of autophagy-derived LDs in amino acid-starved HeLa cells suggesting the importance of lipolysis in this work.
- Our research illustrated that inhibition of autophagy and depletion of LDs is lethal to severely starved cancer cells, suggesting their critical role in promoting cancer cell survival under nutrient stress conditions.
- LD turnover occurs continuously during prolonged serum deprivation via DGAT-mediated LD biogenesis and ATGL-mediated lipolysis.
- Inhibition of ATGL-mediated lipolysis does not lead to cancer cell death during serum starvation nor during more severe, amino acid and serum starvation.

The results of this dissertation have advanced our understanding of lipid metabolism, bringing us closer to a more comprehensive grasp of the subject. The findings are important for both fields of LD metabolism and cancer research and are published in *Cancers* journal.

Overall, our findings underscore the need for more investigation into the intricate interplay between lipid metabolism and autophagy, which may ultimately provide new insights into the development of more effective cancer treatments.

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Publications Related to the Thesis

Jusović, M., Starič, P., Jarc Jovičić, E., & Petan, T. (2023). The combined inhibition of autophagy and diacylglycerol acyltransferase-mediated lipid droplet biogenesis induces cancer cell death during acute amino acid starvation. *Cancers*, *15*(19), 4857. https://doi.org/10.3390/cancers15194857

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Selected Conference Contributions

Jusović M., Jarc Jovičić E., Koren Š., Kump Ana., Starič P., Petan T. Autophagy-driven lipid droplet formation protects cancer cells from nutrient stress. In: Molecules of life: towards new horizons: FEBS 2021: the 45th FEBS Congress : 3-8 July 2021, Ljubljana, Slovenia: virtual: abstracts: Federation of European Biochemical Societies. (poster presentation).

Jusović M., Jarc Jovičić E., Koren Š., Kump Ana., Starič P., Petan T. Crosstalk between autophagy and lipid droplets during severe and mild starvation in cancer cells. In: Book of abstracts. 12th Jožef Stefan International Postgraduate School Students' Conference and 14th Young Researchers' Day, 15th May 2020. Ljubljana: Jožef Stefan International Postgraduate School: Jožef Stefan Institute. Str. 39. (poster presentation).

Jusović M., Jarc Jovičić E., Kump A., Guštin E., Eichmann T.O., Zimmermann R., Petan T. Lipid droplets are targets for reducing cancer resistance to stress. In: Proceedings. 10th Jožef Stefan International Postgraduate School Students' Conference and 12th Young Researchers' Day 10th and 11th May 2018, Piran, Slovenia. Ljubljana, Jožef Stefan International Postgraduate School, Jožef Stefan Institute. (poster presentation).

Biography

The author of this thesis Maida Jusović was born on March 15, 1992 in Prijepolje (Serbia). She finished her primary school in Bosanska Krupa (Bosnia and Herzegovina). In pursuit of her education, she relocated to Sarajevo, the capital city of Bosnia and Herzegovina, at a very young age, where she completed high school. Following the culmination of her secondary education in 2011, four years later, Maida earned her bachelor's degree from the International University of Sarajevo at the Department of Genetics and Bioengineering, discovering her passion for science. Recognized as one of the top students, Maida was awarded the GreenTech WB *Erasmus Mundus* Scholarship, enabling her to pursue a Master of Science degree with a specialization in Biotechnology in Sofia (Bulgaria) at the University of Chemical Technology and Metallurgy. This eighteen-month scholarship resulted in her first scientific publication in 2017.

Driven by her passion for scientific research, Maida embarked on her PhD journey in 2017 at the Jožef Stefan International Postgraduate School in Ljubljana (Slovenia). Her PhD work focused on investigating lipid metabolism in cancer cells, specifically emphasizing lipid droplets. Utilizing *in vitro* cancer cell lines and employing various biochemical and molecular methods, she manipulated specific metabolic processes in cancer cells, examining lipid droplet dynamics and their impact on cancer cell survival. Throughout her PhD, she participated in multiple conferences. Her contribution to science in this field is evident through her recognition as the first author in a scientific article and her involvement as a co-author in a review paper.