THE ROLE OF LIPID DROPLETS IN CANCER CELL STRESS RESISTANCE

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Doctoral Dissertation
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VLOGA LIPIDNIH KAPLJIC PRI ODPORNOSTI RAKAVIH CELIC NA STRES

Doktorska disertacija

Supervisor: Assist. Prof. Dr. Toni Petan

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To my lovely family.

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During the past four years, I have been privileged to have experienced excellent science, which I found to be my true passion. I really enjoyed every part of this amazing journey and I am very excited to be able to continue my science mission in the future. It is not just about science, this period gave me confidence, unforgettable moments, pride, friendships and new opportunities, making me grateful and honored. All these things could not have happened without the people that I want to thank here.

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Abstract

Lipid droplets have recently been recognized as dynamic organelles found in most cells from prokaryotes to mammals. They are major regulators of lipid metabolism, trafficking and signaling, but their synthesis is also induced in cells exposed to various conditions of stress. Lipid droplets accumulate in cancer cells and are emerging as important factors for the plasticity of cancer metabolism and resilience to stress. However, mechanisms that drive lipid droplet turnover in cancer cells are far from being understood. The major objective of this dissertation was to define the principal ways in which lipid droplets are associated with cell survival and the function of aggressive triple-negative breast cancer (TNBC) cells during metabolic stress. By examining the roles of the major enzymes involved in lipid droplet turnover, diacylglycerol acyltransferase (DGAT) and adipose triglyceride lipase (ATGL), we have identified the contribution of lipid droplets to the resistance of TNBC cells against (1) nutrient and (2) lipotoxic stress and their ability to (3) drive the production of inflammatory lipid mediators. We show here that ATGL is involved in lipid droplet breakdown during starvation, but surprisingly, it is not essential for TNBC cell survival during starvation-induced nutrient stress. On the contrary, we found that ATGL-mediated lipolysis is an essential source of polyunsaturated fatty acids (PUFAs), which are used as precursors for the synthesis of eicosanoids and specialized pro-resolving mediators in starved TNBC cells, suggesting a novel role for lipid droplets in inflammatory signalling in cancer. Furthermore, our results reveal that the breakdown of lipid droplets induced by high concentrations of PUFAs is associated with oxidative stress-dependent cell death in TNBC cells, while low concentrations of PUFAs have the opposite effect – they increase the resistance of TNBC cells to starvation-induced stress. In line with a protective role of lipid droplets against the surge of exogenous PUFAs, we found that DGAT-mediated lipid droplet biogenesis reduces PUFA lipotoxicity. Most importantly, we have discovered two novel mechanisms through which lipid droplets protect cells from PUFA lipotoxicity. The first is the stimulation of lipid droplet biogenesis by the activity of secreted phospholipase A_2, which hydrolyses membrane phospholipids and induces the incorporation of monounsaturated acyl chains in TAGs to effectively sequester PUFAs within lipid droplets. The second is the ability of ATGL to modulate PUFA-induced cell damage by regulating the release of PUFAs from lipid droplets. Our results reveal that lipid droplets integrate metabolic and signalling pathways that are associated with cancer cell stress resistance and are thus potential targets in the fight against aggressive breast cancer.

Povzetek

Lipidne kapljice so nedavno odkriti dinamični organeli, ki jih najdemo v večini celic, od prokariontov do sesalcev. So pomembni regulatorji presnove, transporta in signalizacije lipidov, njihova sinteza pa se poveča, ko so celice izpostavljene različnim stresnim pogojem. Lipidne kapljice se kopičijo v rakavih celicah in prispevajo k plastičnosti metabolizma in odpornosti rakavih celic na stres. Mehanizmi, ki so vpleteni v nastanek in razgradnjo lipidnih kapljic v rakavih celicah, pa še zdaleč niso jasni. Glavni cilj tega doktorskega dela je bil opredeliti glavne mehanizme, ki povezujejo lipidne kapljice z odpornostjo celic agresivnega trojno negativnega raka dojke na metabolični stres. S proučevanjem vlog glavnih encimov, ki sodelujejo pri presnovi lipidnih kapljic, diacilglicerol aciltransferaze (DGAT) in adipozne trigliceridne lipaze (ATGL), smo ugotavljali, kako lipidne kapljice prispevajo k preživetju celic raka dojke pri (1) pomanjkanju hranil in (2) lipotoksičnem stresu ter njihovo sposobnost, da (3) poganjajo proizvodnjo vnetnih lipidnih mediatorjev. Pokazali smo, da je lipaza ATGL vpletena v razgradnjo lipidnih kapljic tekom stradanja, vendar presenetljivo ni kritičnega pomena za preživetje celic raka dojke ob pomanjkanju hranil. Ravno nasprotno pa smo ugotovili, da je z ATGL uravnavana lipoliza ključen vir polinenasičenih maščobnih kislin (PNMK) za sintezo eikozanoidov in specializiranih lipidnih mediatorjev v stradanih celicah raka dojke, kar razkriva novo vlogo lipidnih kapljic v vnetni signalizaciji pri raku. Naši rezultati prav tako kažejo, da je razgradnja lipidnih kapljic, ki nastanejo ob izpostavitvi celic visokim koncentracijam PNMK, povezana z oksidativnim stresom, ki vodi v celično smrt. Po drugi strani pa imajo nizke koncentracije PNMK ravno nasprotni učinek – povečajo odpornost celic raka dojke na pomanjkanje hranil in jih zaščitijo tekom stradanja. V skladu z zaščitno vlogo lipidnih kapljic smo ugotovili, da z DGAT posredovana sinteza lipidnih kapljic zmanjšuje lipotoksičnost PNMK. Odkrili smo dva nova mehanizma, preko katerih lipidne kapljice ščitijo pred lipotoksičnostjo. Prvi mehanizem vključuje stimulacijo sinteze lipidnih kapljic s sekretorno fosfolipazo A_2, ki hidrolizira membranske fosfolipide in poveča vgradnjo mononenasičenih acilnih verig v trigliceride ter tako "ujame" PNMK v lipidnih kapljicah. Drugi mehanizem izpostavlja sposobnost ATGL, da z uravnavanjem sproščanja PNMK iz trigliceridov prepreči poškodbe celic, ki jih povzročijo PNMK. Naši rezultati tako razkrivajo, da lipidne kapljice povezujejo presnovne in signalne poti, ki so vpletene v odpornost rakavih celic na stres in so zato potencialne tarče v boju proti agresivnemu raku dojke.

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Abbreviations

AA ... Arachidonic acid
ABHD ... α/β hydrolase domain-containing protein
ACAT ... Acyl-CoA:cholesterol *O*-acyltransferase
ACC ... Acetyl-CoA carboxylase
ACS ... Acyl-CoA synthetase
ACLY ... ATP citrate lyase
AGPAT ... 1-acylglycerol-3-phosphate *O*-acyltransferases
Akt ... Protein kinase B
AMPK ... 5'-AMP-activated protein kinase
ATGL ... Adipose triglyceride lipase
CCT ... CTP:phosphocholine cytidylyltransferase
CE ... Cholesterol ester
CGI-58 ... Comparative gene identification-58
CoA ... Coenzyme A
DAG ... Diacylglycerol
DGAT ... Diacylglycerol acyltransferase
DHA ... Docosahexaenoic acid
EPA ... Eicosapentaenoic acid
ER ... Endoplasmic reticulum
FA ... Fatty acid
FABP ... Fatty acid-binding protein
FIT ... Fat storage-inducing transmembrane protein
FSP27 ... Fat-specific protein 27
GPAT ... Glycerol-3-phosphate acyltransferase
G0S2 ... G0/G1 switch gene protein 2
HER2 ... Human epidermal receptor 2
HFD ... High-fat diet
HIF1α ... Hypoxia-inducible factor-1α
HILPDA ... Hypoxia-inducible lipid droplet-associated protein
HSL ... Hormone sensitive lipase
LAL ... Lysosomal acid lipase
LPA ... Lysophosphatidic acid
LPCAT ... Lysophosphatidylcholine acyltransferase
MAG ... Monoacylglycerol
MAGL ... Monoacylglycerol lipase
MBOAT ... Membrane-bound O-acyltransferase
MGAT ... Acyl-CoA:monoacylglycerol acyltransferase
mTOR ... Mammalian target of rapamycin
NADPH ... Nicotinamide adenine dinucleotide phosphate
NLSDM ... Neutral lipid storage disease with myopathy
OA ... Oleic acid

OXPHO ... Oxidative phosphorylation
PA ... Phosphatidic acid
PC ... Phosphatidylcholine
PE ... Phosphatidylethanolamine
PEDF ... Pigment-epithelium derived factor
PI ... Phosphatidylinositol
PI3K ... Phosphatidylinositol 3-kinase
PLA_2 ... Phospholipase A_2
PLIN ... Perilipin
PPAR ... Peroxisome proliferator-activated receptor
PUFA ... Polyunsaturated fatty acid
ROS ... Reactive oxygen species
SCD ... Stearoyl-CoA desaturase
SREBP ... Sterol regulatory element-binding protein
TAG ... Triacylglycerol
TNBC ... Triple-negative breast cancer

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Chapter 1

Introduction

1.1 Work Motivation

The incidence of malignant diseases is increasing worldwide at an alarming rate, maybe even reaching pandemic proportions. Almost 15 million of new cancer cases and over 8 million deaths occurred in 2012 worldwide based on the GLOBOCAN 2012 statistics. Lung and breast cancers are the most frequently diagnosed cancers and a leading cause of cancer death among men and women. In Central and Eastern Europe, breast cancer incidence is estimated to be lower in comparison with Western and Northern Europe, and this variation was suggested to reflect differences in risk factors and the availability of early diagnosis (Torre et al., 2015). According to the World Health Organization (WHO), almost 50% of cancer deaths could be prevented by efficient cancer management, including cancer prevention, early diagnosis and treatment (World Health Organization, 2019). In Slovenia, we have an effective program of early diagnosis and prevention of breast cancer known as Dora, but the incidence of breast cancer is still increasing (Cancer Patient's Association Slovenia, 2019). It is estimated that every eighth woman will get breast cancer in the future, intensifying the need to develop effective detection and treatment for this malignant disease.

Triple-negative breast cancer (TNBC) is one of the most aggressive cancers with no treatment currently available. Enormous efforts have been put in developing effective cancer therapies in the last several decades, but it has become obvious that genetic and phenotypical heterogeneity of cancer cells and their ability to adapt to therapeutic insult restrict our success in fighting the disease. In the last two decades, advances in cancer research have brought a revived interest in cancer metabolism. Alterations in tumor metabolism are caused by genetic aberrations, but, importantly, stressful conditions in the tumor microenvironment force cancer cells to adapt by reprogramming their metabolism and signaling (Beloribi-Djefaflia, Vasseur, & Guillaumond, 2016; Pavlova & Thompson, 2016; Ward & Thompson, 2012). Therefore, even genetically distinct cancers that are exposed to similar stress conditions may have common metabolic vulnerabilities, thereby opening a therapeutic opportunity that is more advantageous than targeting oncogenic signalling pathways (Hanahan & Weinberg, 2011; Martinez-Outschoorn, Peiris-Pagés, Pestell, Sotgia, & Lisanti, 2017; Vander Heiden & DeBerardinis, 2017).

The importance of lipid metabolism in cancer is emerging (Currie et al., 2013; Röhrig & Schulze, 2016). It is becoming evident that many cancer types are dependent on lipids for their survival. Targeting processes regulating lipid metabolism may thus reduce lipid availability and affect cancer cell survival, but we are still far from understanding the lipid metabolic pathways that are critical for the survival, growth and metastasis of cancer cells. Lipid droplets integrate lipid metabolism with cellular stress responses and may thus be

the nexus between cancer metabolism and resilience to stress. Their accumulation in cancer cells has been associated with higher tumour aggressiveness and chemotherapy resistance (Beloribi-Djefaflia et al., 2016; Nieva et al., 2012; Rak et al., 2014). Mechanisms involved in lipid droplet metabolism regulate lipid availability and define the adaptability strategies of cancer cells. TNBC cells are extremely resistant to chemotherapy (Sirois et al., 2019) and can survive in nutrient depleted conditions for prolonged times (Przybytkowski et al., 2007; Pucer et al., 2013). This resistant phenotype is dependent on lipid droplet metabolism, whose targeting may reduce TNBC cell proliferation and survival. Finding the critical mechanisms used by cancer cells to exploit lipid droplets will boost basic and clinical research in the field and lead to new therapeutic and preventive strategies.

This dissertation provides original results and new knowledge in lipid droplet and cancer biology. Our results and proposed mechanisms have a potential to be further evaluated for therapeutic interventions to treat aggressive breast and other cancers, whose therapeutic targeting is still a major challenge. In the following sections, a theoretical background of lipid droplets is briefly presented with particular focus on lipid droplet roles in cellular stress response mechanisms. Since many of the lipid droplet-driven mechanisms have been described in non-cancer cells, we categorised the roles of lipid droplets according to the type of cellular stress and applied this knowledge to cancer metabolism. Chapter 2 presents the aims and major objectives of the dissertation, followed by Chapters 3 and 4, whereby three relevant scientific publications are included in addition to our latest work, which represents an article in preparation. These papers reveal novel mechanisms that link lipid droplets with breast cancer cell resistance and emphasize the metabolic and signalling roles of lipid droplets in cancer. Chapter 5 presents our major conclusions based on our results and aims of the dissertation. Finally, appendices A, B and C provide additional information about lipid droplets, which has been discussed in our recent review papers (Jarc & Petan, 2019b, 2019a; Petan, Jarc, & Jusović, 2018).

1.2 Lipid Droplets

Lipid droplets have recently been recognized as neutral lipid storage organelles found in most cells from prokaryotes, plants, fungi to mammals (Murphy, 2012). They are most apparent in adipocytes (Fig. 1A), where one big lipid droplet can fill the whole cytoplasm and store large amounts of energy in the form of triacylglycerols (TAGs). These lipid droplets can reach "giant" sizes and have diameters of up to 100 µm. In comparison, lipid droplets in other cell types are much smaller with diameters between 100–200 nm (Walther & Farese, 2012). We often see them in clusters, but they can dynamically disperse during lipogenesis or nutrient starvation (Harris et al., 2011; Rambold, Cohen, & Lippincott-Schwartz, 2015). The lipid droplet structure is unique among other organelles, consisting of a central hydrophobic core of TAGs and cholesterol esters (CEs), surrounded by a monolayer of phospholipids and proteins (Fig. 1B). Their lipid and protein composition is not exclusive and can vary within single cell or different cell types with regard to metabolic states and environmental conditions (Farese & Walther, 2009; Jarc & Petan, 2019b; Thiam & Beller, 2017). Their major role is to balance lipid uptake, storage and release according to cellular needs. Recent evidence suggests that they play a pivotal role in the management of cellular stress and are thus involved in many pathophysiological processes, including cancer (Farese & Walther, 2009; Fujimoto & Parton, 2011; Henne, Reese, & Goodman, 2018; Olzmann & Carvalho, 2019; Petan et al., 2018; Welte & Gould, 2017).

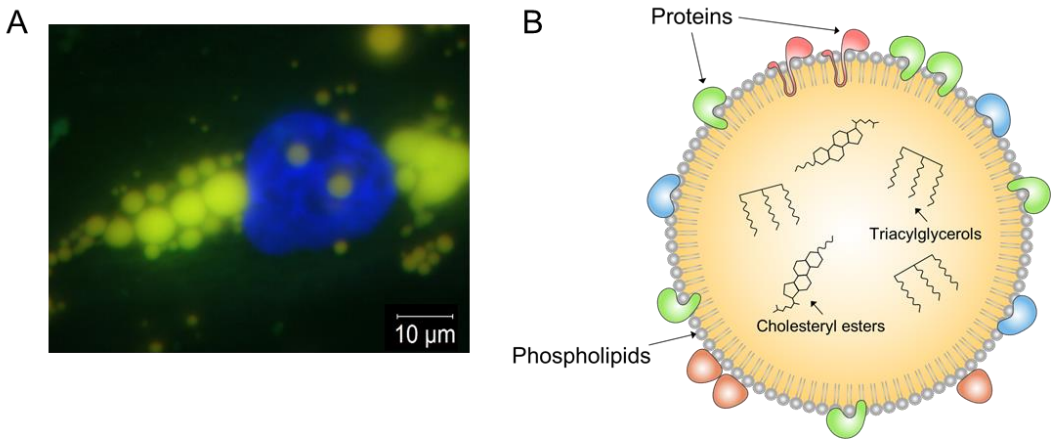

Figure 1: Lipid droplet structure. (A) Lipid droplets in differentiated adipocytes *in vitro*, originating from an adipose cell strain of a patient with the human Simpson-Golabi-Behmel syndrome. Lipid droplets were visualized in fixed cells using BODIPY 493/503 and nuclei with DAPI. (B) Lipid droplets are composed of neutral core that mainly consists of triacylglycerols (TAG) and cholesteryl esters (CE) and is surrounded by a single layer of phospholipids in which numerous proteins are embedded.

1.2.1 Lipid droplet biogenesis

Despite advances and efforts being made in the recent years, the molecular mechanism of lipid droplet formation is still not clear. Several models of lipid droplet biogenesis have been proposed but none of them validated yet. Here, we present the most cited model that includes oil lens formation and budding into the cytosol (Olzmann & Carvalho, 2019; Walther, Chung, & Farese, 2017; Walther & Farese, 2012; Wilfling, Haas, Walther, & Farese, 2014).

In eukaryotes, TAG and CE are synthesized predominantly in the endoplasmic reticulum (ER) (Buhman, Chen, & Farese, 2001). The primary step of TAG synthesis includes the Kennedy pathway, whereas glycerolipids are formed from glycerophosphate and fatty acyl-coenzyme A (CoA) or the monoacylglycerol pathway, whereby products of TAG degradation, including diacylglycerols (DAG) and monoacylglycerols (MAG) are re-esterified back into TAGs by acyltransferases (Walther et al., 2017). Fatty acid (FA) esterification with CoA is an ATP-dependent process regulated by acyl-CoA synthetases (ACS) among which three isoforms are related to lipid droplets ACSL1, ACSL3 and ACSL4 (Pol, Gross, & Parton, 2014). TAG synthesis occurs in four sequential steps, including the enzymatic activities of glycerol-3-phosphate acyltransferases (GPATs), 1-acylglycerol-3-phosphate O-acyltransferases (AGPATs), phosphatidic acid phosphatases (PAPs; also called lipins) and diacylglycerol acyltransferases (DGATs) (Fig. 2). Interestingly, some enzyme isoforms have been found on lipid droplets, including DGAT2, AGPAT3, lipins and GPAT4, and may prefer different sources of lipids, while others are permanently or transiently localized in the ER (Pol et al., 2014; Wilfling et al., 2013). This raises the possibility that neutral lipids are also formed locally on lipid droplets, thereby enabling lipid droplet growth by ER-independent synthesis. Depletion of lipid droplet-associated DGAT2, AGPAT3 and GPAT4 results in a major decrease of the large lipid droplet population. In contrast, depletion of the ER resident DGAT1 enzyme has a strong impact on cellular TAG level, but does not prevent the formation of large lipid droplets (Wilfling et al., 2013), suggesting different roles of enzyme isomers in lipid droplet synthesis.

The synthesis of CE is mediated by acyl-CoA:cholesterol O-acyltransferases (ACAT). Most cells contain a mixture of TAG and CE in their lipid droplets, but some specialized cells accumulate CE-rich lipid droplets, such as foam macrophages and Leydig cells in the adrenal cortex. In addition, retinyl-rich lipid droplets may be found in hepatic stellate and retinal cells, suggesting a wide diversity in the lipid composition of lipid droplets (Kraemer, Khor, Shen, & Azhar, 2013; Molenaar, Vaandrager, & Helms, 2017; Orban, Palczewska, & Palczewski, 2011; Ouimet et al., 2011).

In the second step of lipid droplet biogenesis, newly synthesized neutral lipids accumulate within the bilayer of the ER. When the TAG concentration exceeds the capacity of the bilayer an oil lens is formed to accommodate the high amount of TAG and prevent membrane disruption. Important factors that determine lens formation are ER structure and proteins that regulate ER shape and TAG partitioning. The ER consists of membrane sheets and tubules (Shibata, Voeltz, & Rapoport, 2006; Voeltz, Prinz, Shibata, Rist, & Rapoport, 2006). Lipid droplet formation occurs in ER tubules, where curvatures in the ER membrane may contribute to lens formation by locally disturbing phospholipid composition. Whether oil lenses form in the ER sheets, which lack curvatures in their shape, remains unclear (Walther et al., 2017). Depletion of proteins involved in shaping of the tubular ER network, such as atlastin-1 and receptor expression-enhancing protein 1, affects lipid droplet size and number (Renvoisé et al., 2016; Walther et al., 2017). In addition, depletion of lipid-binding fat storage-inducing transmembrane proteins (FITs) that are involved in TAG partitioning results in the absence of lipid droplet formation (Kadereit et al., 2008).

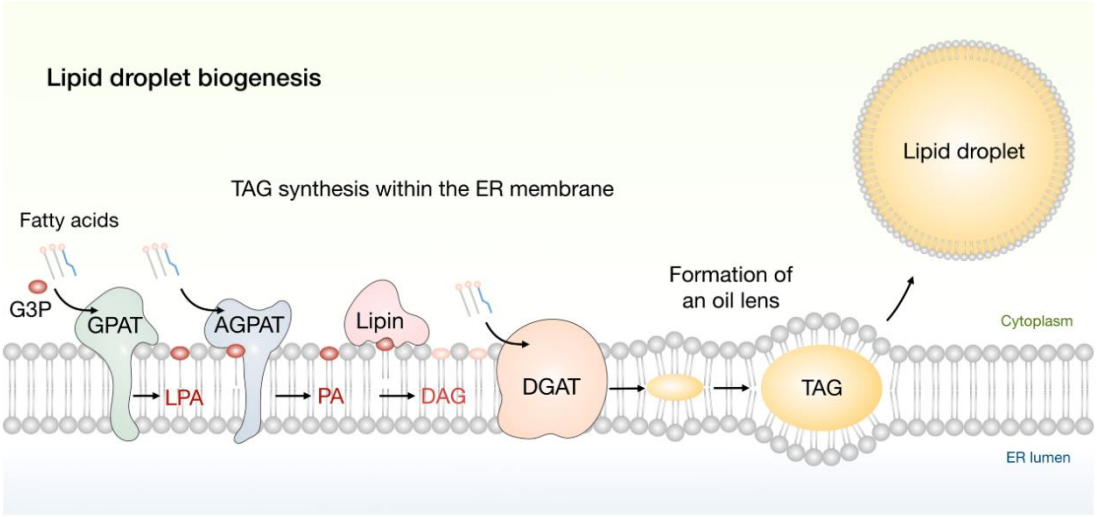

Figure 2: Lipid droplet formation occurs in the ER. The initial step starts by the addition of fatty acids (in their activated acyl-CoA form) to a glycerol-3-phosphate backbone, yielding lysophosphatidic acid (LPA), phosphatidic acid (PA) and diacylglycerol (DAG). These reactions are catalyzed by glycerol-3-phosphate acyltransferase (GPAT), acylglycerol-3-phosphate acyltransferase (AGPAT) and phosphatidic acid phosphatase (lipin) enzymes. The final step in TAG synthesis is mediated by diacylglycerol acyltransferases (DGATs) (Jarc & Petan, 2019b).

In the final step, the growth of the oil lens results in budding of a nascent lipid droplet from the membrane, which is released into the cytosol and becomes known as cytosolic lipid droplet. The budding mechanism is still poorly understood, but seipin protein seems

to be required for the process. Seipin is a conserved ER transmembrane protein involved in lipid droplet budding. In addition, it is required for the formation of ER–lipid droplet membrane contact sites by stabilizing membrane bridges. Seipin depletion results in delayed lipid droplet formation and formation of lipid droplets with aberrant morphology (Wang et al., 2016). Intriguingly, recent evidence suggests that for sufficient lipid droplet budding and release in the cytosol cells require a constant refill of phospholipids at the cytosolic site of ER membrane. In the case of phospholipid deficiency, lipid droplets emerge on both membrane sides of the ER, which may affect the ER structure and function (Chorlay et al., 2019).

Lipid droplet maturation includes cytosolic lipid droplet growing and expansion. Lipid droplets expand through lipid droplet fusion, through transfer of lipids via ER–lipid droplet contact sites or through TAG synthesis on the lipid droplet surface (Olzmann & Carvalho, 2019). They may also expand by Ostwald ripening-mediated diffusion, where giant lipid droplets can form by depletion of oil in the adjacent second lipid droplet (Walther et al., 2017). Besides the ER, lipid droplets form multiple contact sites with other organelles, including mitochondria and peroxisomes, which may contribute to lipid droplet growth (Barbosa & Siniossoglou, 2017; Jarc & Petan, 2019b; Olzmann & Carvalho, 2019; Schuldiner & Bohnert, 2017).

During lipid droplet expansion, phospholipid content must also increase. In mammalian cells, the lipid droplet surface mainly consists of phosphatidylcholine (PC), followed by phosphatidylethanolamine (PE) and phosphatidylinositol (PI) (Walther & Farese, 2012). The synthesis of PC is under control of CTP:phosphocholine cytidylyltransferase α (CCTα), whose activation may occur at the lipid droplet surface (Krahmer et al., 2011). Finally, different proteins have been employed in regulation of lipid droplet growth and expansion. Many of them localize on the lipid droplet surface, thereby forming the diverse lipid droplet proteome that contains numerous lipid metabolic enzymes, regulatory scaffold proteins, proteins involved in lipid droplet metabolism and proteins with a currently unknown function. Despite establishing the lipid droplet proteome is still challenging, the fact that its composition varies with metabolic changes and depending on cellular needs is undeniable (Bersuker & Olzmann, 2017; Kory, Farese, & Walther, 2016; Thiam & Beller, 2017; Walther et al., 2017).

To summarize, lipid droplet biogenesis is a complex and energy-consuming process that occurs at the ER and requires the cooperation of numerous proteins and enzymes. The rate-limiting step in lipid droplet biogenesis is regulated by DGAT enzymes, which are essential for TAG synthesis.

1.2.1.1 DGAT enzymes are essential for *de novo* lipid synthesis

The two DGAT enzymes catalyze the same biochemical reaction, but belong to distinct protein families and display significant structural differences. The gene encoding human DGAT1 comprises 17 exons on chromosome 8 and is a member of the membrane-bound O-acyltransferase (MBOAT) gene family. It shares a similarity with the ACAT family of enzymes. In contrast, the gene encoding human DGAT2 comprises 8 exons on chromosome 11 and is a member of the DGAT2 gene family, displaying a similarity with acyl-CoA:monoacylglycerol acyltransferases (MGAT). Human DGAT1 and DGAT2 share less than 25% of protein sequence similarity (according to the BLAST alignment tool). DGAT1 localizes exclusively to the ER, where it exists as dimers or tetramers that form functional units (Walther et al., 2017; Yang et al., 2012; Yen, Stone, Koliwad, Harris, & Farese, 2008). Its topology reveals many hydrophobic regions and multiple transmembrane domains, while DGAT2 possesses only a hydrophobic region, possibly a hairpin, that is embedded in the ER membrane bilayer (Walther et al., 2017; Yen et al., 2008). This key difference in the

structure of DGAT2 may also explain why only DGAT2 localizes to lipid droplets (Stone et al., 2009; Wilfling et al., 2013). Interestingly, stimulating lipid droplet synthesis by the addition of oleate promotes DGAT2 localization on lipid droplets and co-localization with mitochondria (Stone et al., 2009). This indicates that DGAT2 is either a mitochondrial enzyme or it associates transiently with mitochondria. It is also possible that DGAT2 is present on lipid droplets and linked with mitochondria through lipid droplet–mitochondria contact sites. Besides the differences in their structure, DGAT enzymes have different substrate preferences, subcellular localizations and physiological roles. TAGs are predominant DGAT1 products, but DGAT1 exhibits activity also towards retinyl esters and long-chain alcohols. In contrast, DGAT2 does not possess multiple acyltransferase activities, but displays a higher activity towards its substrates for TAG synthesis than DGAT1 (Yen et al., 2008).

Animal studies have revealed important differences in the physiological roles of DGAT enzymes. Mice lacking DGAT1 are viable and have body fat, but are lean and resistant to high-fat diet (HFD)-induced obesity, diabetes and liver steatosis (H. C. Chen, Stone, Zhou, Buhman, & Farese, 2002; Smith et al., 2000). In contrast, mice lacking DGAT2 die shortly after birth due to a lipid defect in the skin impairing its barrier function (Stone et al., 2004). Furthermore, DGAT2 KO mice completely lack TAG, suggesting DGAT2 to be the major enzyme for TAG synthesis in mammals. A recent study reported that mice lacking DGAT2 in adipose tissue (ADGAT2 KO mice) remain viable, in comparison with mice lacking DGAT2 in the epidermis, which die postnatally (Chitraju, Walther, & Farese, 2019). This indicates that DGAT2 is essential for skin function, but the molecular mechanism remains unclear. Under basal conditions when mice were on chow diet, no differences in phenotype and lipid mass were observed between ADGAT2 KO and mice lacking DGAT1 in adipose tissue (ADGAT1 KO), indicating a compensatory relationship between DGATs in murine adipose tissue. However, only ADGAT1 KO mice were protected from HFD-induced obesity and diabetes. Importantly, HFD feeding provoked a lipotoxic response by upregulating gene expression of ER stress and inflammatory markers in ADGAT1 KO but not in ADGAT2 KO mice, indicating that DGAT1 has a crucial role in preventing lipotoxicity. These results suggest that DGAT2 functions when FA concentrations are low and is preferentially associated with *de novo* lipogenesis, whereas DGAT1 responds to high levels of FAs and prevents lipotoxicity. In line with these findings, DGAT1 was found essential for protection from lipotoxicity in various conditions, including increased adipose tissue lipolysis during fasting (Chitraju et al., 2017), the presence of excess exogenous saturated lipids (Listenberger et al., 2003) and during high autophagic flux in the cell, which leads to a sudden rise in endogenous lipids (Nguyen et al., 2017).

DGAT1 deficiency in humans causes severe clinical features with increased sensitivity to lipid-induced lipotoxicity, leading to vomiting, diarrhea and fat intolerance (van Rijn et al., 2018). Interestingly, a compensatory relationship between DGATs has also been reported in human intestinal stem cell-derived organoids, but more importantly, stimulating DGAT2 overexpression in organoids fully prevented FA-induced lipotoxicity and compensated for the loss of DGAT1, indicating that induced DGAT2 expression has a therapeutic potential in DGAT1-deficient humans and could prevent lipotoxic injury (van Rijn et al., 2019).

To summarize, DGAT enzymes have distinct biochemical properties, but are both indispensable for lipid droplet biogenesis. They also differ in their physiological function, DGAT1 being essential for protection from lipotoxicity and DGAT2 being crucial for de novo lipogenesis. Studying the role of lipid droplets in preventing lipotoxicity is an important part of our studies and of this work, and is discussed in more detail in Section 1.3.1.

1.2.2 Lipid droplet breakdown

1.2.2.1 Lipolysis

Lipolysis is a widely recognized process of lipid hydrolysis mediated by various lipases (Lass, Zimmermann, Oberer, & Zechner, 2011; Schreiber, Xie, & Schweiger, 2018; Zechner, Madeo, & Kratky, 2017; Zechner et al., 2012). Lipolysis may occur intra- and extracellularly and is catalyzed by neutral or acidic lipid hydrolases, the latter being necessary for lysosomal lipid hydrolysis. Extracellular lipolysis occurs in the lumen of the gastrointestinal tract and blood vessels, enabling the breakdown of dietary lipids and lipoproteins. Arguably the most appreciated form of intracellular lipolysis occurs in adipose tissue, where the breakdown of lipid droplets enables the supply of other tissues with FAs. However, lipolysis does not occur only in adipocytes, but most likely in every cell of our body in response to cellular needs for FAs.

Lipid droplets are broken down by neutral lipases (Fig. 3). The hydrolytic degradation primarily occurs in three consecutive steps, including the activity of adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL). The majority of TAG lipolysis in adipose tissue is regulated by ATGL and HSL, while their expression in other tissues is significantly lower, raising the possibility that other lipases or processes may contribute to TAG degradation (Zechner et al., 2012). For a long time, HSL was considered the main TAG lipase. In 2004, three independent groups reported that the lipase ATGL (also called PNPLA2; encoded by the *PNPLA2* gene) is in fact the major TAG lipase in adipose and non-adipose tissues (Smirnova et al., 2006; Zechner, 2015; Zechner et al., 2017, 2012; Zimmermann et al., 2004). Lipid droplet lipolysis is initiated with TAG degradation by ATGL, followed by DAG degradation by HSL and monoacylglycerol (MAG) degradation by MAGL. In each step, FAs are released into the cytosol. FAs are major energy substrates, precursors for membrane lipids, hormones and bioactive lipids, such as eicosanoids (Zechner, 2015). Different studies suggest that lipolysis is involved in the regulation of mitochondrial β-oxidation, membrane and ER homeostasis, cell growth and the inflammatory response (Bosma et al., 2014; Dichlberger, Schlager, Maaninka, Schneider, & Kovanen, 2014; Fuchs et al., 2012; Haemmerle et al., 2011; Kurat et al., 2009; Ong, Mashek, Bu, Greenberg, & Mashek, 2011; Rambold et al., 2015).

1.2.2.2 Lipophagy

Lipophagy is a recently discovered form of autophagy, whereby via an as yet unknown mechanisms, lipid droplets are recognized by the autophagic machinery and delivered to the lysosomes for degradation (Jaishy & Abel, 2016; Petan et al., 2018; Schulze, Sathyanarayan, & Mashek, 2017; Zechner et al., 2017). In the lysosomes, the activity of lysosomal acid lipase (LAL) contributes to lipid droplet degradation (Fig. 3). Interestingly, lipolysis and lipophagy are transcriptionally regulated by common pathways and their actions often overlap, suggesting a dynamic interplay and complementarity between the two processes (Jarc & Petan, 2019b; Zechner et al., 2017).

Of note, autophagy can also be involved in lipid droplet synthesis. During severe cellular starvation, a high autophagic breakdown of membrane lipids may occur resulting in a burst of cytosolic FAs, thereby promoting lipid droplet biogenesis and preventing mitochondrial damage. Intriguingly, this also replenishes the pool of existing lipid droplets for a subsequent "gentler" lipid droplet breakdown by lipolysis (Nguyen et al., 2017; Rambold et al., 2015). The lack of specific methods for lipophagy detection, which would enable a discrimination between classical autophagy and lipophagy, hampers current efforts in establishing the exact role of autophagy in lipid droplet metabolism. In this work we focus

on the role of lipolysis in breast cancer cell resistance, therefore, mechanisms linking lipid droplets and autophagy/lipophagy are not presented here. However, they have been discussed in our recent review papers (Jarc & Petan, 2019b; Petan et al., 2018).

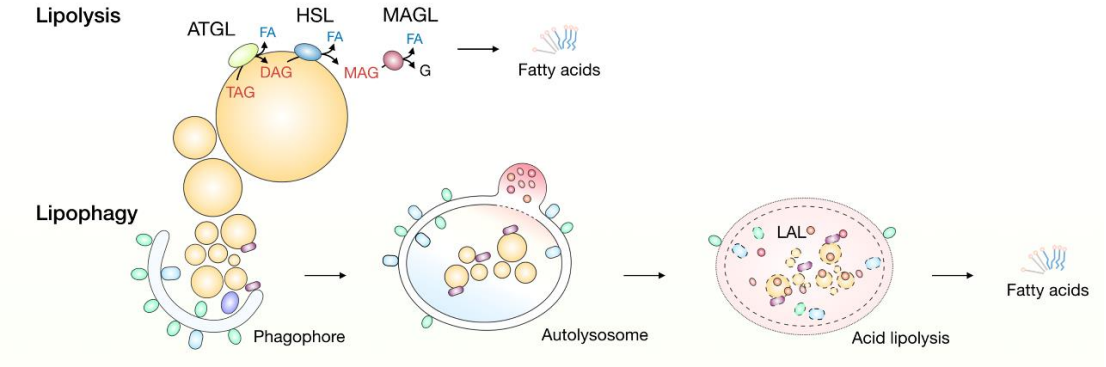

Figure 3: Lipid droplet breakdown occurs through lipolysis and/or lipophagy. TAG lipolysis is carried out by sequential actions of adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL), and leads to the release of glycerol and FAs in the cytosol. Lipophagy is a selective form of autophagy, where the autophagic machinery recognizes lipid droplets for lysosomal degradation by lysosomal acid lipase (LAL) and leads to the release of free cholesterol and FAs (Jarc & Petan, 2019b).

1.2.2.3 ATGL is a key enzyme in lipid homeostasis

ATGL belongs to the patatin-like phospholipase domain-containing protein (PNPLA) family. It preferentially hydrolyses TAG species containing long-chain FAs at the *sn*-2 and *sn*-1 positions, but not at the *sn*-3 position, and exhibits only a minor activity towards DAG, MAG and CE substrates (Eichmann et al., 2012). In addition, it possesses weak phospholipase activity (Notari et al., 2006), retinyl ester activity (Taschler et al., 2015) and DAG transacylase activities, generating TAG and MAG independently from acyl-CoA (Jenkins et al., 2004). To exhibit full TAG activity, ATGL requires activation by its endogenous activator α/β hydrolase domain-containing protein 5 (ABHD5), also known as comparative gene identification-58 (CGI-58) (Lass et al., 2006). Lipid droplet-associated proteins of the perilipin family regulate ATGL activation by interacting with CGI-58 and preventing its binding under nutrient rich conditions, but facilitating it during starvation (Granneman, Moore, Mottillo, Zhu, & Zhou, 2011; Pollak et al., 2015; Sanders et al., 2015). In addition, pigment epithelium-derived factor (PEDF) has been shown to promote lipolysis in adipocytes and FA metabolism in skeletal cells in an ATGL-dependent manner, but the exact mechanism remains unknown (Borg et al., 2011). G0/G1 switch protein 2 (G0S2) is an endogenous ATGL inhibitor, which also directly binds to the enzyme, but does not compete with CGI-58 for binding (X. Yang et al., 2010). Recent evidence suggests that hypoxia-inducible lipid droplet-associated protein (HILPDA) is another ATGL endogenous inhibitor, which shares a similar structure with G0S2 and directly interacts with ATGL (Padmanabha Das et al., 2018). In human adipocytes, fat-specific protein 27

(FSP27) interacts with ATGL, leading to reduced lipolysis and enhanced lipid storage capacity. However, the exact mechanism, as well as whether this interaction occurs at the lipid droplet surface, remains unclear (Grahn et al., 2014).

The results of studies on animal models with genetic knockouts and transgenic modifications provide important insights into the (patho)physiological role of ATGL. They have recently been listed and comprehensively presented by Schreiber et al. (Schreiber et al., 2018). Briefly, mice with systemic ATGL deletion accumulate TAG in many tissues and exhibit lethal cardiac failure due to a massive TAG accumulation in the heart (Haemmerle et al., 2006). Unexpectedly, these mice are only moderately obese and display improved insulin sensitivity despite TAG accumulation in various tissues (Haemmerle et al., 2006; Hoy et al., 2011; Kienesberger et al., 2009). This phenotype was explained by mice with systemic ATGL deletion, but with rescued ATGL expression in their heart. These mice were HFD-induced obesity resistant and had improved glucose homeostasis, but displayed impaired lipogenic signaling through peroxisome proliferator-activated receptor (PPAR) γ and sterol regulatory element-binding protein 1c (SREBP-1c) (Schreiber et al., 2015). These results indicate that reduced lipogenesis may contribute to the resistance of ATGL-deficient mice to obesity and obesity-induced insulin resistance. Recently, an ATGL-specific inhibitor of Atglistatin has been developed (Mayer et al., 2013). Pharmacological inhibition of ATGL by Atglistatin in mice effectively reduces adipose tissue lipolysis and protects from HFD-induced obesity, and most promisingly, it does not cause cardiac steatosis and dysfunction as observed in animal models lacking ATGL (Schweiger et al., 2017). This indicates that ATGL inhibition has a great potential to be used as a treatment for obesity and obesity-associated diseases.

Humans with ATGL mutations develop a neutral lipid storage disease with myopathy (NLSDM). NLSDM is a rare autosomal disorder with around 30 different mutations reported so far. The clinical features of NLSDM are progressive cardiomyopathy and excessive TAG accumulation in leukocytes (also called Jordan's anomaly) and multiple tissues, including liver, skin, skeletal muscles and pancreas (Fischer et al., 2007; Zechner et al., 2017). In comparison with ATGL-deficient mice, the impact on whole-body insulin sensitivity and FA metabolism is minor in ATGL-deficient humans (Natali et al., 2013). ATGL activity is required for supplying working muscle with FAs from adipose tissue during exercise (Schoiswohl et al., 2010). It also enables FA transfer from lipid droplets to mitochondria and supports mitochondrial oxidative metabolism in embryonic fibroblasts, hepatocytes and cardiomyocytes (Haemmerle et al., 2011; Ong et al., 2011; Rambold et al., 2015). Finally, products of ATGL-mediated lipolysis may act as bioactive lipids, suggesting its signaling role in lipid metabolism and the inflammatory response (Dichlberger et al., 2014; Haemmerle et al., 2011). Therefore, ATGL regulates several physiological processes and its functions extend far beyond energy metabolism.

1.3 Lipid Droplets and the Management of Nutrient and Oxidative Stress

Nutrient homeostasis is essential for all living organisms. Cellular nutrient stress arises from imbalances in nutrient availability, either from nutrient excess that may cause toxic accumulation of lipids, or from nutrient starvation that may lead to energy depletion and cell death. AMP-activated protein kinase (AMPK) and mammalian target of rapamycin (mTOR) are fundamental reciprocal regulators of cellular metabolism that coordinate metabolic responses during stress (Efeyan, Comb, & Sabatini, 2015; Hardie, Ross, & Hawley, 2012; Jarc & Petan, 2019b). Their activity is controlled by nutrient availability and oxidative status. In conditions of nutrient or oxidative stress, AMPK is activated and promotes FA oxidation and autophagy by inhibiting mTOR (Hardie, 2011; Hardie et al., 2012). Their activity also regulates lipid droplet turnover in stressed cells (Nguyen et al., 2017; Seo et al., 2017; Zhu et al., 2019). The biogenesis of lipid droplets is commonly induced in cells exposed to excess lipids, but, paradoxically, it also occurs in a variety of stressful conditions, such as nutrient starvation, oxidative stress, infection and inflammation (Fig. 4) (Henne et al., 2018; Jarc & Petan, 2019b; Koizume & Miyagi, 2016; Petan et al., 2018). These states are also characteristic of the tumor microenvironment. Altered lipid droplet turnover may support the survival of cancer cells under conditions of nutrient and oxidative stress through several different mechanisms, including the removal of excess lipids, the provision of FAs for energy production and for the synthesis of structural and signaling lipids. These aspects of lipid droplet metabolism are briefly discussed in the following subsections.

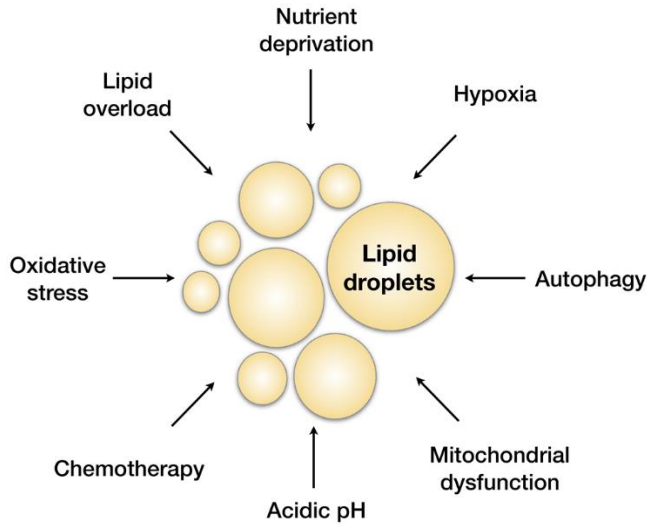

Figure 4: Lipid droplet synthesis is induced in stressed cells. Imbalances in energy metabolism and redox homeostasis, including hypoxia, starvation, oxidative stress, lipid overload, chemotherapy, acidic pH, mitochondrial dysfunction, high autophagic flux, lead to lipid droplet synthesis (Petan et al., 2018).

1.3.1 Lipid droplet biogenesis protects from lipotoxicity

Lipotoxicity is a condition that occurs when cellular mechanisms fail to overcome cell damage caused by lipids and may lead to cell death (Schaffer, 2003). Cellular mechanisms of protection against lipotoxicity act on the principle of eliminating surplus lipids by storing them into inert storage pools, disposing them by oxidation or removing them from the cell. Lipid droplets are major regulators of lipid metabolism. They are able to take up large amounts of lipids and release them gradually when needed in response to cellular demands (Jarc & Petan, 2019b). This delayed release of lipids protects from organellar damage and supports energy and redox homeostasis. In general, lipid droplets protect the cell from accumulation of FAs, DAGs, cholesterol and ceramides by storing them into their less hazardous forms within the lipid droplet core. They are stored within lipid droplets in the form of TAGs, CEs and acylceramides, respectively.

Emerging evidence suggests that DGAT1-mediated lipid droplet biogenesis protects cells and tissues from lipotoxicity by mitigating the overload of exogenous and endogenous lipids (Bosma, Kersten, Hesselink, & Schrauwen, 2012; Chitraju et al., 2017; Listenberger et al., 2003; Nguyen et al., 2017). In starved mouse embryonic fibroblasts, DGAT1-mediated TAG synthesis is essential for protection of mitochondria from the surge of FAs released by the high autophagic flux induced by nutrient starvation (Nguyen et al., 2017). In macrophages, elevated DGAT1 expression increases their capacity for TAG storage and protects from diet-induced insulin resistance and inflammation. In the endothelium, DGAT1-mediated TAG storage protects from the lipotoxicity of postprandial surges of FAs (Koliwad et al., 2010; Kuo, Lee, & Sessa, 2017). Interfering with DGAT activity when cells are exposed to an excess of saturated lipids leads to cell death (Bosma et al., 2014; Listenberger et al., 2003), revealing a metabolic vulnerability and confirming the importance of lipid droplet biogenesis for the protection against lipotoxicity.

1.3.2 Dysregulated lipid droplet breakdown leads to lipotoxic injury

Lipid overload is also linked with dysregulated breakdown of lipid droplets, leading to the uncontrolled release of FAs into the cytosol. ATGL is the rate-limiting enzyme in TAG hydrolysis. Its activity fuels cellular energetic needs during nutrient starvation (Rambold et al., 2015; Zechner et al., 2012) and regulates lipid metabolism and inflammatory responses (Dichlberger et al., 2014; Haemmerle et al., 2011). Stimulation of ATGL-mediated lipolysis may lead to lipotoxicity by increasing the pool of free FAs in the cytosol, altering signaling pathways and organelle homeostasis, overstimulating mitochondrial oxidative metabolism and ROS production (Chitraju et al., 2017; Haemmerle et al., 2011; Wang et al., 2015). In these conditions, preserved lipid droplet biogenesis may contribute to the protection against lipolysis-induced lipotoxicity. In adipocytes, DGAT1-mediated lipid droplet biogenesis overcomes the lipotoxic damage and lipotoxicity-induced ER stress during fasting by simultaneous esterification of ATGL-derived FAs into the pool of lipid droplets (Chitraju et al., 2017). Proteins that regulate ATGL activity, such as perilipin 5 (PLIN5), also prevent lipotoxicity by providing a lipolytic barrier against ATGL lipolysis and uncontrolled TAG lipolysis (Laurens et al., 2016; Pollak et al., 2013; Wang et al., 2015; Zheng et al., 2017).

1.3.3 Lipid droplets fuel cellular energetic needs by acting as metabolic and signaling hubs

Lipid droplet biogenesis is a general response to lipid overload, but it also occurs when cells are exposed to nutrient starvation. This has been demonstrated in various cell types, including in cancer cells (Cabodevilla et al., 2013; Gubern et al., 2009; Nguyen et al., 2017; Rambold et al., 2015). The need for this ATP consuming process of lipid droplet synthesis in the absence of nutrients can be justified by two main reasons: (1) to prevent the lipotoxicity of FAs released by autophagy and (2) to enable efficient transfer of FAs from lipid droplets to mitochondria for oxidation (Nguyen et al., 2017; Petan et al., 2018; Rambold et al., 2015). Emerging evidence suggests that FAs stored in lipid droplets are mobilized by lipolysis, but not lipophagy, to fuel mitochondrial oxidation and enable cell survival during severe and acute amino acid starvation of mouse embryonic fibroblasts (Nguyen et al., 2017; Rambold et al., 2015). ATGL activity was found essential for supplying mitochondria with lipid droplet-derived FAs in these conditions (Rambold et al., 2015). Aside from providing substrates for energy production, ATGL is involved in the signaling regulation of mitochondrial oxidative metabolism and inflammation. It was found that ATGL-derived FAs directly mediate the transcription of PPAR-regulated genes and thus affect signaling regulation of mitochondrial and lipid metabolism (Haemmerle et al., 2011; Mottillo, Bloch, Leff, & Granneman, 2012; Ong et al., 2011). Thus, ATGL-mediated lipolysis fuels cellular energy metabolism through metabolic and signaling pathways.

1.3.4 Lipid droplets are involved in lipid mediator synthesis

Lipid mediators are bioactive lipids that are produced locally in a variety of cells in response to extracellular stimuli. They act as autocrine or paracrine signaling agents through binding to G protein-coupled membrane receptors on target cells. Almost 40 years ago eicosanoids were identified as the first lipid mediators. Ever since, several lipid classes have been identified as lipid mediators and are grouped according to their structure and primary source. Eicosanoids are widely recognized "fire-causing" derivatives of the ω-6 FA arachidonic acid (AA) involved in inflammation. In the resolution phase of inflammation, a temporal lipid mediator class switch occurs and leads to the production of "fire-extinguishing" derivatives of the ω-3 eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, the maresins, protectins and resolvins, also known as anti-inflammatory specialized pro-resolving lipid mediators (SPMs). The balance between the production of pro- or anti-inflammatory lipid mediators defines the outcome of a particular inflammatory response and disease development (Chiurchiù, Leuti, & Maccarrone, 2018; Murakami, 2011; Serhan, 2014).

Eicosanoid synthesis has been classically linked with the activity of phospholipase A_2 (PLA_2) enzymes, which release FAs from the *sn*-2 position of glycerophospholipids from extracellular and intracellular membranes and membranous organelles (Dennis, Cao, Hsu, Magrioti, & Kokotos, 2011). However, recent evidence suggests that FAs stored in TAG and the activity of ATGL are an important source for eicosanoid synthesis in immune cells (Dichlberger et al., 2014; Schlager et al., 2015). In human mastocytes, blocking ATGL-mediated lipolysis resulted in lipid droplet accumulation and reduced levels of eicosanoids, such as prostaglandin D_2 and leukotriene C_4, suggesting that TAG synthesis is a source of their precursor AA (Dichlberger et al., 2014). Similarly, inhibition of ATGL activity in murine neutrophils led to a significant decrease in the release of several eicosanoids. In addition, exudates from ATGL deficient mice displayed reduced amounts of eicosanoids, indicating a role for ATGL in inflammatory signaling *in vivo* (Schlager et al., 2015). Thus,

lipid droplets are sources of precursors for lipid mediator synthesis and may play a role in the regulation of inflammatory responses.

1.4 Cancer

Cancer is essentially a disease of uncontrolled cell proliferation (Michalopoulou, Bulusu, & Kamphorst, 2016). Most human tumors are genetically heterogeneous, driven by activation of tumor oncogenes or loss of tumor suppressor genes, and by alterations in cellular signaling (Beloribi-Djefaflia et al., 2016; Pavlova & Thompson, 2016). This genetic variation makes cancer therapy greatly challenging. However, different alterations in oncogenes and tumor suppressor genes often induce a common metabolic phenotype, and in fact many tumors exhibit similar alterations in metabolism. These enable the adaptation of cancer cells to severe conditions of nutrient and oxidative stress and sustainment of cell proliferation and growth under unhospitable conditions. Most aggressive tumors are resistant to chemotherapy and this resistance often arises from their metabolic plasticity (Martinez-Outschoorn et al., 2017; Vander Heiden & DeBerardinis, 2017). Since the identification of the first metabolic hallmark of cancer almost a century ago, the increased glucose uptake, glycolysis and lactate production (also called the Warburg effect), several specific cancer-related metabolic alterations have been proposed: (1) dysregulated glucose and amino acid metabolism, (2) use of opportunistic modes of nutrient acquisition, (3) increased demand for nitrogen, (4) dependence on lipid metabolism and NADPH production, (5) pro-tumorigenic interactions with the microenvironment and (6) alterations in epigenetics (Pavlova & Thompson, 2016). The requirement for tight nutrient balance might be a vulnerability of many tumors and provides an opportunity for therapeutic interventions (Martinez-Outschoorn et al., 2017).

1.4.1 Breast cancer

Breast cancer is the most prevalent cancer in women worldwide. There are at least four subtypes of the disease, which differ in the expression of several markers, including estrogen receptor (Er), progesterone receptor (Pr) and human epidermal growth factor receptor 2 (HER2), that define disease aggressiveness and availability of treatment options. The most aggressive triple negative breast cancer (TNBC) tumors do not express any of these receptors (Er–/Pr–/HER2–) and are characterized by high tumor proliferation rates and frequent detrimental metastases to the lung and brain. There are different subtypes of TNBC that can be ranked according to gene expression profiling: basal like-1, basal like-2, mesenchymal-like, mesenchymal stem-like, luminal androgen receptor, immunomodulatory and unstable TNBC. Molecular profiling provides discovery of new promising targets, but the discovery of key molecular targets in TNBC is particularly difficult because of its genetic and phenotypic heterogeneity that is driven by different oncogenic pathways (Hwang, Park, & Kwon, 2019; Kalimutho et al., 2015). Currently, there are no specific treatment options for TNBC, apart from conventional chemotherapy and radiation. However, despite different oncogenic backgrounds, TNBC may exhibit similar metabolic requirements that are crucial for their adaptation to adverse environmental conditions and their metastatic potential (Martinez-Outschoorn et al., 2017). For studying breast cancer, several established cell lines are commercially available (Neve et al., 2006). Most of our research work for this thesis was done on the Ras (also called KRAS, RAS) oncogene-driven MDA-MB-231 cell line, which is a basal-like, invasive and highly tumorigenic model

of TNBC. We also performed some experiments on the T-47D cell line, which expresses Er, and Pr receptors and represents a less aggressive, hormone-responsive type of breast cancer.

1.4.2 Metabolic challenges of cancer cells

Cells within tumors can be exposed to different amounts of oxygen and nutrients, depending on the proximity of the bloodstream and conditions in the tumor environment (Fig. 5). Cancer cells located closer to the vasculature have some access of nutrients and oxygen, produce ATP through glycolysis and oxidative phosphorylation (OXPHOS) and exhibit upregulated anabolism, which supports fast proliferation. However, accelerated OXPHOS drives the production of reactive oxygen species (ROS) and causes oxidative stress. The latter initiates the onset of oxidative stress-induced changes in the metabolism of cancer cells and surrounding stromal cells, leading to stromal–cancer metabolic coupling. Increased ROS generation in cancer cells stimulates stromal cells to upregulate autophagy and the production of lactate and ketones, which are taken up by cancer cells to fuel mitochondrial FA metabolism and ATP production (also called reverse Warburg effect) (Martinez-Outschoorn et al., 2017; Pavlides et al., 2012). Fast proliferating cancer cells thus switch their metabolism from using glucose to the utilization of FAs for energy production (Carracedo, Cantley, & Pandolfi, 2013). Similarly, cells experiencing hypoxia and nutrient depletion are dependent on autophagy and the utilization of alternative sources of FAs for energy production. Hypoxia affects *de novo* FA production by compromising the activity of stearoyl-CoA desaturase 1 (SCD1), required for the production of oleate, which is the most abundant unsaturated FA in mammalian cells. This forces cells to increase FA uptake to satisfy their high metabolic demands. Importantly, Ras-driven tumors recapitulate this hypoxic phenotype, which makes them resistant to SCD1 inhibition (Kamphorst et al., 2013). Recent evidence suggests that most aggressive cancer cells rely on altered lipid metabolism and have greater capabilities to acquire extracellular lipids, possess an increased capacity for FA accumulation in lipid droplets, and upregulate mitochondrial β-oxidation to resist nutrient and oxidative stress (Beloribi-Djefaflia et al., 2016; Currie et al., 2013; Petan et al., 2018; Santos & Schulze, 2012). Altered lipid metabolism thus supports cancer proliferation and tumor growth.

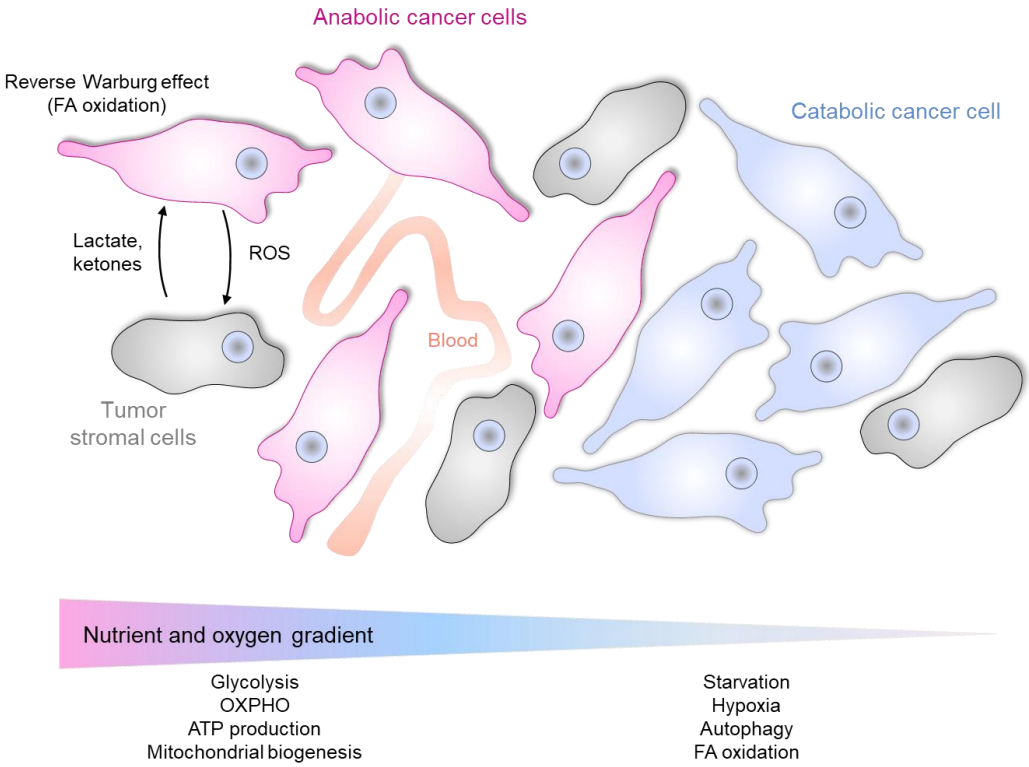

Figure 5: The availability of nutrients and oxygen affects metabolism in cancer cells. Cancer cells display different metabolic states depending on the proximity of the vasculature and the particular properties of the tumor microenvironment. Proliferating anabolic cancer cells have a stable supply of nutrients and oxygen from the vasculature, and generate ATP through glycolysis and oxidative phosphorylation, leading to increased ROS production. This increased oxidative stress stimulates neighboring tumor stromal cells to release lactate and ketones, which are taken up by anabolic cancer cells and used to fuel mitochondrial metabolism and ATP production (reverse Warburg effect). Nutrient depletion and hypoxia upregulate alternative metabolic pathways in catabolic cancer cells located further from the vasculature, whose survival depends on altered lipid metabolism, autophagy and alternative modes of nutrient acquisition from the surrounding tissues. The picture was re-designed from the original one published by Martinez-Outschoorn *et al.* (Martinez-Outschoorn et al., 2017).

1.4.3 Alterations in cancer lipid metabolism

Lipids are essential for cancer growth and metastasis. When access to circulating lipids is disrupted, cancer cells may acquire lipids from at least three lipid sources: (1) extracellular lipids, taken up by alternative and opportunistic modes of lipid acquisition, including membrane phospholipid or TAG hydrolysis in neighboring cells; (2) endogenous FA synthesis and (3) mobilization of endogenous, structural lipids via breakdown of membranous organelles by autophagy and/or membrane phospholipid hydrolysis by PLA_2s (Petan et al., 2018). These mechanisms enable cancer cell proliferation and survival under conditions of nutrient and oxidative stress.

In healthy humans, FA synthase (FASN) is expressed in various tissues, but *de novo* lipid synthesis is generally restricted to the liver, adipose tissue and mammary glands. The activity of FASN is transcriptionally regulated through hormone signaling and nutritional

status, and is downregulated during starvation. Increased FASN expression was found in several cancers and in the blood of cancer patients, suggesting that high FA synthesis in cancer supports increased metabolic demands of cancer cells. Interestingly, FA synthesis in cancer cells is not dependent on the level of dietary lipids, but is driven by oncogenic and signaling alterations. Upregulated growth-promoting signaling pathways, such as phosphatidylinositol 3-kinase (PI3K)/protein kinase B (Akt) and mTOR-mediated signaling, contribute to increased lipogenesis in cancer cells. In addition, the expression of lipogenic enzymes, such as FASN, acetyl-CoA carboxylase (ACC), ATP citrate lyase (ACLY) and the SREBP transcription factors, is increased in several cancers (Beloribi-Djefaflia et al., 2016; DeBerardinis & Chandel, 2016; Santos & Schulze, 2012).

On the other hand, mitochondrial FA oxidation is an important source of ATP and NADPH, and supports cancer survival during metabolic and oxidative stress. The production of NADPH is essential for the maintenance of redox homeostasis. Many cancers display increased ROS levels, but they have developed effective antioxidant mechanisms to prevent oxidative damage (Barrera, 2012). Furthermore, most aggressive cancer cells use alternative modes of nutrient acquisition by scavenging extracellular lipid sources to sustain energy production under conditions of nutrient starvation or hypoxia (Kamphorst et al., 2013; Michalopoulou et al., 2016). Macropinocytosis is a feeding mechanism driven by actin-mediated endocytosis (Recouvreux & Commisso, 2017) and may provide the uptake of opportunistic sources of lipids such as damaged cells or necrotic bodies (Kim et al., 2018). Cancer cells also act as metabolic parasites by sequestering metabolites from neighboring tissues. They are often engaged in symbiotic relationships with adipocytes to obtain lipids for energy production (Balaban et al., 2017; Nieman et al., 2011; Wang et al., 2017). Several signaling lipids, such as sphingolipids, FAs and their derivatives, affect cancer cell proliferation, migration, metastasis and immune responses (Röhrig & Schulze, 2016; Sulciner, Gartung, Gilligan, Serhan, & Panigrahy, 2018; Wang & Dubois, 2010). Lipid composition and lipid saturation levels are also two important aspects that determine membrane and organelle homeostasis, and may affect cancer cell survival during stress (Ackerman et al., 2018).

Finally, lipid droplets are emerging as regulatory hubs in lipid cancer metabolism and key players in the management of nutrient and oxidative stress. They can assume different roles in cancer cells, which may govern cancer cell adaptability and resistance to stress.

1.4.4 Lipid droplets in cancer cells

Since the 1950s, various studies have reported the accumulation of "lipid vesicles" in cancer tissues (Abbas, Yoo, & Viles, 1982; Aboumrad, Horn, & Fine, 1963; Lantos, 1975; Novikoff, 1957; Ramos & Taylor, 1974; Spence & Rubinstein, 1975). At that time, it was not yet known that these vesicles are organelles with many functions. However, it was reported that accumulation of lipid vesicles correlates with tumor aggressiveness (Ramos & Taylor, 1974). Today we know that lipid droplets accumulate in various human cancers, including breast, colon, cervical and prostate cancer (Petan et al., 2018). Elevated lipid droplet accumulation has been linked with poorer prognosis and lipid droplets have been proposed as tumor markers in cancer diagnostics (Beloribi-Djefaflia et al., 2016). Conditions of nutrient and oxidative stress lead to increased lipid droplet accumulation in many tumors (Bozza & Viola, 2010; Delikatny, Chawla, Leung, & Poptani, 2011; Koizume & Miyagi, 2016; Petan et al., 2018), suggesting a link between microenvironmental cellular stress and lipid droplet synthesis. Indeed, recent evidence suggests that lipid droplets are important players in the management of nutrient and oxidative stress, which has a strong impact on cancer cell proliferation and tumor growth. They have been implicated in several stress

response mechanisms, but the molecular basis of these mechanisms in cancer cells is still poorly understood. Their functions in stressed cells (Fig. 6) include, but are not limited to, regulating energy and redox homeostasis, FA channeling to mitochondria, ER and membrane homeostasis, autophagy, lipid mediator production and protection from lipotoxicity (Petan et al., 2018; Shyu, Wong, Crasta, & Thibault, 2018; Tirinato et al., 2017). We have comprehensively reviewed the current knowledge on the function of lipid droplets in cancer in our recent manuscript (Petan et al., 2018), but their roles relevant for this work are also presented in the following chapter.

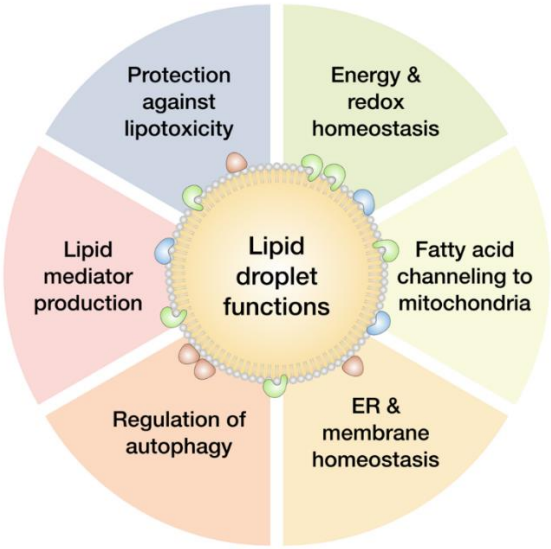

Figure 6: Lipid droplet functions in stressed cells. Lipid droplets have been implicated in several stress response mechanisms, including the regulation of energy and redox homeostasis, FA channeling to mitochondria, ER and membrane homeostasis, autophagy, lipid mediator production and protection against lipotoxicity (Petan et al., 2018).

1.5 Targeting Lipid Droplet Metabolism in Cancer

DGAT enzymes are emerging as potential targets for reducing cancer growth. Targeting these enzymes may cause cancer cell damage by several mechanisms, including the increase in lipotoxicity, impairment of membrane and organelle homeostasis, and abolishment of the beneficial effects of lipid droplets in energy and redox homeostasis (see Section 1.5.1). On the contrary, the role of ATGL in cancer is less clear (Currie et al., 2013; Petan et al., 2018). There are conflicting reports, arguing between a pro-tumorigenic and a tumor suppressor role of ATGL in cancer (see Section 1.5.4). However, it is very likely that ATGL displays pleotropic functions in cancer metabolism and a universal, either pro-tumorigenic or tumor suppressor, role should not be expected (Petan et al., 2018). Since lipid droplets are involved in stress response mechanisms and support cell survival during nutrient and oxidative stress, targeting lipid droplet metabolism in cancer has a great potential for reducing cancer growth and chemoresistance. Currently, this idea is still in its infancy due

to the lack of mechanistic knowledge on how cancer cells exploit lipid droplet turnover for tumor growth and metastasis. This is also in line with the fact that specific inhibitors of lipid droplet synthesis or lipid droplet breakdown have not yet been developed or tested for the use in cancer therapy. The following sections provide examples of how lipid droplets contribute to the survival of cancer cells in different stressful conditions. These studies also reveal the numerous open questions in the investigation of lipid droplet biology in cancer.

1.5.1 Impaired lipid droplet accumulation reduces cancer growth and chemoresistance

In prostate cancer cells, DGAT enzymes are overexpressed and contribute to lipid droplet accumulation, which is important for cancer cell growth (Mitra, Le, Gorjala, & Goodman, 2017). DGAT1 inhibition results in reduced aggressiveness of prostate cancer cells and tumor growth *in vivo* (Nardi et al., 2019). Blocking the expression of DGAT1 similarly inhibits lipid droplet synthesis and leads to prostate cancer cell death by triggering autophagy (Mitra et al., 2017). Although autophagy is now generally considered a mechanism of cell survival and not a type of cell death (Kroemer, Mariño, & Levine, 2010), recent studies suggest that autophagy may cause cell death when lipid droplet formation is compromised, leading to the accumulation of toxic acylcarnitines and mitochondrial dysfunction (Nguyen et al., 2017). In accordance, silencing of DGAT1 and DGAT2 in conditions of low oxygen and nutrient availability leads to overproduction of toxic ceramides and acylcarnitines, and to disruption of TAG and phospholipid membrane composition, reducing renal cancer cell proliferation and tumor growth *in vivo* (Ackerman et al., 2018).

Lipid droplets are also sites of CE storage. CE accumulation has been associated with increased uptake of exogenous lipids, cell proliferation, migration and survival in various cancer cells, including TNBC cells (Antalis et al., 2010; Antalis, Uchida, Buhman, & Siddiqui, 2011; de Gonzalo-Calvo et al., 2015; Geng et al., 2016; Yue et al., 2014). Elevated CE accumulation is also an indicator for cancer aggressiveness and poorer prognosis in breast cancer patients (de Gonzalo-Calvo et al., 2015; Peck & Schulze, 2014). Inhibition of cholesterol esterification by ACAT1 depletion leads to suppressed tumor growth and metastasis by increasing intracellular levels of free cholesterol, causing ER stress and cell death (Li et al., 2016). Therefore, the ability of cancer cells to remove cholesterol from its bioactive pool is beneficial for cancer progression and provides a therapeutic opportunity for reducing cancer growth.

The activity of DGAT2, but not DGAT1, has been linked with the removal of toxic ceramides. Silencing of DGAT2 led to enhanced 5-fluorouracil (5-FU)-induced ceramide accumulation and cancer cell death, while stimulation of acylceramide synthesis correlated with lipid droplet accumulation and reduced apoptosis of colorectal carcinoma cells (Senkal et al., 2017). These results suggest that DGAT2 acyltransferase activity is essential for the conversion of toxic ceramides into their less toxic form of acylceramides, which may improve cancer resistance to chemotherapy. Another mechanism that links chemoresistance and lipid droplets has been recently proposed. In colorectal cancer cells, treatments with 5-FU and oxaliplatin lead to lysophosphatidylcholine acyltransferase 2 (LPCAT2)-dependent lipid droplet accumulation (Cotte et al., 2018). Cotte *et al.* found that the expression of LPCAT2 in various cancer cell lines correlates with the inhibition of chemotherapy-induced ER stress and chemoresistance *in vitro* and *in vivo*. They also found that lipid droplet accumulation correlates with impaired CD8+ T-cell infiltration and chemotherapy-induced immunogenic cell death. The mechanism includes the sequestration of calreticulin to lipid droplets, thereby reducing the exposure of this danger-associated

molecular pattern (DAMP) molecule for T-cell recognition and impairs the inflammatory response. Thus, lipid droplets mediate chemoresistance by alleviating chemotherapy-induced ER stress and suppressing inflammation in the tumor microenvironment.

1.5.2 Lipid droplets may affect the response of cancer cells to PUFA treatment

Polyunsaturated fatty acids (PUFAs) have been investigated in the context of using these FAs, ω-3 PUFAs in particular, as adjuvant therapy in cancer (Calviello, Serini, & Piccioni, 2007; Chénais & Blanckaert, 2012; Schmitz & Ecker, 2008). In general, ω-6 PUFAs, such as AA, promote inflammation and display pro-tumorigenic effects, while ω-3 PUFAs, such as DHA and EPA, display anti-tumorigenic effects. However, most *in vitro* studies have not been performed in physiologically relevant conditions and have used high micromolar concentrations of PUFAs, suggesting that their apparent toxicity may be largely exaggerated and could hide their potential underlying effects (Petan et al., 2018). PUFAs are easily oxidized by enzymatic and non-enzymatic reactions, leading to the production of oxygenated products that cause membrane dysfunction and enter into lipid peroxidation chain reactions that may potentially damage proteins and DNA and lead to cell death. The sensitivity of cancer cells to PUFAs may be related to their different capacity to maintain redox balance and remove oxidized lipid from membranes, preventing lipid peroxidation and cell death. Ferroptosis is a recently discovered type of programmed cell death dependent on iron and characterized by accumulation of lipid peroxides. One of the mechanisms involved in ferroptosis initiation includes the loss of glutathione peroxidase 4 (GPX4) activity caused by impaired cysteine import and a lack of glutathione synthesis required for GPX4 activity, leading to the accumulation of lipid peroxides and cell death (Feng & Stockwell, 2018). A recent study has reported that the activity of ACSL4 is responsible for sensitizing cells to ferroptosis by promoting PUFA incorporation into cellular membranes, making them susceptible to oxidation. Furthermore, ACSL4 was preferentially expressed in a basal-like cancer cell lines, such as the MDA-MB-231 TNBC cell line, and its expression strongly correlated with sensitivity of cancer cells to ferroptosis (Doll et al., 2017). This suggests that the response of cancer cells to PUFA lipotoxicity is dependent on their resistance to ferroptosis, i.e. their ability to prevent or neutralize lipid peroxidation and thus maintain the cellular redox balance when challenged with PUFAs.

Two mechanisms have recently been suggested to regulate the sensitivity of cancer cells to ferroptotic cell death. The first is based on the effect of monounsaturated FAs, whose incorporation into membrane phospholipids reduces membrane PUFA content and thus limits unwanted lipid peroxidation reactions. The mechanism requires the activity of ACSL3, but is not dependent on lipid droplet formation (Magtanong et al., 2019). In contrast, a second ACSL-independent mechanism has been suggested, which likely depends on alterations in FA metabolism and TAG saturation levels mediated by cell density (Panzilius et al., 2018). It was found that the sensitivity of breast cancer cells to ferroptosis increases with low cell density, while high cell density supports cancer cell resistance to ferroptosis. The expression level of ATGL and enzymes required for mitochondrial oxidation, in addition to SCD1-mediated PUFA uptake, inversely correlated with cell density. Lipidomic analyses revealed an enrichment of PUFAs in TAG species, suggesting that PUFA-TAG accumulation might be a compensatory mechanism that reduces PUFA toxicity caused by the elevated PUFA uptake at low cell density. However, a similar trend of PUFA enrichment was also observed in phospholipid species, which may explain why these cells were more sensitive for ferroptosis in comparison with cells cultured at high cell density, enriched with saturated and monounsaturated FAs in their phospholipid species

(Panzilius et al., 2018). Importantly, a mechanism of lipid droplet-mediated protection against PUFA lipotoxicity has recently been shown in *Drosophila*. It was reported that the sequestration of membrane-derived PUFAs within TAGs of lipid droplets reduces PUFA lipotoxicity, which is essential for larval neuronal development during stress (Bailey et al., 2015). Interestingly, the composition of TAG at high cell density in ferroptosis-resistant cancer cells showed an enrichment in saturated and monounsaturated FAs (Panzilius et al., 2018). This suggests a correlation between lipid droplets, TAG composition and vulnerability of cancer cells towards ferroptosis.

1.5.3 Lipid droplets contribute to the resistance of cancer cells to hypoxia

Cancer cells may experience oxidative stress during both oncogene-driven fast proliferation in nutrient replete conditions and during nutrient and oxygen deprivation in poorly vascularized tumors (Harris, 2002; Wilson & Hay, 2011). Several studies have found increased lipid droplet accumulation in various cancer cells exposed to hypoxia, a condition of low oxygen tension (Bensaad et al., 2014; Koizume & Miyagi, 2016; Mylonis et al., 2012; Schlaepfer et al., 2015). Low oxygen availability affects de novo lipogenesis, forcing cells to upregulate FA uptake in order to compensate for the inhibition of the oxygen-dependent desaturase SCD1 and satisfy metabolic demands (Ackerman & Simon, 2014). Recently, it was suggested that hypoxia-induced lipid droplet accumulation occurs due to increased FA uptake and supports cancer cell survival in conditions of hypoxia and hypoxia-reoxygenation (Bensaad et al., 2014). Hypoxia-induced lipid droplet biogenesis was dependent on hypoxia inducible factor 1α (HIF1α)-mediated signaling, occurring along with an upregulated expression of FA-binding proteins (FABPs), which are essential for FA uptake, and of perilipin 2 (PLIN2), a protein involved in lipid droplet turnover. Silencing of FABP3, FABP7 or PLIN2 led to reduced lipid droplet storage, which compromised the survival of glioblastoma and breast cancer cells after reoxygenation. In addition, silencing of FABP3 or FABP7 led to impaired growth of tumor xenografts *in vivo*. In conditions of hypoxia and nutrient starvation, lipid droplets may also protect cancer cells by supplying TAG-derived unsaturated FAs for neutralizing the elevated, hypoxia-induced membrane FA saturation levels and to maintain the production of membrane phospholipids (Ackerman et al., 2018). Renal cancer cells exposed to high levels of extracellular lipids and/or oleate (OA) accumulate large amounts of lipid droplets enriched in OA-containing TAGs (Ackerman et al., 2018). The lipolytic release of OA from lipid droplets then maintains the membrane phospholipid unsaturation balance during hypoxia and nutrient deprivation, thereby opposing the accumulation of saturated lipids, which may lead to the production of toxic ceramides and acylcarnitines (see Section 1.5.1). Thus, lipid droplets contribute to cancer cell survival in various stressful conditions with fluctuating oxygen levels and nutrient availability, which are often found in the tumor environment.

1.5.4 The role of lipid droplet lipolysis and ATGL in cancer

Most *in vitro* studies suggest a pro-tumorigenic role for ATGL in cancer. Namely, several studies have shown that ATGL depletion leads to growth inhibition in various cancer cell lines (Ou et al., 2014; Zagani, El-Assaad, Gamache, & Teodoro, 2015). Additionally, studies have suggested that its activity in both tumor cells and neighboring adipocytes contributes to the adipocyte-induced metabolic reprogramming and aggressiveness of different cancers, including breast cancer (Balaban et al., 2017; Grace et al., 2017; Nieman, Romero, Van Houten, & Lengyel, 2013; Wang et al., 2017). In accordance, the expression of ATGL is

elevated in high-grade mammary tumors and breast cancer cell lines *in vitro* (Wang et al., 2017). However, recent *in vivo* studies suggest a tumor suppressor role for the enzyme, showing that its expression is reduced in several human cancers, including breast invasive carcinoma, and it correlates with a reduced patient survival (Al-Zoughbi et al., 2014). In addition, mice lacking ATGL spontaneously develop lung tumors (Al-Zoughbi et al., 2014), and an adipose tissue-specific deletion of ATGL and HSL leads to the development of liposarcoma (Wang, Fu, McNerney, & White, 2014). The story is additionally complicated by the contribution of CGI-58 and G0S2, endogenous regulators of ATGL activity, to cancer. They have been suggested to play a tumor suppressor role and may also display ATGL-independent roles (Vegliante, Di Leo, Ciccarone, & Ciriolo, 2018).

The pleotropic functions of ATGL activity may have a broad influence on various processes linked with tumorigenesis, such as inflammation, oxidative stress and autophagy. As mentioned above, ATGL fuels mitochondrial FA oxidation and regulates the PPAR and SREBP signaling axes, thereby affecting essential cellular processes, such as FA uptake, lipogenesis, mitochondrial oxidative metabolism and lipid oxidation (Haemmerle et al., 2011; Ong, Mashek, Davidson, & Mashek, 2014; Rambold et al., 2015). Furthermore, ATGL is involved in the synthesis of pro-inflammatory eicosanoids in immune cells (Dichlberger et al., 2014; Schlager et al., 2015). There is a clear association between elevated eicosanoid signaling and tumorigenesis (Wang & Dubois, 2010), but the role of ATGL in the production of eicosanoids in cancer remains to be investigated. Finally, recent evidence suggests that ATGL regulates autophagy and lipophagy in the liver (Sathyanarayan, Mashek, & Mashek, 2017). Whether the correlation between ATGL and autophagy also exists in cancer cells and is important for cancer resistance is not known.

Chapter 2

Aims and Hypothesis

Breast cancer is the most common cancer in women and represents a leading cause of cancer death among women (Torre et al., 2015). The recently revealed common metabolic vulnerabilities and adaptations of different types of cancer cells to cellular stress provide an opportunity for the development of novel therapies that may reduce the growth of currently incurable cancers, including most aggressive triple-negative breast cancer (TNBC) (Hwang et al., 2019; Martinez-Outschoorn et al., 2017). The importance of lipid metabolism in cancer is emerging (Currie et al., 2013; Röhrig & Schulze, 2016), but we are still far from understanding the lipid metabolic pathways that are critical for the survival, growth and metastasis of cancer cells. Lipid droplets integrate lipid metabolism with cellular stress responses and may thus be the nexus between cancer metabolism and resilience to stress. However, the mechanisms of lipid droplet turnover and the connecting survival advantages of cancer cells are not clear.

Our research was based on the following hypothesis: The survival of aggressive breast cancer cells under conditions of nutrient and lipotoxic stress is dependent on lipid droplet turnover controlled by DGAT and ATGL enzymes that govern cancer cell resistance to stress through metabolic and signaling pathways.

The aim of this doctoral thesis was to determine the major molecular pathways that link lipid droplets with TNBC cell survival during starvation-induced nutrient stress. Our recent studies have shown that lipid droplet breakdown correlates with cell survival during prolonged starvation of TNBC cells (Pucer et al., 2013). However, the mechanism of lipid droplet breakdown and that connecting breakdown with cell survival remained unknown. The major TAG lipase ATGL is involved in lipid droplet breakdown in most cell types and fuels mitochondrial FA oxidation by providing the optimal delivery of FAs to mitochondria and regulating underlying signaling pathways, thereby affecting essential cellular processes (Haemmerle et al., 2011; Rambold et al., 2015; Smirnova et al., 2006). Our major goal was to determine whether ATGL contributes to lipid droplet breakdown in TNBC cells and thus supports cancer cell survival during starvation.

Our second aim was to determine the involvement of lipid droplets in the protection of TNBC cells from the lipotoxicity of excess exogenous lipids. Emerging evidence suggests that lipid droplets act as buffers that reduce the harmful effects of excess FAs. Dysregulated lipid droplet turnover, including DGAT-mediated lipid droplet biogenesis and/or ATGL-mediated lipid droplet breakdown may lead to lipotoxicity and cell death, suggesting that the precise regulation of these pathways is critical for the protection against lipotoxicity (Aflaki et al., 2011; Bosma et al., 2014; Listenberger et al., 2003; Nguyen et al., 2017; Petan et al., 2018). We hypothesized that lipid droplets regulate intracellular FA trafficking, which may be critical for cancer cell survival during stress. Our major goal was to determine

the importance of DGAT and ATGL activities in the cellular distribution and lipotoxicity of different FAs in TNBC cells.

Finally, our third aim was to establish whether lipid droplets regulate the production of bioactive lipid mediators, which may be important for both metabolic and inflammatory responses in stressed cancer cells. Recent studies have revealed that ATGL-mediated lipolysis drives the production of eicosanoids in immune cells, suggesting a role for lipid droplets in eicosanoid production (Dichlberger et al., 2014; Schlager et al., 2015). However, whether ATGL-mediated lipolysis is also involved in the production of eicosanoids or other bioactive lipids in cancer cells remains unknown. Our major goal was to establish the relationship between lipid droplets and bioactive lipid mediator production in TNBC cells, which may affect cancer cell resistance to stress.

Chapter 3

Results

This chapter describes our latest and yet unpublished work on the role of lipid droplets in the synthesis of bioactive lipids. Previously, we have reported that lipid droplets are involved in the protection of TNBC cells from nutrient and oxidative stress, whereby we explored two novel mechanisms that regulate lipid droplet turnover and cancer cell survival (Jarc, Kump, et al., 2018). Here, we focused on mechanisms that link lipid droplet metabolism with the production of inflammatory lipid mediators in cancer cells.

3.1 Lipid Droplets are Sources of Polyunsaturated Fatty Acids for Lipid Mediator Production in Starving Breast Cancer Cells

3.1.1 Introduction

Lipid droplets are conserved organelles that play pivotal roles in the intracellular lipid metabolism. Their biogenesis is a rapid and highly regulated process that is often activated in response to cellular stress. Diacylglycerol acyltransferase (DGAT) enzymes catalyze the last step in triacylglycerol (TAG) synthesis in the ER and are rate-limiting for lipid droplet formation (Thiam, Farese, & Walther, 2013; Walther et al., 2017). Compromised DGAT-dependent lipid droplet biogenesis leads to lipotoxicity and other forms of cellular stress, *e.g.* the activation of ER stress response pathways (Chitraju et al., 2017; Listenberger et al., 2003; Nguyen et al., 2017). The lipid droplet core is mainly composed of TAGs and cholesterol esters (CE), which are broken down by TAG and CE lipases depending on cellular needs for FAs and cholesterol (Henne et al., 2018; Jaishy & Abel, 2016; Jarc & Petan, 2019b; Petan et al., 2018). Besides being energetic substrates, FAs activate different signaling pathways in immune and other cells by binding to cell surface receptors, including G-protein coupled receptors (GPCRs) and toll-like receptors (TLRs), fatty acid transport proteins and nuclear receptors, such as the peroxisome proliferator-activated receptors (PPARs) (Papackova & Cahova, 2015; Schmitz & Ecker, 2008; Zechner et al., 2012). The involvement of lipid droplet-derived FAs in the activation of these signaling pathways is poorly understood (Jarc & Petan, 2019a).

Adipose triglyceride lipase (ATGL) is the major TAG lipase in adipose and non-adipose tissues and is essential for lipolysis initiation (Smirnova et al., 2006; Zechner et al., 2012; Zimmermann et al., 2004). Its activity results in the release of FAs from TAGs stored within lipid droplets and their transfer to mitochondria, thereby supporting cell survival during nutrient deprivation (Rambold et al., 2015). Recent studies have shown that the products of ATGL-mediated lipolysis are able to activate the family of PPARs, nuclear

receptors that control the transcription of genes involved in lipogenesis and oxidative lipid metabolism in metabolically active tissues (Ahmadian et al., 2011; Haemmerle et al., 2011; Ong et al., 2011; Schreiber et al., 2015; Schweiger et al., 2017). Furthermore, recent evidence suggests that ATGL acts as a node of metabolic and inflammatory pathways, activating Sirtuin 1 (SIRT1)-mediated signaling and promoting oxidative metabolism, while possibly suppressing inflammatory responses (Chen et al., 2015; Khan et al., 2015; Young & Zechner, 2013). ATGL activity has also been implicated in the production of bioactive lipid mediators from the eicosanoid family, whose origin has been traditionally attributed to phospholipase A_2 activity on cell membranes but not to lipid droplet metabolism (Dichlberger et al., 2014; Riederer, Lechleitner, Köfeler, & Frank, 2017; Schlager et al., 2015).

The production of PUFA-derived bioactive lipids, also called lipid mediators, is controlled by the availability of their precursors. Glycerophospholipid hydrolysis by phospholipase A_2 (PLA_2) enzymes is considered the main source of PUFAs for lipid mediator production. Among the PLA_2 enzymes, cytosolic phospholipase $A_2\alpha$ ($cPLA_2\alpha$) has been recognized as the primary source of arachidonic acid (ω-6; 20:4 AA), whose oxidation by cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 epoxygenase enzymes leads to the production of pro-inflammatory eicosanoids (Dennis & Norris, 2015). Interestingly, the same enzymes are employed for the conversion of ω-3 PUFAs, such as docosahexaenoic (ω-3; 22:6 DHA) and eicosapentaenoic (ω-3; 22:5 EPA) acid, into specialized pro-resolving mediators (SPMs), which are essential for the proper resolution of inflammation (Serhan, 2014; Serhan, Dalli, Colas, Winkler, & Chiang, 2015). The family of secreted phospholipases A_2 ($sPLA_2$s), whose activity results in the release of various monounsaturated and polyunsaturated FAs besides AA, has also been implicated in the generation of lipid mediators. The group X $sPLA_2$ (GX $sPLA_2$) is the most potent mammalian $sPLA_2$ in hydrolyzing phosphatidylcholine-rich membranes (Singer et al., 2002), thereby releasing a mixture of unsaturated FAs, including ω-3 PUFAs, from the surface of lipoproteins and the plasma membrane of intact cells (Jarc, Kump, et al., 2018; Lambeau & Gelb, 2008; Murakami, 2017). Studies have suggested the involvement of GX $sPLA_2$ in the production of anti-inflammatory lipid mediators responsible for suppression of colitis (Murase et al., 2016) and in the production of eicosanoids, which affect allergic responses, inflammation and tumorigenesis (Nolin et al., 2017; Schewe et al., 2016; Surrel et al., 2009).

Lipid droplets are sources of various lipid species, inclusive of PUFAs that could in principle be used for lipid mediator synthesis. They have also been proposed to function as platforms for transient storage of numerous proteins and enzymes, including those that are involved in lipid signaling and inflammation, such $cPLA_2\alpha$ (Yu et al., 1998), COXs (Accioly et al., 2008) and LOXs (Silva et al., 2009). $cPLA_2\alpha$ was found on the outer lipid droplet surface and may contribute to the release of AA from the phospholipid monolayer (Moreira et al., 2009; Yu et al., 1998), but this has not been demonstrated so far (Bozza, Bakker-Abreu, Navarro-Xavier, & Bandeira-Melo, 2011). Besides the phospholipid monolayer, PUFAs in lipid droplets are also stored in TAGs, whose synthesis drives lipid droplet formation and dynamically responds to inflammatory stimuli (Bozza et al., 2011; Triggiani, Oriente, & Marone, 1994; Triggiani et al., 1995). Activated immune cells accumulate lipid droplets, enriched with AA-containing TAG species. The storage of excess AA within neutral lipids may be a way for the cell to reduce its availability in the phospholipid pool, thereby limiting eicosanoid production and suppressing the inflammatory response (Johnson et al., 1999; Triggiani, Oriente, & Marone, 1994; Triggiani et al., 1995). TAGs could also serve as reservoirs for the replenishment of the phospholipid pool with AA (Bozza et al., 2011). Finally, TAG lipolysis could in fact be a *bona fide* source of AA and other

PUFAs for direct conversion into lipid mediators by COX and LOX enzymes, thereby bypassing the phospholipid pool.

Intriguingly, recent studies have shown that ATGL-mediated TAG lipolysis is involved in eicosanoid synthesis in activated mast cells, neutrophils and vascular endothelial cells (Dichlberger et al., 2014; Riederer et al., 2017; Schlager et al., 2015). Silencing of ATGL led to lipid droplet accumulation in human activated mastocytes and importantly, it significantly reduced the release of prostaglandin D_2 and leukotriene C_4 (Dichlberger et al., 2014). Similarly, treating mouse neutrophils with an ATGL inhibitor led to a reduced release of various COX-, LOX and CYP-derived lipid mediators. Reduced concentrations of lipid mediators were also found in peritoneal lavages from ATGL deficient mice (Schlager et al., 2015). Intriguingly, silencing of $cPLA_2\alpha$ in human activated mastocytes caused a similar reduction in the release of eicosanoids as silencing ATGL, indicating that both enzymes contribute to the release of AA for eicosanoid synthesis (Dichlberger et al., 2014). However, it is not yet clear whether ATGL-released AA is directly used by COX and LOX enzymes, or it is incorporated back in the phospholipid monolayer of lipid droplets and then released by $cPLA_2\alpha$. Interestingly, lipidomic data from ATGL depleted neutrophils showed increased PUFA-TAG accumulation, while no significant changes in phospholipid species were observed (Schlager et al., 2015), suggesting that phospholipid hydrolysis is not important for the function of ATGL in eicosanoid synthesis. Although these studies establish ATGL-mediated TAG hydrolysis as a major and physiologically relevant source of precursors for lipid mediator synthesis in immune cells, the possibility that $cPLA_2\alpha$ complements or replenishes its activity is highly probable and requires further examination. To establish a general role for lipid droplets as sources of precursors for lipid mediator synthesis additional evidence is required, including studies on non-immune cells, comprehensive lipidomic analyses of cellular lipid composition and specific environmental conditions, relevant for different physiological and pathophysiological cellular states.

Several studies suggest the association between eicosanoid metabolism, tumorigenesis and tumor progression (Dennis & Norris, 2015; Wang & Dubois, 2010). Eicosanoids have been shown to modulate cancer cell proliferation, differentiation and apoptosis through multiple signaling pathways (Wang & Dubois, 2010), but mechanisms that regulate eicosanoid production in cancer and in tumor-associated immune cells are not completely understood. Prostaglandin E_2 (PGE_2) is the most abundant eicosanoid in various human cancers, including breast, neck and colon cancer. Increased release of PGE_2 is associated with cancer invasiveness and poorer prognosis (Karpisheh et al., 2019; Wang & Dubois, 2010). Limited information is currently available regarding the mechanisms that drive lipid mediator production in cancer. Clinical studies with inhibitors of COX and PLA_2 have not provided promising results in suppressing eicosanoid synthesis in cancer (Dennis et al., 2011; Karpisheh et al., 2019; Sulciner, Gartung, et al., 2018), indicating other mechanisms might be involved. There is a great need for their discovery to improve current knowledge on targeting cancer metabolism and signaling, and to reveal new therapeutic options for reducing cancer growth and invasiveness. It has been reported that increased PGE_2 synthesis in colon cancer cells is linked with lipid droplet accumulation and COX-2 overexpression (Accioly et al., 2008). Treating cells with aspirin and the FA synthase inhibitor C75 resulted in reduced lipid droplet content and PGE_2 synthesis, indicating that lipid droplets might be involved in PGE_2 synthesis in cancer. The abovementioned studies in immune cells have shown that ATGL-mediated lipid droplet breakdown is an important source of AA for eicosanoid synthesis (Dichlberger et al., 2014; Schlager et al., 2015). Whether lipid droplet breakdown is a source of precursors that drive lipid mediator synthesis in cancer cells is not known.

We have recently shown that lipid droplets are involved in the protection of triple-negative breast cancer (TNBC) cells from nutrient and oxidative stress (Jarc, Kump, et

al., 2018; Pucer et al., 2013). We found that lipid droplet breakdown supports TNBC cell survival during nutrient depletion. ATGL activity was found to be required for lipid droplet breakdown, but surprisingly, it was not essential for the pro-survival effect of lipid droplets during starvation. Intriguingly, our lipidomic data revealed an enrichment with highly unsaturated PUFA species in TAGs in lipid droplets (Pucer, 2014). The significance of this enrichment remains unknown, but it indicates that lipid droplets in TNBC cells are reservoirs of PUFA species.

In this study, we hypothesized that breakdown of PUFA-rich lipid droplets affects lipid mediator synthesis in TNBC cells. Our major goal was to establish the role of ATGL lipolysis in the synthesis of eicosanoids and other lipid mediators, particularly under conditions of starvation-induced nutrient stress. Our second goal was to explore whether cPLA$_2$α contributes to ATGL-mediated eicosanoid production and lipid droplet turnover in TNBC cells. Finally, we asked whether lipid droplets are necessary for eicosanoid production. To answer this question we suppressed DGAT-mediated TAG synthesis and determined the effects on lipid mediator production. By interfering with DGAT, ATGL and cPLA$_2$α activities we established their role in lipid droplet metabolism and eicosanoid synthesis under lipolytic conditions in TNBC cells.

3.1.2 Materials and methods

3.1.2.1 Materials

Breast cancer cells (MDA-MB-231) and culture medium (RPMI-1640) were obtained from ATCC (USA), fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS), TrypLE Select and Opti-MEM from Life Technologies (USA). Arachidonic acid and prostaglandin E_2 (PGE_2) standards were from Cayman Chemical (USA), Lipofectamine RNAiMAX, lipofectamine 3000 and High-Capacity cDNA Reverse Transformation kit from Thermo Fisher Scientific (USA). Human ATGL-targeting siRNAs, human cPLA2α-targeting siRNAs and the AllStars Negative Control siRNA were from Qiagen (Germany); T863 (DGAT1 inhibitor), PF-06424439 (DGAT2 inhibitor), essentially fatty acid-free (EFAF) bovine serum albumin (BSA) (cat. no. A7511) and Nile red were from Sigma-Aldrich (USA). The High Pure RNA Isolation Kit was from Roche (Germany), horseradish peroxidase (HRP)-labelled secondary antibodies from Jackson ImmunoResearch Laboratories (USA), ATGL antibodies (#2138) from Cell Signaling Technology (USA) and β-actin antibodies (NB600-532) from Novus Biologicals (UK). The recombinant wild-type human group X $sPLA_2$ was prepared as described previously (Petan, Križaj, Gelb, & Pungerčar, 2005; Singer et al., 2002). The full-length cDNA coding for human cPLA$_2$α was cloned into the pcDNA3.1/D-V5-His-TOPO vector (Life technologies, USA) according to manufacturer's instructions and prepared by Dr. Anja Pucer Janež. The full-length cDNA coding for human ATGL cloned into the pcDNATM4/HisMaxC vector (Thermo Fisher Scientific, USA) was a kind gift from Dr. Robert Zimmermann (University of Graz). All other chemicals were of at least analytical grade and purchased from Sigma-Aldrich (USA) or Serva (Germany).

3.1.2.2 Cell culture and treatments

MDA-MB-231 cells were cultured in RPMI-1640 medium supplemented with 10% FBS and adherent cells detached using TrypLE Select. Unless otherwise indicated, cells were seeded in 24-well plates at a concentration of 6×10^4 cells/well and grown for 48 h in complete medium followed by 24 h of serum deprivation in RPMI-1640 medium containing 0.02%

EFAF-BSA. Aliquots of stock solutions of AA and the PGE_2 standard in absolute ethanol were stored under argon at –80 °C. Prior to addition to cell culture, AA was complexed to EFAF-BSA in RPMI-1640 medium at a molar AA:BSA ratio of 4:1 by incubating the mixtures for 1 h at room temperature. T863 and PF-06424439 were added to cells 2 h prior to the addition of recombinant hGX $sPLA_2$ or AA, and were present in the medium for the duration of the treatment.

3.1.2.3 Silencing of ATGL and cPLA$_2$α expression using small interfering RNA (siRNA)

MDA-MB-231 cells were reverse transfected at a concentration of 6 × 104 cells/well in 24-well plates, or 3 × 105 cells/well in 6-well plates, with 20 nM mixture of two siRNAs for ATGL silencing and with 40 nM mixture of 4 siRNAs for $cPLA_2\alpha$ silencing, or with 20 nM or 40 nM AllStars Negative Control siRNA (Qiagen). Transfection complexes were generated using 1 µl/well of Lipofectamine RNAiMAX in 24-well plates, and 7.5 µl/well in 6-well plates, and Opti-MEM medium according to manufacturer's instructions.

3.1.2.4 Transient expression of ATGL and cPLA$_2$α

For transient protein (over)expression experiments, cells were seeded in complete medium in 24-well plates at a concentration of 6×10^4 cells/well, or in 6-well plates at 3×10^5 cells/well, and incubated 48 h in complete medium. After 48 h, cells were washed, transfected and left in RPMI-1640 medium containing 0.02% EFAF-BSA for 24 h. Cells were transfected with 0.5 µg/well of plasmid DNA in 24-well plates or 2.5 µg/well in 6-well plates using Lipofectamine 3000 and Opti-MEM medium according to manufacturer's instructions.

3.1.2.5 Real-time quantitative PCR (qPCR)

qPCR analysis was performed as described previously (Jarc, Kump, et al., 2018; Pucer et al., 2013). Briefly, cells were seeded in complete medium in 6-well plates at 3 × 105 cells/well and left for 48 h in complete medium followed by 24 h of serum deprivation in RPMI-1640 medium containing 0.02% EFAF-BSA. Total RNA was isolated from cell lysates and first strand cDNA generated according to the manufacturer's instructions. qPCR analysis was performed for all genes of interest and two reference genes in each sample. Relative gene expression was calculated upon normalization to two reference genes, corrected for primer-specific PCR efficiency and considering error propagation.

3.1.2.6 Western blot analysis

Cells were seeded in complete medium in 6-well plates at 3 × 105 cells/well and reverse transfected with siRNA and/or transiently transfected with pDNA as described above. Cell lysates were prepared by scrapping adherent cells in Tris-Glycine SDS Sample Buffer (2X) (Novex by Life Technologies, USA) containing 800 mM DTT (Sigma-Aldrich, USA) with the addition of Halt Protease Inhibitor Cocktail (Thermo Scientific, USA). Lysates were incubated at 95 °C for 10 min and stored on ice. Total protein concentration was determined using Pierce 660 nm Protein Assay (Thermo Scientific, USA). Ten µg of total protein were separated on 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane (Serva, Germany). After the transfer, the membrane was blocked for 1 h in 5% non-fat dry milk in TBS/0.1% Tween-20 (TBST) (for ATGL) or in 1% Western Blocking Reagent (WBR) (Roche Applied Science, Germany) in TBS (for β-actin) and incubated overnight at 4 °C in the presence of rabbit anti-human primary antibodies for ATGL

(1:1000 dilution) in 5% non-fat dry milk in TBST and rabbit anti-human primary antibodies for β-actin (1:5000 dilution) in 0.5% WBR in TBS. After washing with TBST, membranes were incubated for 1 h with HRP-conjugated secondary antibodies (1:10,000 dilution) in 5% non-fat dry milk in TBST for ATGL or 0.5% WBR in TBS for β-actin. After washing, the signal was visualized using Lumi-Light Western Blotting Substrate (Roche Applied Science, Germany) on a Gel Doc XR system (Bio-Rad, USA).

3.1.2.7 Lipidomic analyses of lipid mediators in cell supernatants

Cells were seeded in complete medium in 6-well plates at 3×10^5 cells/well and reverse transfected with siRNA as described above. After 24 h, cells were treated with 10 nM hGX $sPLA_2$ for 48 h, washed with DPBS and left for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. The cells were lysed and total protein concentrations determined as described above. Cell supernatants were collected in low-binding microcentrifuge tubes, centrifuged at 4 °C (16,000 *g*,10 min) and stored at –80 °C before shipping on dry ice. For comprehensive lipidomic analysis of lipid mediators in cell supernatants, a novel breakthrough methodology was employed and performed in collaboration with our partners from the University of Jena, Dr. Oliver Werz and Dr. Jana Gerstmeier as described previously (Werz et al., 2018). Briefly, the supernatants were put in ice-cold methanol containing deuterium-labelled internal standards to facilitate quantification and sample recovery. The samples were kept at –20 °C for 1 h to allow protein precipitation and then centrifuged before solid-phase extraction using C18 columns. After extraction, the samples were dried and immediately resuspended in a methanol/water (1:1) solution before LC-MS-MS analysis. To monitor each lipid mediator an MRM method was used (Dalli & Serhan, 2012). Calibration curves were obtained for each lipid mediator using authentic compound mixtures and deuterium-labelled standards of lipid mediators at a concentration range of 3.12–200 pg. Internal standard recoveries, matrix effect and limit of detection were determined in biological matrix.

3.1.2.8 ELISA-based quantification of PGE_2 in cell supernatants

The amount of PGE2 released into culture media was analyzed using commercial enzyme immunoassay (Prostaglandin E2 Express ELISA Kit, Cayman Chemicals, USA) according to the manufacturer's protocol. Cells were seeded in 24-well plates and reverse transfected with siRNA as described above. After 24 h, the cells were treated with 10 nM hGX $sPLA_2$ and/or 10 µM AA and/or a 40 µM mixture of T863 and PF-06424439 DGAT inhibitors (in a molar ratio of 1:1) for 48 h in complete medium followed by 24 h starvation in RPMI-1640 medium containing 0.02% EFAF-BSA, when ATGL and $cPLA_2\alpha$ were transiently expressed as described above. Cell supernatants were collected in low-binding microcentrifuge tubes, centrifuged at 4 °C (16,000 g, 10 min) and immediately used for analysis or stored at –80 °C for up to 7 days. For analysis, 50 µl of undiluted samples was used and standard curves prepared by diluting the PGE_2 standard in culture medium.

3.1.2.9 Nile red staining of lipid droplets

Cellular neutral lipid amount was quantified by flow cytometry as described previously (Pucer et al., 2013). Floating and adherent cells were harvested, centrifuged at 300 *g* for 10 min and the pellet resuspended in 500 µl of 1 µg/ml Nile Red solution in DPBS. After 10 min incubation in the dark, cell analysis was performed by flow cytometry using the FL-1 (530/30) filter on at least 2×10^4 events per sample.

3.1.2.10 Statistical analysis

Statistical analyses were performed using Prism 8.0 (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.

3.1.3 Results

3.1.3.1 Lipid droplet levels correlate with increased PGE_2 synthesis under lipolytic conditions

We have previously found that lipid droplets are gradually broken down during serum starvation, which improves energy and redox status of the cells and protects from cell death (Pucer et al., 2013). In this study, we focused on the signaling role of lipid droplets in TNBC cells. We hypothesized that the breakdown of lipid droplets stimulates the production of eicosanoids and other lipid mediators. To show that lipid droplets are involved in the synthesis of eicosanoids, we first induced lipid droplet biogenesis in nutrient-rich conditions and then their breakdown by nutrient starvation. For this purpose, we treated MDA-MB-231 cells with recombinant hGX $sPLA_2$ and/or with AA, a precursor for PGE_2 synthesis, followed by starvation in the absence of these agents, and ending with the determination of cellular lipid droplet content and PGE_2 levels in cell supernatants (Fig. 7A). As expected (Jarc, Kump, et al., 2018; Pucer et al., 2013), we found that the addition of hGX $sPLA_2$ increases the levels of lipid droplets remaining after the starvation phase in breast cancer cells (Fig. 7B). A similar effect was observed when cells were treated with AA, while a combined addition of hGX $sPLA_2$ and AA further augmented lipid droplet accumulation. Notably, the breakdown of hGX $sPLA_2$ or AA-induced lipid droplets was associated with an increase in PGE_2 production under lipolytic conditions (Fig. 7C), suggesting a link between lipid droplets and lipid mediator production in TNBC cells. The release of PGE_2 was most significantly increased in cells co-treated with hGX $sPLA_2$ and AA (Fig. 7C). These results indicate that the breakdown of lipid droplets, induced in MDA-MB-231 cells by $sPLA_2$ or AA in nutrient-rich conditions, may have provided AA for the synthesis of PGE_2 during serum starvation.

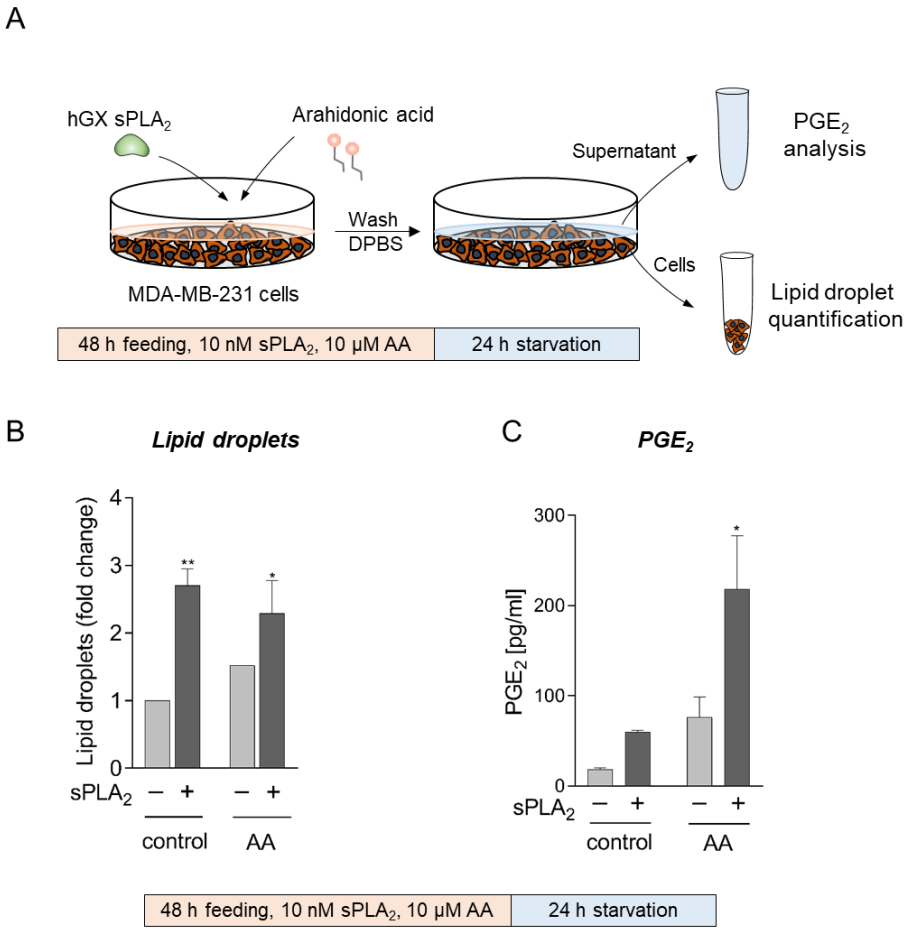

Figure 7: PGE_2 generation in TNBC cells correlates with lipid droplet accumulation. (A) Adherent MDA-MB-231 cells were treated with 10 nM $sPLA_2$ and/or 10 µM AA for 48 h in complete medium. After 48 h, the cells were washed with DPBS and serum-starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. Cell supernatants were used for PGE_2 analysis, while adherent cells were prepared for flow cytometry to determine lipid droplet levels. (B) The amount of neutral lipids in TNBC cells was determined by staining cells with 1 µg/ml of Nile Red and flow cytometry analysis. (C) The release of PGE_2 was determined as described in Methods. Values on the graphs are means ± SEM of at least three experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni adjustment (B, C)).

3.1.3.2 Blocking ATGL activity reduces the release of PGE_2 in starving TNBC cells

Recent evidence suggests that ATGL-mediated lipolysis is involved in the regulation of inflammatory responses by regulating the production of lipid mediators (Dichlberger et al., 2014; Schlager et al., 2015). We have previously reported that ATGL is involved in lipid droplet breakdown in MDA-MB-231 cells, but surprisingly, it is not essential for the pro-survival effect of hGX $sPLA_2$ (Jarc, Kump, et al., 2018). Here we asked whether ATGL-mediated lipolysis is involved in lipid mediator production in TNBC cells and perhaps mediates the effect of hGX $sPLA_2$ on PGE_2 synthesis. To answer this question, we blocked lipid droplet breakdown by knocking down ATGL to suppress lipolysis as described

previously (Jarc, Kump, et al., 2018) and measured cellular PGE_2 production. We found that TNBC cells depleted in ATGL accumulated more lipid droplets in comparison with control cells (Fig. 8A). Furthermore, ATGL depletion led to prominent lipid droplet accumulation in hGX $sPLA_2$-treated cells, suggesting a reduced breakdown of hGX $sPLA_2$-induced lipid droplets (Fig. 8A). Remarkably, ATGL depletion significantly reduced the release of PGE_2 from both untreated and hGX $sPLA_2$-treated cells (Fig. 8B), suggesting that ATGL-mediated lipolysis is involved in both basal and $sPLA_2$-elicited PGE_2 synthesis in TNBC cells.

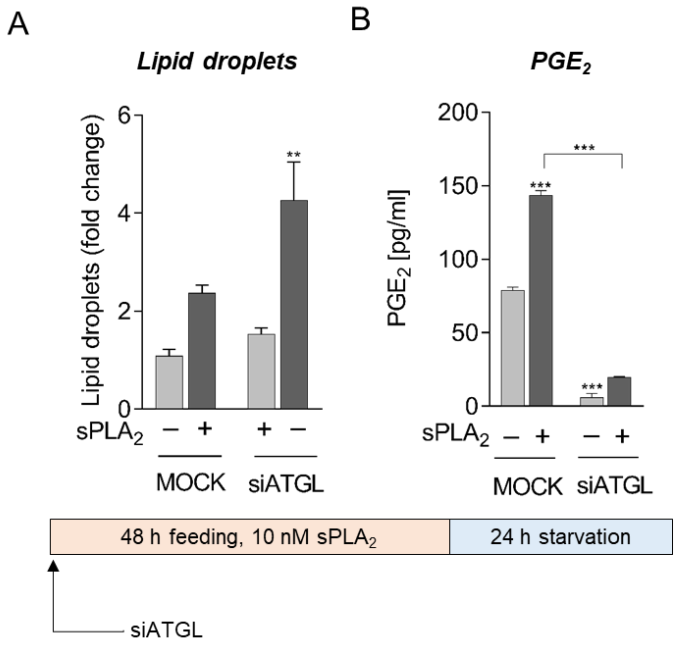

Figure 8: PGE_2 synthesis in TNBC cells is dependent on ATGL-mediated lipid droplet breakdown. MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) or with transfection reagent (MOCK) and treated with 10 nM $sPLA_2$ for 48 h in complete medium and then serum starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. Neutral lipid content was determined by Nile Red staining (A) and PGE_2 release with a commercial enzymatic immunoassay (B). Values on the graphs are means ± SEM of at least three experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni adjustment).

3.1.3.3 Starvation-induced lipid droplet breakdown contributes to the production of eicosanoids and specialized pro-resolving lipid mediators

We next examined whether ATGL depletion not only affects PGE_2 production, but also the release of other lipid mediators. Based on our previous results, showing TAG enrichment with highly unsaturated FAs (Pucer, 2014), we hypothesized that lipolysis may contribute to the release of different PUFA species, including AA, DHA and EPA, and thus affect the production of a wide range of eicosanoids and SPMs. The latter are related to the resolution of inflammation, but their dysregulated synthesis causes chronic inflammation and increases the risk for tumorigenesis (Serhan, 2014; Sulciner, Gartung, et

al., 2018). We employed a state-of-the-art lipidomic LC-MS-MS technology that enables the analysis of a broad spectrum of lipid mediators, including their definitive identification and quantification in biological samples, where the limit of detection is below pg/ml. Targeted lipidomics of cell supernatants using LC-MS-MS revealed that treating TNBC cells with hGX sPLA$_2$ increased the release of numerous lipid mediators, derived from different FA precursors and enzymatic pathways. In Fig. 9, we have summarized the major pro-inflammatory and pro-resolving lipid mediator biosynthetic pathways.

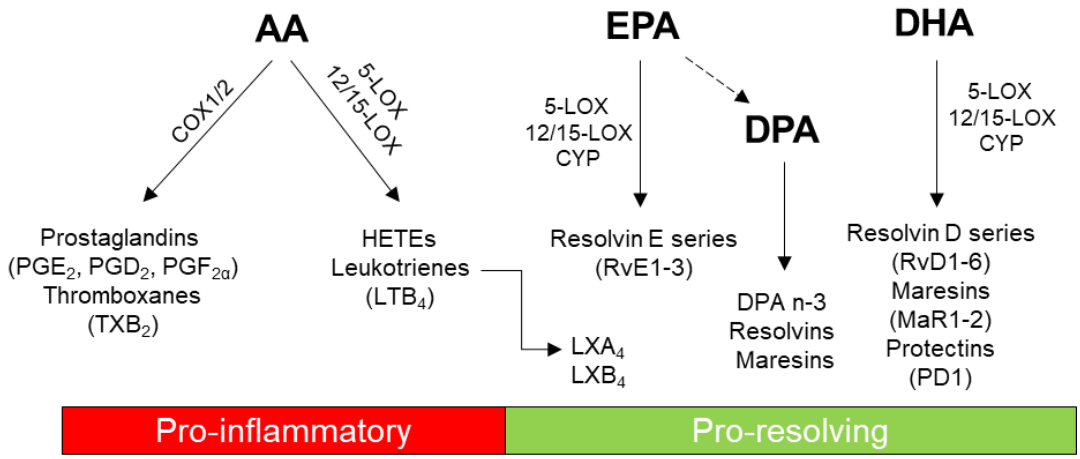

Figure 9: The major enzymatic pathways involved in pro-inflammatory and pro-resolving lipid mediator synthesis. COX enzymes convert arachidonic acid (AA) into pro-inflammatory prostaglandins and thromboxanes, while 5-LOX and 12/15-LOX enzymes convert AA to hydroxyeicosatetraenoic acids (HETEs) and leukotrienes. The latter are precursors for the synthesis of pro-resolving lipoxins. Ω-3 PUFAs, eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, are converted by 5-LOX, 12/15-LOX and CYP oxidases into several hydroxyeicosapentaenoic acids (HEPEs) and hydroxydocosahexaenoic acids (HDHAs), respectively, which are precursors for the synthesis of specialized pro-resolving lipid mediators, including resolvins, maresins and protectins. Docosapentaenoic acid (DPA) is an intermediate in the conversion of EPA to DHA and a precursor for the synthesis of DPA$_{n\text{-}3}$ resolvins and maresins that also exhibit pro-resolving effects in inflammation.

We identified 86 different lipid mediators and among 57 species we observed changes induced by hGX sPLA$_2$. Surprisingly, we found that hGX sPLA$_2$ activity predominantly promotes the synthesis of EPA-derived products, but also contributes to the production of AA- and DHA-derived lipid mediators (Fig. 10A). By grouping lipid mediators according to the enzymatic pathways of their synthesis, we found that hGX sPLA$_2$ activity mostly affects the production of 12/15-LOX-derived EPA and DHA products (51%; 29/57), followed by the cytochrome P450 oxygenase (CYP) derivatives (14%; 8/57), COX derivatives (16%; 9/57), 5-LOX derivatives (14%; 8/57) and SPMs (5%; 3/57) (Fig. 10B). hGX sPLA$_2$-induced changes in lipid mediator production relative to untreated cells were averaged within each enzymatic pathway group and are presented in the heat map (Fig. 10C). We found that EPA-derived hydroxyeicosapentaenoic acid metabolites (HEPEs) were the most abundant group of products, followed by leukotrienes, prostaglandins, hydroxydocosahexaenoic acid metabolites (HDHAs), hydroxyeicosatetraenoic acid metabolites (HETEs) and resolvins, while the level of lipoxins remained unchanged. Among

COX-derived lipid mediators, PGE_2 was the most abundant product (Fig. 10D), followed by the 5-LOX-derived 5-hydroxyeicosapentaenoic acid (5-HEPE) (Fig. 10E) and 12/15-LOX-derived products of EPA and DHA (Fig. 10F). Surprisingly, we found a significant increase in the release of 14-HDPA (Fig. 10F), a lipid mediator that derives from the n-3 docosapentaenoic acid (DPA) and is a precursor of a group of recently discovered immunoresolvents (Dalli, Colas, & Serhan, 2013). Among CYP-derived products and SPMs, we found a significant increase in the production of the bioactive EPA metabolite, 18-HEPE (Fig. 10G), whose activity was recently shown to reduce metastasis of pulmonary carcinoma (Li et al., 2018). Intriguingly, the release of the prostaglandin H_2 metabolite, 12-hydroxyheptadecatrienoic acid (12-HHT), was also significantly increased (Fig. 10G). 12-HHT is an endogenous ligand of leukotriene B_4 receptor 2, whose activation leads to the production of tumor necrotic factors and interleukins (Okuno & Yokomizo, 2018). Finally, we observed a very low production of DHA- and EPA-derived maresin and resolvin SPMs in TNBC cells. In cells treated with hGX $sPLA_2$ there was an increase in the amount of resolvin D6 (RvD6) and a decrease in the production of resolvin E1 (RvE1) (Fig. 10F). Both lipid mediators have been implicated in the regulation of inflammatory responses (Serhan, 2017), but their specific roles and actions are currently not completely understood.

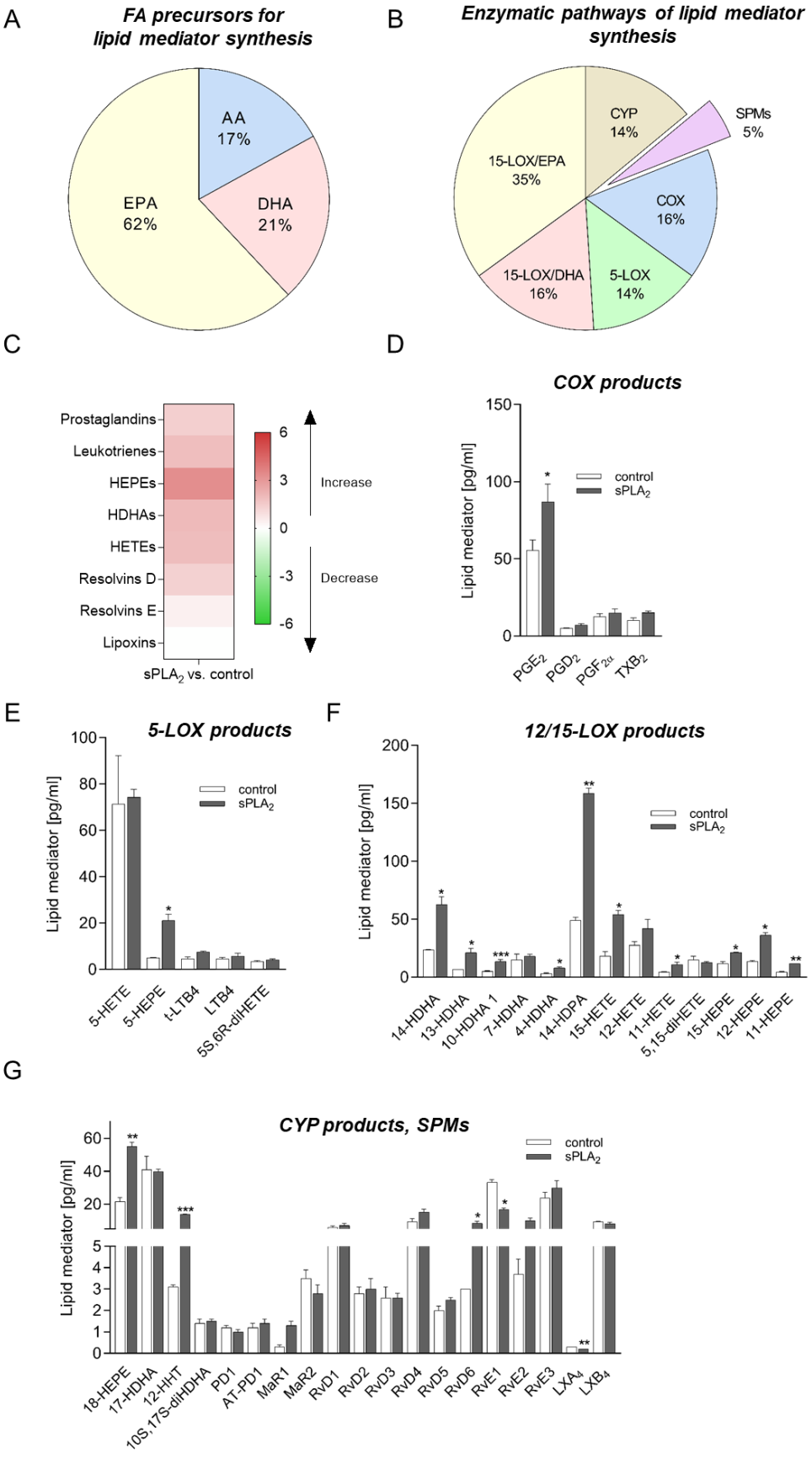

Figure 10: Starvation-induced lipid droplet breakdown leads to the release of various COX, LOX and CYP metabolites in TNBC cells. (A–G) MDA-MB-231 cells were treated

with 10 nM $sPLA_2$ for 48 h in complete medium and then serum-starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. Lipid mediator species were extracted from cell supernatants and analyzed by LC-MS-MS analysis as described in Methods. (A) Percentages of fatty acid (FA) precursors that contribute to $sPLA_2$-induced lipid mediator production in TNBC cells. (B) Percent contributions of enzymatic pathways involved in $sPLA_2$-induced lipid mediator production in TNBC cells. (C) Heat map of averaged changes in the release of prostaglandins, leukotrienes, HEPEs, HDHAs, HETEs, resolvins E, resolvins D and lipoxins induced by $sPLA_2$ activity, relatively to untreated cells. Identified changes in the release of COX-derived lipid mediators (D), 5-LOX-derived metabolites (E), 12/15-LOX-derived lipid mediators (F), products of CYP oxidases and specialized pro-resolving lipid mediators (G), induced by $sPLA_2$ activity. Values on the graphs are means ± SEM of at least two experiments; statistically significant differences in mean values are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired t-test).

3.1.3.4 ATGL depletion suppresses basal and $sPLA_2$-induced release of pro-inflammatory and pro-resolving lipid mediators in breast cancer cells

We next analyzed whether ATGL activity is necessary for lipid mediator synthesis in untreated and in hGX $sPLA_2$-treated cells. In ATGL-depleted cells, which were not treated with the $sPLA_2$, we found significantly lower levels of almost all major groups of lipid mediators (Fig. 11), suggesting a role for ATGL-mediated lipolysis in the basal production of pro-inflammatory and pro-resolving lipid mediators in TNBC cells. We analyzed changes in lipid mediator synthesis in cells treated with non-targeting siRNA (SCR) and cells treated with ATGL-targeting siRNA, and found reduced levels of prostaglandins, leukotrienes, HEPEs, HDHAs, HETEs, resolvins D and E, and lipoxins, caused by ATGL depletion (Fig. 11A). Among COX-derivatives, PGE_2, prostaglandin D_2 (PGD_2) and thromboxane B_2 (TXB_2) concentrations were significantly lower in ATGL depleted cells (Fig. 11B), confirming that the results of PGE_2 analysis with ELISA (Fig. 8B) are in line with the LC-MS-MS analysis. Among 5-LOX derivatives, we found significantly lower levels of 5-HEPE and *trans*-leukotriene B_4 (t-LTB_4) (Fig. 11C). Similarly, several derivatives of 12/15-LOX were significantly lower, including 7-HDHA, 4-HDHA, 8-HETE and 15-HEPE (Fig. 11D). The products of CYP450 oxygenases, including 21-HDHA, 21-HDPA, 18-HEPE and 12-HHT, were also significantly lower (Fig. 11E).

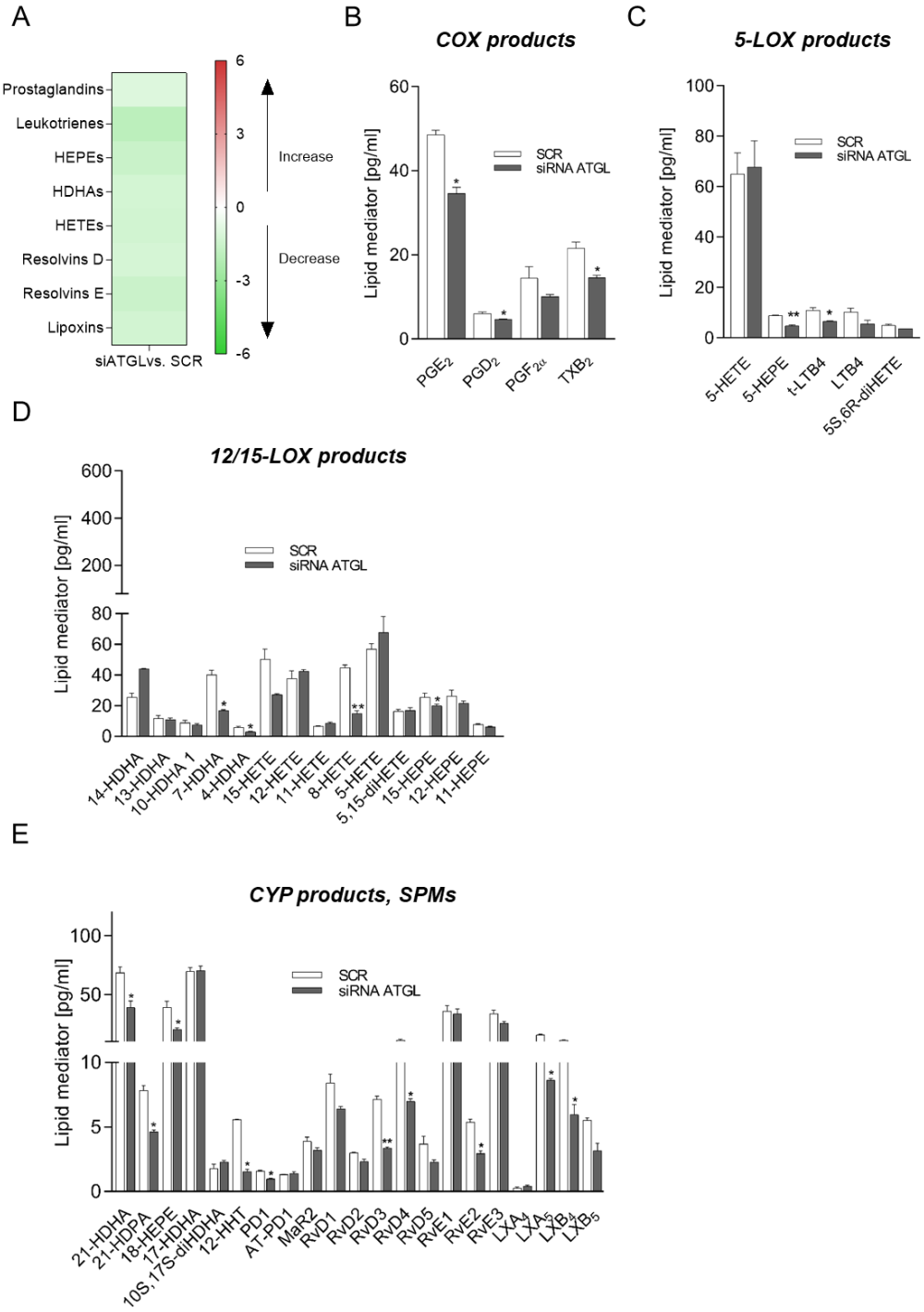

Figure 11: Blocking lipolysis by ATGL depletion prevents the release of several lipid mediators in starving TNBC cells. (A–E) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) or non-targeting scrambled siRNA (SCR) and cultured 48 h in complete medium and then serum-starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. Lipid mediator species were extracted from cell supernatants and analyzed by LC-MS-MS analysis as described in Methods. (A) Heat map of averaged changes in the release of prostaglandins, leukotrienes, HEPEs, HDHAs, HETEs, resolvins E, resolvins D and lipoxins induced by ATGL depletion, relatively to SCR-treated control cells. Identified changes in the release of COX-derived lipid mediators (B), 5-LOX-derived metabolites (C), 12/15-LOX-derived lipid mediators (D),

products of CYP oxidases and specialized pro-resolving lipid mediators (E), caused by ATGL depletion. Values on the graphs are means ± SEM of at least two experiments; statistically significant differences in mean values are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired t-test).

Among SPMs, we found reduced levels of protectin D1 (PD1), RvD3 RvD4, RvE2, lipoxin A_5 (LXA5) and LXB5 (Fig. 11E). Therefore, inhibition of lipolytic lipid droplet breakdown by ATGL depletion leads to a reduced production of numerous lipid mediators in TNBC cells, providing, to the best of our knowledge, the first evidence that lipid droplets and TAG lipolysis are involved in the synthesis of bioactive lipid mediators in cancer.

Next, we examined whether ATGL is involved in the production of the various lipid mediators released from hGX $sPLA_2$ stimulated cells (Fig. 10), in addition to PGE_2 (Fig. 8B). Importantly, we found that ATGL knockdown almost fully blocked hGX $sPLA_2$-induced lipid mediator release, including the release of prostaglandins, leukotrienes, HEPEs, HDHAs, HETEs, resolvins and lipoxins (Fig. 12A). The most prominent changes were found in the reduction of COX-derived PGE_2 and PGD_2 (Fig. 12B), 5-LOX-derived 5-HEPE and 5S,6R-diHETE (Fig. 12C), several 12/15-LOX derived HDHAs, HEPEs and HETEs (Fig. 12D), CYP-derived 21-HDHA, 18-HEPE and 12-HHT, and most of SPM species (Fig. 12E). Taken together, ATGL activity is required for the effect of hGX $sPLA_2$ on lipid mediator synthesis in TNBC cells.

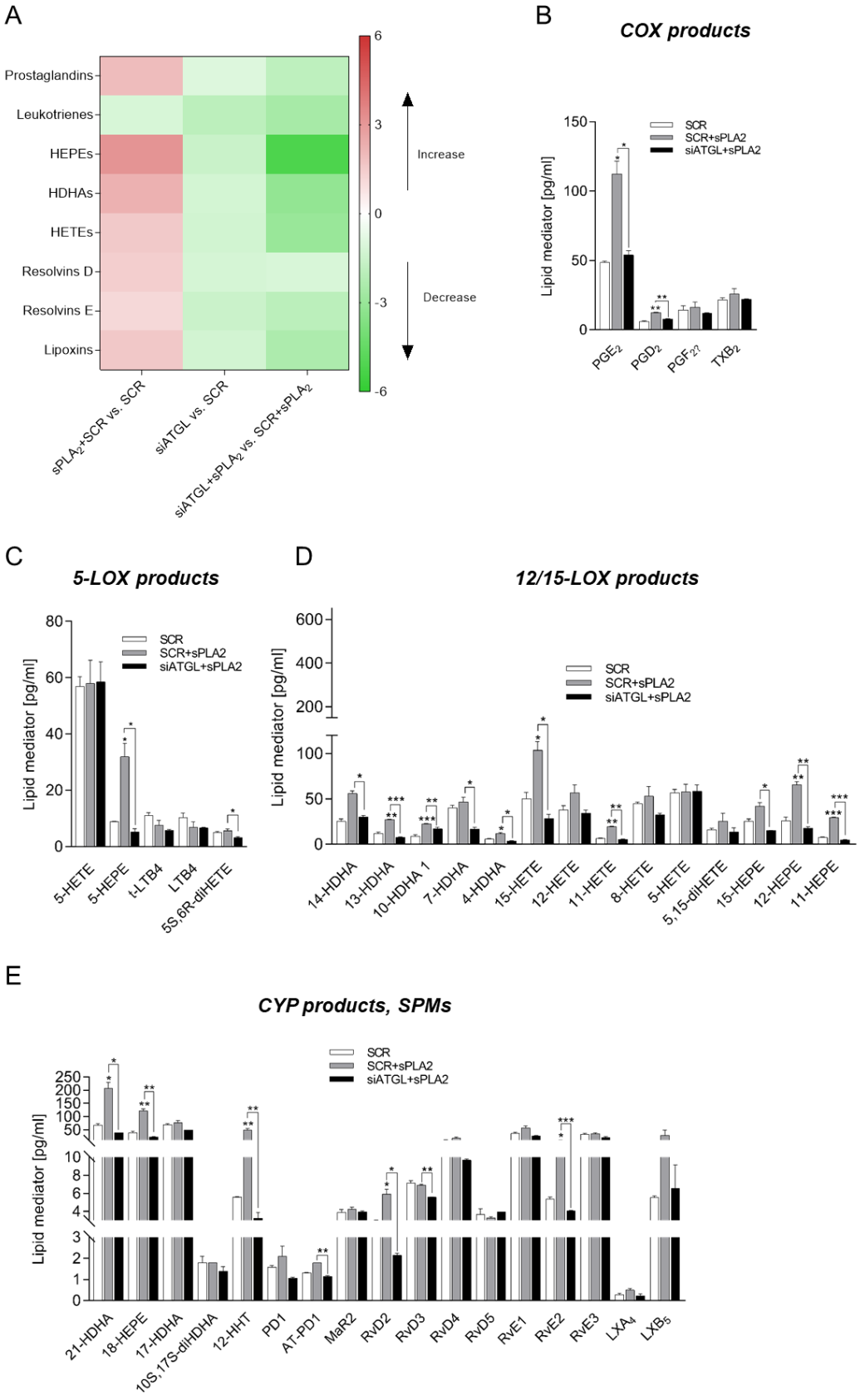

Figure 12: ATGL is essential for the effect of hGX sPLA2 on lipid mediator generation in TNBC cells. (A–E) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) or non-targeting scrambled siRNA (SCR) and treated with 10 nM

sPLA2 for 48 h in complete medium and then serum-starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. Lipid mediator species were extracted from cell supernatants and analyzed by LC-MS-MS analysis as described in Methods. (A) Heat map of averaged changes in the release of prostaglandins, leukotrienes, HEPEs, HDHAs, HETEs, resolvins E, resolvins D and lipoxins induced by sPLA2 activity and ATGL depletion, relatively to SCR control cells. Identified changes in the release of COX-derived lipid mediators (B), 5-LOX-derived metabolites (C), 12/15-LOX-derived lipid mediators (D), products of CYP oxidases and specialized pro-resolving lipid mediators (E), induced by sPLA2 activity and ATGL depletion. Values on the graphs are means ± SEM of at least two experiments; statistically significant differences in mean values are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired t-test).

3.1.3.5 Stimulation of ATGL-mediated lipolysis augments PGE_2 production

Our results show that ATGL depletion results in a reduced capacity of breast cancer cells to produce PGE_2 (Fig. 8B) and it also affects the production of a broad spectrum of lipid mediators, derived from COX-, LOX- and CYP-signaling pathways, in both untreated (Fig. 11) and in $sPLA_2$-stimulated cells (Fig. 12). To provide further support for our findings, we overexpressed ATGL in TNBC cells and determined the effect on cellular lipid droplet content and PGE_2 release. Plasmid-based transfection with human ATGL cDNA resulted in a potent increase of ATGL protein levels in TNBC cells under lipolytic conditions (Fig. 13A). Importantly, co-transfection with ATGL-targeting siRNA efficiently suppressed plasmid-driven ATGL overexpression, providing more evidence for the specificity of ATGL downregulation in our experimental system. Interestingly, ATGL overexpression did not significantly affect lipid droplet levels (Fig. 13B). We observed only a slight decrease in the amount of neutral lipids in ATGL overexpressing cells upon co-transfection with siRNA. However, in both control and in $sPLA_2$-treated cells, ATGL overexpression cells caused a significant increase in the release of PGE_2 (Fig. 13C), suggesting that stimulation of ATGL-mediated lipolysis promotes PGE_2 synthesis in starving TNBC cells. Furthermore, overexpression of ATGL in ATGL-depleted cells almost fully restored PGE_2 production back to control levels, suggesting that ATGL is the predominant source for PGE_2 synthesis under lipolytic conditions.

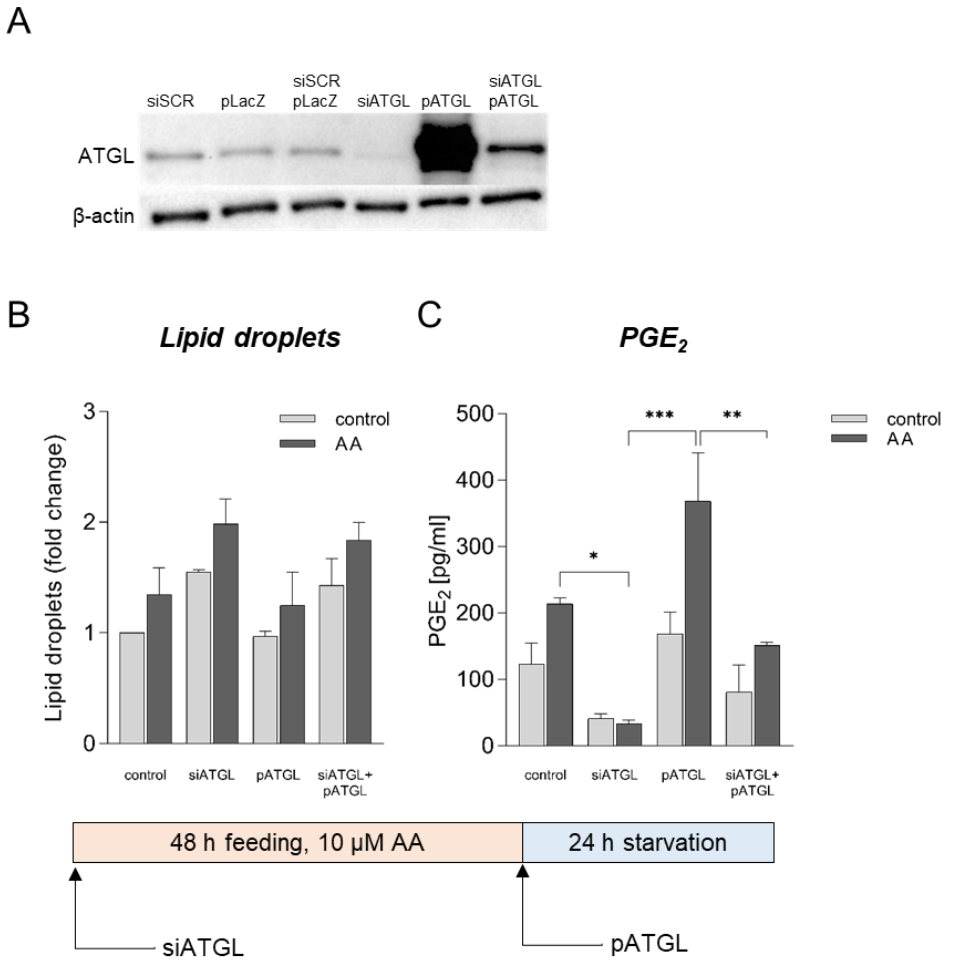

Figure 13: Stimulation of ATGL-mediated lipolysis augments PGE_2 synthesis in starving TNBC cells. (A–C) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) or non-targeting scrambled siRNA (siSCR) and treated with 10 µM AA for 48 h, and then transiently transfected with plasmid DNA containing ATGL cDNA (pATGL) and starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. (A) Cell lysates were analyzed for the presence of ATGL protein and β-actin by western blotting. Neutral lipid droplet content (B) was determined by Nile Red staining and flow cytometry and PGE_2 release (C) using a commercial enzymatic immunoassay. Values on the graphs are means ± SEM of at least three experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni adjustment). Plasmid containing the gene for β-galactosidase (pLacZ) was used as negative control of ATGL overexpression.

3.1.3.6 Starving breast cancer cells depleted of lipid droplets display impaired PGE_2 synthesis

The results presented above suggest that lipid droplets are sources for precursors that drive PGE_2 synthesis in starving TNBC cells. Promotion of neutral lipid storage by the addition of exogenous AA or hGX $sPLA_2$ followed by ATGL-mediated lipolysis leads to increased PGE_2 synthesis, suggesting the importance of both lipid droplet accumulation and lipid droplet breakdown. We next hypothesized that inhibiting TAG synthesis and thus lipid

droplet biogenesis would likely have the opposite effect on PGE_2 production. To elucidate the role of TAG synthesis, we inhibited DGAT1- and DGAT2-dependent TAG synthesis using the specific inhibitors T863 and PF-06424439 (Cao et al., 2011; Futatsugi et al., 2015). We determined lipid droplet content twice, after feeding and after starvation, in order to follow net lipid droplet biogenesis and lipolysis. Incubating TNBC cells with DGAT inhibitors during feeding led to a significant decrease in the amount of neutral lipids (measured at the end of the feeding phase) in both control and in AA-treated cells (Fig. 14A). Interestingly, DGAT inhibition during the feeding phase did not affect lipid droplet content following cell starvation, indicating that blocking TAG synthesis during feeding likely leads to its compensatory stimulation during starvation, resulting in increased lipid droplet accumulation by the end of the starvation phase (Fig. 14B). Interestingly, we have found that even when inhibitors were present during both phases of the experiment, their effect on lipid droplet levels is less prominent after starvation (data not shown; Petra Hruševar, MSc study in progress). Importantly, inhibition of lipid droplet biogenesis during feeding led to a blockade in PGE_2 production measured after the subsequent starvation period (Fig. 14C). Furthermore, it completely abolished AA-stimulated PGE_2 generation (Fig. 14C), suggesting that the esterification of FA precursors in lipid droplet-stored TAGs is a required step for lipid mediator production. Interestingly, ATGL depletion in the presence of DGAT inhibitors further reduced PGE_2 production, reaching lower levels than DGAT inhibition itself (Fig. 14D). One possible explanation is that the lipid droplets formed via the compensatory biogenesis mechanism suggested above also contribute to PGE_2 release. Nevertheless, this result further strengthens the fact that ATGL activity is necessary for PGE_2 production. Collectively, these results reveal that the presence of lipid droplets at the onset of starvation is necessary for the capacity of cells to produce lipid mediators during starvation and further establish lipid droplets and lipolysis as major sources of lipid mediators in TNBC cells.

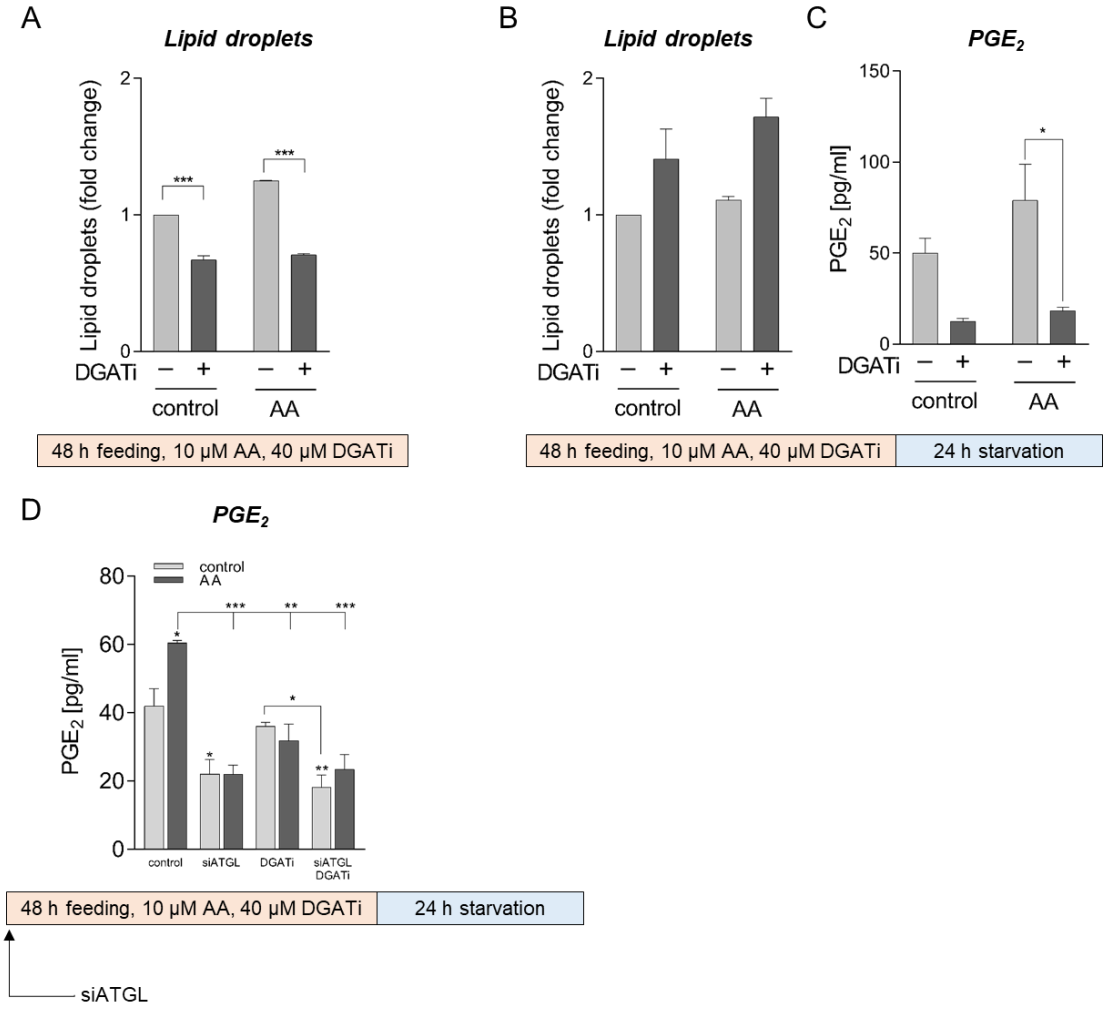

Figure 14: The presence of lipid droplets is necessary for the production of PGE_2 under lipolytic conditions in TNBC cells. (A–C) MDA-MB-231 cells were treated with 10 µM AA and/or 40 µM mixture in the ratio 1:1 of T863 DGAT1 and PF-06424439 DGAT2 inhibitors for 48 h in complete medium, and then serum-starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. (D) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) or non-targeting scrambled siRNA (SCR) and treated with 10 µM AA and/or 40 µM mixture in the ratio 1:1 of T863 DGAT1 and PF-06424439 DGAT2 inhibitors, and then serum starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. Neutral lipid content was determined by Nile Red staining and flow cytometry (A, B) and PGE_2 release with commercial enzymatic immunoassay (C, D). Values on the graphs are means ± SEM of at least three experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni adjustment).

3.1.3.7 The production of PGE_2 in starving TNBC cells is dependent on ATGL and $cPLA_2\alpha$ activities

Three possible pathways have recently been suggested to mediate the production of eicosanoids that derive from lipid droplet lipids, including the activity of ATGL and

cPLA$_2$α (Dichlberger et al., 2014). Besides directly contributing to eicosanoid synthesis, TAG-derived ATGL-liberated AA may be transiently incorporated in the lipid droplet phospholipid monolayer or other membranes and subsequently released by, as currently accepted, the major AA-releasing enzyme cPLA$_2$α. The involvement of cPLA$_2$α in liberating lipid droplet-derived AA for eicosanoid synthesis in breast cancer cells is possible, since cPLA$_2$α, and several other eicosanoid pathway enzymes, have been found localized to lipid droplets. Moreover, PGE$_2$ immunolocalization studies have also suggested that lipid droplets are sites for PGE$_2$ synthesis (Accioly et al., 2008; Bozza et al., 2011; Moreira et al., 2009; Murakami et al., 2011). Whether cPLA$_2$α releases AA from lipid droplets in TNBC cells and contributes to eicosanoid synthesis in cooperation with ATGL activity or independently is not known. Both enzymes may also work independently on their own respective target lipids and cooperate through unknown mechanisms. The results shown above indicate that ATGL is a major source of AA for PGE$_2$ synthesis in TNBC cells under lipolytic conditions. Here, we investigated the role of cPLA$_2$α in lipid droplet metabolism and PGE$_2$ synthesis in TNBC cells.

We first analyzed the expression of some of the major genes involved in lipid droplet turnover and the production of eicosanoids. Our qPCR analyses showed that starving TNBC cells express low levels of cPLA$_2$α (encoded by the *PLA2G4A* gene) and COX-2 (encoded by the *PTGS2* gene), while the expression of ATGL, COX-1, DGAT1 and DGAT2 (encoded by the *PNPLA2, PTGS1, DGAT1* and *DGAT2* genes, respectively) is substantially higher (Fig. 15A). Even though *PLA2G4A* gene expression was very low, we were able to further suppress its expression using siRNA (Fig. 15B). cPLA$_2$α depletion did not significantly change the amount of neutral lipids (Fig. 15C), but importantly, it slightly reduced PGE$_2$ production (Fig. 15D), indicating the involvement of cPLA$_2$α in the production of PGE$_2$ in starving TNBC cells. In comparison with the effect of ATGL depletion on PGE$_2$ synthesis, the effect of cPLA$_2$α depletion was less prominent (Fig. 15D). cPLA$_2$α overexpression did not affect lipid droplet content (Fig. 9C), but it slightly increased PGE$_2$ production (Fig. 15D), reaching a similar level to that found in ATGL overexpressing cells. TNBC cells depleted in both cPLA$_2$α and ATGL exhibited increased lipid droplet accumulation (Fig. 15C), more so than in cells depleted only of ATGL, suggesting that cPLA$_2$α somehow contributes to lipid droplet breakdown. These double knockdown cells displayed a significantly suppressed PGE$_2$ generation, but this was not lower than that observed in single ATGL knockdown cells (Fig. 15D). Conversely, overexpression of both cPLA$_2$α and ATGL augmented PGE$_2$ synthesis relative to control untransfected cells (Fig. 15D). These results suggest that PGE$_2$ synthesis in starving TNBC cells is mainly dependent on ATGL, but cPLA$_2$α also contributes to lipid mediator production, possibly by affecting lipid droplet breakdown through an as-yet-unknown mechanism.

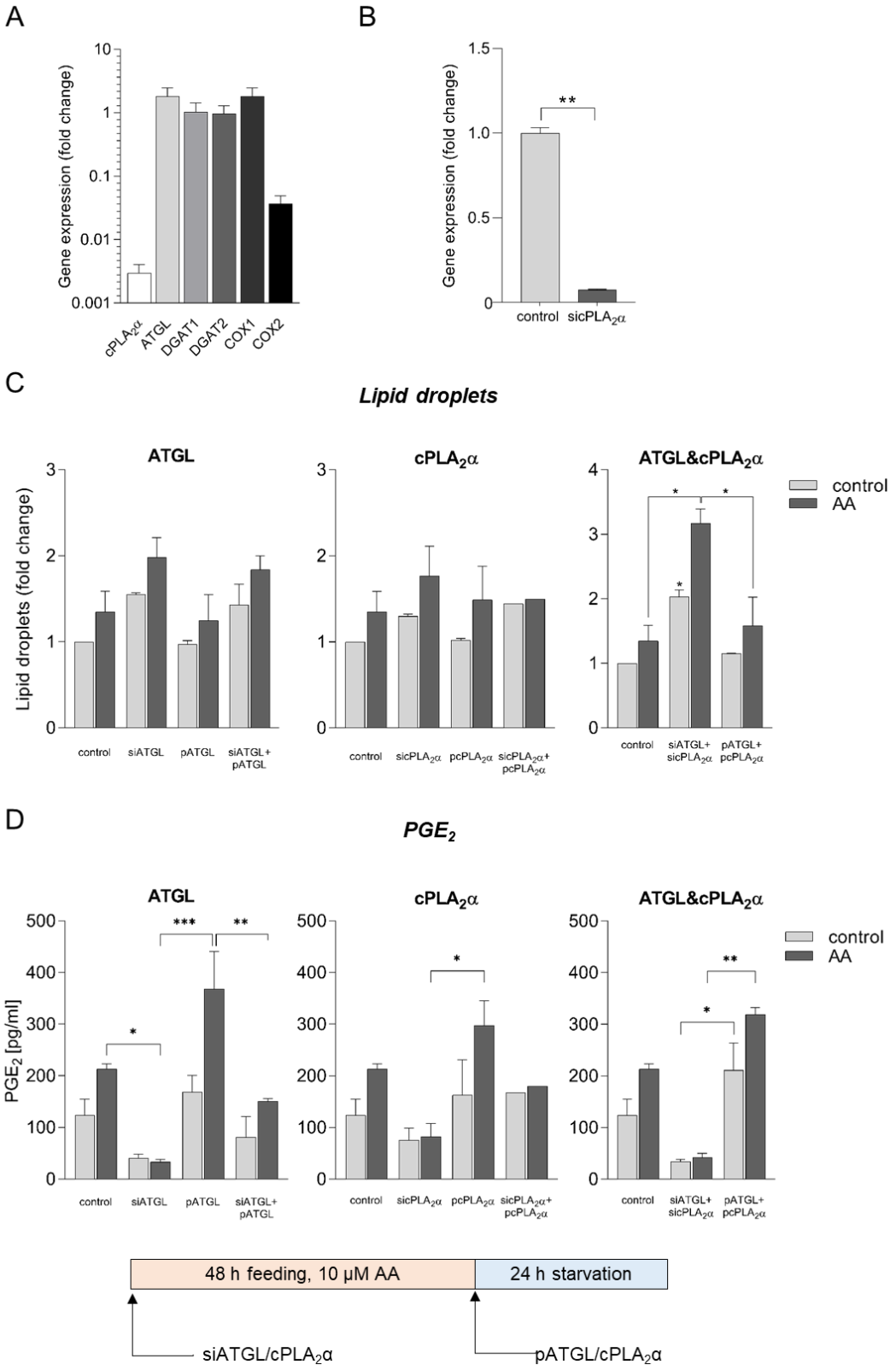

Figure 15: ATGL and cPLA$_2$α regulate PGE$_2$ production in starving TNBC cells. (A) MDA-MB-231 cells were seeded and cultured in complete medium for 48 h, and then serum-starved in RPMI-1640 medium containing 0.02% EFAF-BSA for 24 h. (B) MDA-MB-231 cells were reverse transfected with siRNA-targeting cPLA$_2$α (sicPLA$_2$α) or non-targeting siRNA (control) and cultured 48 h in complete medium, and then serum-

starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. (C, D) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) and/or siRNA-targeting cPLA$_2$α (sicPLA$_2$α) or non-targeting scrambled siRNA (control) and treated with 10 µM AA for 48 h in complete medium, and then transiently transfected with plasmid DNA containing ATGL (pATGL) and/or cPLA$_2$α (pcPLA$_2$α) cDNA and starved for 24 h in RPMI-1640 medium containing 0.02% EFAF-BSA. (A, B) The relative gene expression levels of target genes were determined by qPCR. Neutral lipid content was quantified by flow cytometry (C) and PGE_2 release determined with commercial enzymatic assay (D). Values on the graphs are means ± SEM of at least two experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; unpaired t-test (B); two-way ANOVA with Bonferroni adjustment (C, D)).

3.1.4 Discussion

Lipid droplets are central regulators of lipid uptake, metabolism, trafficking and signaling in the cell. Their functions in energy and redox homeostasis have been broadly recognized, whereas their roles in lipid signaling are less studied (Farese & Walther, 2009; Ohsaki, Suzuki, & Fujimoto, 2014; Olzmann & Carvalho, 2019; Petan et al., 2018; Welte, 2015; Welte & Gould, 2017). However, recent studies have implicated lipid droplets in a variety of signaling pathways involved in energy and redox homeostasis, transcriptional regulation, inter-organelle communication and the inflammatory response (Arrese, Saudale, & Soulages, 2014; Jarc & Petan, 2019b; Papackova & Cahova, 2015; Petan et al., 2018; Zechner et al., 2012). These features make lipid droplets important modulators of stress response mechanisms particularly in "challenged" cells that fight to survive in various nutrient- and oxygen-deprived conditions, such as those found in the tumor microenvironment (Henne et al., 2018; Petan et al., 2018).

Tumorigenesis is tightly linked with altered lipid metabolism and signaling. Emerging evidence suggests that alterations in the levels of pro-inflammatory lipids, such as eicosanoids, modulate cancer cell proliferation, apoptosis and migration, and also affect immune cell recruitment and activation (Wang & Dubois, 2010). However, the currently available non-steroidal anti-inflammatory drugs (NSAIDs), which mostly target the COX enzymes to block eicosanoid production, have a limited potential in cancer prevention and therapy due to adverse effects on the cardiovascular and gastrointestinal systems (Bessone, 2010; Kanikarla-Marie et al., 2018). Thus, there is a great need to discover new molecular mechanisms and targets, which may provide an effective way to reduce cancer-associated inflammation and limit the development and progression of cancer.

The production of eicosanoids depends on the release of their AA precursor from phospholipid membranes, involving the activity of cPLA$_2$α and some other PLA$_2$s (Dennis et al., 2011; Dennis & Norris, 2015). However, clinical trials of PLA$_2$ inhibitors for the management of inflammation have failed due to similar side effects to those displayed by NSAIDs, suggesting that other approaches should be considered. Recent studies have shown that cellular stores of neutral lipids are an important source of AA for eicosanoid synthesis in immune cells (Dichlberger et al., 2014; Schlager et al., 2015), suggesting the involvement of neutral lipases in the production of bioactive lipids. The activity of the major TAG lipase ATGL has been implicated as a source of precursors for the synthesis of eicosanoids, however, it is unclear whether ATGL somehow cooperates with cPLA$_2$α or independently affects eicosanoid generation (Dichlberger et al., 2014).

In this study, we focused on the role of lipid droplets and ATGL in the production of eicosanoids in highly tumorigenic TNBC cells. Our results suggest that lipid droplets are sources of PUFA precursors for the production of eicosanoids and other lipid mediators in

breast cancer cells. By releasing PUFAs from TAGs stored in lipid droplets upon starvation-induced stimulation of lipolysis, ATGL emerges as a major regulator of lipid mediator production in breast cancer cells. Moreover, cells depleted of lipid droplets were unable to produce eicosanoids during starvation, suggesting that the esterification of FA precursors in lipid droplet-stored TAGs is a required step for lipid mediator production. Interestingly, our results also implicate cPLA$_2\alpha$ in both lipid droplet metabolism and lipid mediator production in TNBC cells, suggesting a complementary role for cPLA$_2\alpha$ alongside ATGL in these processes.

3.1.4.1 Lipid droplets are sources of PUFA precursors for the synthesis of pro-inflammatory and pro-resolving lipid mediators in TNBC cells

We show here that starvation-stimulated breakdown of hGX sPLA$_2$-induced lipid droplets leads to the release of various pro-inflammatory and inflammatory lipid mediators in TNBC cells (Fig. 12). Our results provide the first evidence of lipid droplet-mediated lipid mediator production in cancer cells. The hypothesis of lipid droplet-mediated lipid mediator production has already been suggested by other authors, according to the evidence of: (1) inflammatory stimuli-dependent lipid droplet enrichment with AA, (2) co-localization of enzymes involved in eicosanoid synthesis with lipid droplets in various cells, including cancer cells, and (3) subcellular compartmentalization of lipid droplets and PGE$_2$ synthesis (Accioly et al., 2008; Bozza et al., 2011; Triggiani et al., 1995; Wooten et al., 2008). However, these studies have not demonstrated that lipid droplet-derived lipids, including neutral lipids in the core and phospholipids from the monolayer, are sources of AA for eicosanoid synthesis, nor have they connected the lipolytic molecular mechanisms of AA release with eicosanoid synthesis. We show that blocking DGAT-mediated TAG synthesis is necessary for PGE$_2$ synthesis (Fig. 14C), indicating that neutral lipids are sources of AA. In addition, we show that impaired TAG lipolysis not only reduces the production of PGE$_2$, but the production of lipid mediators in general (Fig. 11).

Our results are in line with recent breakthrough studies in immune cells, which suggested that TAG in lipid droplets are sources of AA for eicosanoid synthesis (Dichlberger et al., 2014; Schlager et al., 2015). In activated human mastocytes, TAG lipolysis is involved in the synthesis of PGD$_2$ and LTC$_4$ (Dichlberger et al., 2014), while in mouse neutrophils, it drives the secretion of lipid mediators that derive from all major enzymatic pathways, including COX-, LOX- and CYP-derivatives (Schlager et al., 2015). However, a recent study in human foam cells has suggested that neutral lipids, at least in these cells, are not the source of AA for lipid mediator signaling, but may rather constitute an expendable pool for storing excess AA that cannot be incorporated into the phospholipid pool, which exhibits a lower storage capacity and higher affinity for AA in comparison with the TAG pool (Guijas et al., 2019). The release of AA from TAGs in foamy macrophages was not detected even in conditions when cellular levels of AA in TAGs exceeded those in membrane phospholipids. Nevertheless, this study has several drawbacks. To establish whether membrane phospholipids or TAGs are sources of AA, mobilized by zymosan stimulation, inhibitors of cPLA$_2\alpha$ and ATGL activity were used. For ATGL inhibition, the cells were treated with a general inhibitor of patatin-like phospholipases, bromoenol lactone, which does not specifically inhibit only ATGL activity, but affects a wide-range of enzymes from the patatin-like family. Pharmacological agents can also induce various non-specific effects on cell function and metabolism, which may affect the final outcome. In addition, whether phospholipid-derived AA is a source for eicosanoid synthesis in these cells has not been addressed. Our results with ATGL-specific siRNA downregulation and ATGL overexpression strongly suggest that the TAG pool of lipid droplets in TNBC cells

is a major source of PUFAs for the synthesis of various lipid mediators that derive from all three major enzymatic pathways.

3.1.4.2 hGX sPLA$_2$ as a tool for studying lipid droplet metabolism in TNBC cells

In this study, we used recombinant hGX sPLA$_2$ as a tool for inducing lipid droplet synthesis in TNBC cells. Previous studies on the role of group X sPLA$_2$ in cancer revealed that exogenously added mouse group X sPLA$_2$ stimulates cancer cell proliferation through the ability of releasing a significant amount of AA, which leads to increased production of various eicosanoids, with PGE$_2$ being by far the most prominent product in colon cancer cells (Surrel et al., 2009). In contrast, we have found that hGX sPLA$_2$ stimulates cancer cell proliferation by another mechanism that is dependent on the release of fatty acids and lysophospholipids, but does not depend on the stimulation of PGE$_2$ generation (Pucer et al., 2013). Instead, hGX sPLA$_2$ prolongs serum-free survival of TNBC cells in an enzyme activity-dependent manner by driving lipid droplet biogenesis and altering lipid metabolism (Pucer et al., 2013). The hGX sPLA$_2$-induced lipid droplet formation was dependent on the ability of hGX sPLA$_2$ to hydrolyze membrane phospholipids in TNBC cells, leading to the release of various unsaturated FAs, including ω-6 and ω-3 PUFAs (Jarc, Kump, et al., 2018). Lipidomic analyses of hGX sPLA$_2$-induced lipid droplets revealed an enrichment of TAGs with long-chain PUFAs (Pucer, 2014). This suggests that the PUFAs released by hGX sPLA$_2$ are preferentially incorporated into growing lipid droplets. It is very likely that hGX sPLA$_2$ activity affects several mechanisms in lipid metabolism that could be relevant in cancer pathology, however, its effect on lipid droplets is particularly interesting.

3.1.4.3 Lipid droplets are the link between hGX sPLA$_2$ and lipid mediator synthesis

The group X sPLA$_2$ has been shown to release AA and other ω-3 PUFAs from cellular membranes of different intact cells and promote lipid mediator synthesis in various cell types, including lung epithelial cells, lung leukocytes, intestinal cells and cancer cells (Jarc & Petan, 2019a; Murakami, 2011). Importantly, the ability of murine GX sPLA$_2$ to deliver phospholipid membrane-derived precursors for lipid mediator synthesis that affect inflammation and tumorigenesis has also been demonstrated *in vivo* (Murase et al., 2016; Schewe et al., 2016). There have been some studies suggesting its involvement in lipid metabolism in adipogenesis in mice (Li, Shridas, Forrest, Bailey, & Webb, 2010). Historically, the activity of cPLA$_2$α has been considered the major source of AA for eicosanoid synthesis, along with the activities of some sPLA$_2$s and iPLA$_2$s, which have also been implicated in lipid mediator synthesis (Jarc & Petan, 2019a). Novel evidence suggests that PLA$_2$s, including group X sPLA$_2$ and cPLA$_2$α play an important role in lipid droplet metabolism (Guijas, Rodríguez, Rubio, Balboa, & Balsinde, 2014). This opens the possibility that sPLA$_2$- and cPLA$_2$α-mediated lipid mediator synthesis is associated with lipid droplets. cPLA$_2$α plays a regulatory role in lipid droplet synthesis and its activity may contribute to lipid droplet accumulation and eicosanoid synthesis in the liver, causing hepatosteatosis (Gubern et al., 2008; Ishihara et al., 2012). The group X sPLA$_2$ promotes lipid droplet accumulation in breast cancer cells, which enables cancer cells resistance to nutrient and oxidative stress (Jarc, Kump, et al., 2018; Pucer et al., 2013). Its activity also supports cancer progression by mediating the release of AA and eicosanoid synthesis in colon cancer cells (Surrel et al., 2009). Furthermore, elevated expression of the group X sPLA$_2$ and of eicosanoid synthesis enzymes, along with increased levels of PUFA-TAGs have been linked with inflammation in visceral adipose tissue (Liesenfeld et al., 2015). So far there have not been any suggestions or evidence that would link lipid mediator production induced by any (s)PLA$_2$ with lipid droplet metabolism.

In this study we provide the first evidence of an association between hGX $sPLA_2$ activity, lipid droplets and lipid mediator synthesis in TNBC cells. We show that treating TNBC cells with AA or hGX $sPLA_2$ leads to lipid droplet biogenesis and that starvation-induced breakdown of these lipid droplets drives lipid mediator production (Figs. 7, 8, 10). Importantly, our lipidomic analyses enabled the detection of a wide range of lipid mediators, including AA-, DHA- and EPA-derived metabolites. The latter were found to be the most abundant products derived from the breakdown of hGX $sPLA_2$-induced lipid droplets (Fig. 10A). We have previously reported that the major TAG lipase ATGL is not essential for the pro-survival effects of hGX $sPLA_2$ in TNBC cells (Jarc, Kump, et al., 2018), but the results of this study reveal that it is crucial for hGX $sPLA_2$ induced lipid mediator synthesis (Fig. 12), including that of the pro-tumorigenic PGE_2 (Figs. 8B and 12B). These findings indicate that hGX $sPLA_2$- elicited production of PGE_2 and other lipid mediators requires intact lipid droplet metabolism in breast cancer cells. Given the ability of hGX $sPLA_2$ to act on the plasma membranes of most mammalian cells and induce lipid mediator production, this mechanism is very likely to occur in other cell types and tissues as well and could provide novel interpretations for numerous previous studies in the field.

3.1.4.4 Besides PGE_2, 15-LOX metabolites are the most abundant products of hGX $sPLA_2$ activity in TNBC cells

The expression of COX, 5-, 12- and 15-LOX enzymes in aggressive TNBC cells is significantly higher in comparison with that in less tumorigenic breast cancer cell lines (Jiang, Douglas-Jones, & Mansel, 2003). Relatively high amounts of COX-derived PGE_2 were released from TNBC cells under lipolytic conditions (Fig. 10D), and these were further augmented upon the addition of hGX $sPLA_2$. We show that the mRNA expression of the *PTGS1* gene (encoding the COX-1 protein) is higher in comparison with *PTGS2* (COX-2 protein) (Fig. 15A), suggesting a predominant role for COX-1 in the synthesis of PGE_2 in these conditions. PGE_2 is the most abundant prostaglandin species found in various cancers and has an important role in promoting cancer cell growth and proliferation (Wang & Dubois, 2010). To establish the effect of increased PGE_2 levels on the proliferation of TNBC cells, we performed additional experiments and preliminary results indicate that exogenous PGE_2 stimulates TNBC proliferation also in our experimental system (data not shown). However, more experiments are necessary to determine whether lipid droplet breakdown contributes to cell proliferation via the synthesis of PGE_2.

Interestingly, our results show that the breakdown of hGX $sPLA_2$-induced lipid droplets has the most prominent effect on 15-LOX (encoded by the *ALOX15* gene) (Fig. 10B) and 12-LOX (encoded by the *ALOX12* gene) metabolites. Since the 12/15-LOX enzymatic pathway is responsible for the conversion of different precursors, including AA, EPA and DHA, into lipid mediators (Fig. 9), a wide range of effects on cellular signaling and function is to be expected. To date, not many studies have addressed the effects of individual 12/15-LOX metabolites on cancer cells, but *in vivo* evidence suggests that 12-HETE facilitates metastasis of breast tumor xenografts in mice, while metastasis-free survival inversely correlates with the expression of the *ALOX15* gene in tumor cells (Kerjaschki et al., 2011). We observed an increase in the levels of several HDHA, HETE and HEPE metabolites, including 12-HETE, but intriguingly, the release of 14-HDPA was the most prominent (Fig. 10F), DPA is an intermediate in the conversion of EPA to DHA. It has been found in numerous mammalian tissues and it has been proposed to be a precursor for the synthesis of novel immunoresolvents, including $resolvins_{n\text{-}3\ DPA}$ and $maresins_{n\text{-}3\ DPA}$, that share similar functionality with DHA-derived SPMs. Indeed, their production could compensate for a

lower production of DHA-derived SPMs, when DHA availability is reduced, and may enable an efficient resolution of inflammation (Dalli et al., 2013).

Recent evidence suggests an anti-tumorigenic activity of DHA- and EPA-derived SPMs, which counter tumor growth by promoting phagocytosis and suppressing the synthesis of pro-inflammatory cytokines (Sulciner, Serhan, et al., 2018). Our results show that the production of DHA-derived and EPA-derived SPMs in TNBC cells is barely detectable and does not change dramatically by the addition of hGX sPLA$_2$ (Fig. 10G). Among other lipid mediators, we observed a significant increase in the production of 18-HEPE and 12-HHT, which seem to exhibit opposite effects in cancer. EPA-derived 18-HEPE has been shown to protect from melanoma pulmonary metastasis, indicating an anti-tumorigenic activity (Li et al., 2018). On the contrary, 12-HHT-mediated signaling has been implicated in chemotherapy resistance. Splenic macrophages have been shown to mediate chemoresistance in mouse tumor xenograft models by producing 12-HHT following treatments with exogenous platinum-induced fatty acids (PIFAs) (Houthuijzen et al., 2014). PIFA synthesis was shown to be induced by platinum analogues of chemotherapeutics (Roodhart et al., 2011). Genetic loss or chemical inhibition of the leukotriene B4 receptor (BLT2), which specifically binds 12-HHT, prevented 12-HHT-mediated resistance to chemotherapy, suggesting that 12-HHT-mediated resistance is dependent on LTB4 signaling (Houthuijzen et al., 2014). Furthermore, combined therapy of indomethacin, a COX inhibitor, and platinum-based chemotherapy effectively suppressed 12-HHT levels in patients' plasma, suggesting a mechanism that may improve chemosensitivity (van der Velden et al., 2018).

3.1.4.5 ATGL activity is essential for the production of PGE_2 and other lipid mediators in starving TNBC cells

Lipolytic lipid droplet breakdown requires the activity of ATGL, which preferentially hydrolyses TAGs, leading to the release of FAs that may be used for energy production in mitochondria (Rambold et al., 2015) and bind to nuclear receptors, thereby also affecting the transcriptional regulation of mitochondrial energy metabolism (Haemmerle et al., 2011; Zechner et al., 2012). These two fundamentally different, but complementary fates of ATGL-released FAs reveal that the enzyme is important for the integration of metabolic and signaling pathways in the cell. The role of ATGL in cancer is currently not clear and is even controversial, with a pro-tumorigenic role suggested by several in vitro studies and a tumor suppressor role suggested by some in vivo reports (Petan et al., 2018). Nevertheless, current studies on the role of ATGL and lipolysis in cancer are largely focused on their metabolic effects, whereas the signaling aspects of ATGL-mediated lipolysis are still largely neglected. Our results provide the first evidence that ATGL-mediated lipolysis is involved in lipid mediator production in TNBC cells (Fig. 11), revealing a link between ATGL activity and lipid mediator signaling in cancer. These results support the findings in non-cancer cells, including immune (Dichlberger et al., 2014; Schlager et al., 2015) and vascular endothelial cells (Riederer et al., 2017), which first reported that ATGL activity is involved in lipid mediator production by releasing precursors for their synthesis. The idea that lipid droplets are sources of precursors for lipid mediator production is not limited only to the ATGL activity, but extends to other neutral lipases (Gartung et al., 2016) and processes. Recent evidence suggests that lysosomal acid lipase contributes to lipid mediator synthesis in macrophages (Schlager et al., 2017), revealing that lysosomes release TAG-derived FAs that may be used as precursors of lipid mediators. Indeed, lysosomes are also sites of lipid droplet breakdown in the process of autophagy, i.e. lipophagy (Zechner et al., 2017), possibly uncovering yet another mechanism that links lipid droplets with lipid

mediator synthesis. Our recent results indicate that ATGL lipolysis is a predominant, but probably not the sole pathway of lipid droplet breakdown in TNBC cells (Jarc, Kump, et al., 2018). We show here that ATGL depletion significantly reduces the synthesis of a wide range, but not all of lipid mediators (Fig. 11), suggesting that other processes also provide precursors for lipid mediator synthesis when ATGL is missing.

Among COX-derived metabolites, we found a significant decrease in the release of the most abundant prostaglandin in TNBC cells, PGE_2, in addition to reduced levels of PGD_2, $PGF_{2\alpha}$ and TXB_2 (Fig. 11B). ATGL depletion also abolished the effect of hGX $sPLA_2$-induced changes in PGE_2 and PGD_2 levels (Fig. 12B). With the exception of 5-HETE, the release of other metabolites of 5-LOX enzymatic pathway was compromised by ATGL depletion (Fig. 11C). The addition of 5-HETE to breast cancer cells stimulates cancer cell proliferation, while inhibition of 5-LOX activity results in reduced cell growth and increased apoptosis (Avis et al., 2001). Interestingly, the activity of hGX $sPLA_2$ also did not alter the production of 5-HETE (Fig. 12C), suggesting other enzymes and/or processes to regulate its generation.

ATGL depleted cells displayed a reduced release of several 12/15-LOX metabolites, including 7-HDHA, 4-HDHA, 8-HETE and 15-HEPE (Fig. 11D). Surprisingly, ATGL depletion led to an increased release of 14-HDHA in control cells (Fig. 11D), but it abolished the effect of hGX $sPLA_2$ on increased 14-HDHA synthesis (Fig. 12D). We cannot explain this discrepancy and further analyses would be needed to confirm or deny this finding. ATGL depletion also led to reduced levels of the most abundant 12/15-LOX-derived products of the hGX $sPLA_2$ activity (Fig. 12D), suggesting the importance of lipid droplets and ATGL lipolysis for the hGX $sPLA_2$-induced changes on lipid mediator synthesis. Finally, blocking ATGL lipolysis resulted in reduced release of several CYP-derived metabolites and SPM species, including resolvins and lipoxins (Fig. 11E), and in completely abrogated induction of their synthesis by hGX $sPLA_2$ (Fig. 12E). The release of some resolvins, such as RvE1 and RvE3, was not affected by ATGL depletion (Fig. 11E), nor hGX $sPLA_2$ activity (Fig. 12E). These results suggest that the production of some lipid mediators may occur through distinct mechanisms and may not respond to hGX $sPLA_2$ and ATGL activities.

3.1.4.6 Both ATGL and cPLA$_2\alpha$ affect lipid droplet turnover and participate in lipid droplet-mediated lipid mediator synthesis

Several studies have reported that upon cell activation large amounts of AA and other PUFAs are incorporated into TAGs stored in lipid droplets (Bozza et al., 2011; Shmarakov, Jiang, Liu, Fernandez, & Blaner, 2019), leading to lipid droplet breakdown that stimulates the production of various lipid mediators (Dichlberger et al., 2014; Shmarakov et al., 2019). Two distinct pools of lipids stored in lipid droplets have been suggested to be the sources of AA for eicosanoid synthesis, including phospholipids of the lipid droplet monolayer and TAGs in the neutral lipid core (for extended discussion on this topic, see our recent review paper Jarc & Petan, 2019a). cPLA$_2\alpha$ mainly releases AA from perinuclear membranes and the ER, but it has also been localized on lipid droplets in immune cells (Bozza et al., 2011), suggesting that it may catalyze the release of AA from glycerophospholipids that constitute the lipid droplet monolayer. A recent study has proposed several options of lipid droplet-mediated eicosanoid synthesis in immune cells, suggesting that ATGL-released, TAG-derived AA is either directly or indirectly used for eicosanoid synthesis (Fig. 16).

The indirect pathway includes a possibility that ATGL-released AA is transiently re-esterified back into the phospholipid monolayer of lipid droplets and then released by cPLA$_2\alpha$ activity (Dichlberger et al., 2014). As a third option, cPLA$_2\alpha$ could also directly provide phospholipid-derived AA by acting on the lipid droplet monolayer in a process,

which is somehow dependent on ATGL (since it is affected by ATGL knockdown (Dichlberger et al., 2014)), but without the involvement of TAG-derived AA. Our results suggest that both ATGL and cPLA$_2$α are involved in PGE_2 production, but ATGL plays a predominant role (Fig. 15D). Interestingly, the effect of single gene depletion of either ATGL or cPLA$_2$α on lipid droplet levels was similar, while double knockdown of both enzymes led to a significantly elevated lipid droplet accumulation (Fig. 15C), suggesting that both enzymes are involved in lipid droplet breakdown in TNBC cells. These results are in line with the proposed mechanisms in immune cells (Dichlberger et al., 2014), but they also suggest a fourth option: cPLA$_2$α could facilitate ATGL-mediated lipolysis, e.g. possibly by hydrolyzing the phospholipid monolayer on lipid droplets, thereby increasing membrane fluidity and providing an easier access for ATGL to the neutral lipid core underneath the monolayer (Fig. 16). Moreover, other authors have suggested that cPLA$_2$α is also involved in lipid droplet biogenesis in severely nutrient-deprived cells (Gubern et al., 2009, 2008; Guijas et al., 2014). Whether cPLA$_2$α is involved in lipid droplet biogenesis in serum-starved TNBC cells remains to be determined, but it is worth to speculate that the enzyme regulates lipid droplet turnover by different mechanisms that depend on the different metabolic states and requirements of the cell. It is also very likely that ATGL and cPLA$_2$α cooperate and adapt their effects on lipid droplets accordingly.

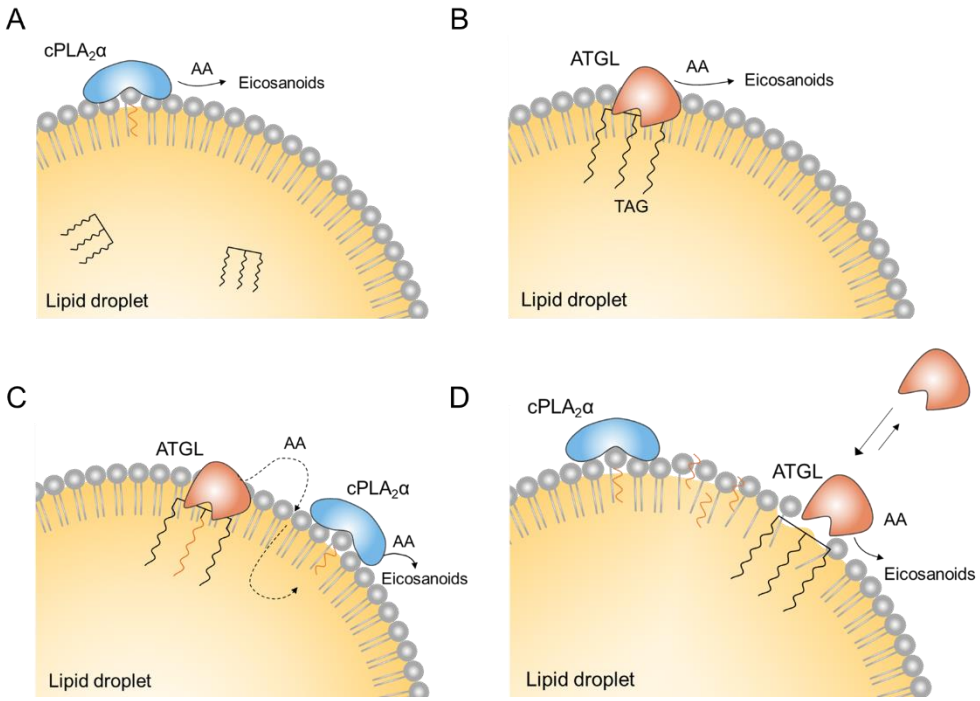

Figure 16: Proposed mechanisms of ATGL- and cPLA$_2$α-mediated release of lipid droplet-derived precursors for lipid mediator synthesis. The release of arachidonic acid (AA) from lipid droplets may occur through distinct mechanisms, which collectively or individually contribute to COX- and LOX-driven eicosanoid synthesis. At least four different options are possible (e.g. mechanisms when cPLA$_2$α acts on other membranes have not been depicted here). (A) cPLA$_2$α activity could directly promote eicosanoid synthesis by releasing AA from the phospholipid monolayer of the lipid droplet. ATGL-mediated lipolysis could affect eicosanoid synthesis by releasing AA from TAG directly (B) or indirectly (C), whereby TAG-derived AA is transiently re-esterified into the phospholipid monolayer and is subsequently released by cPLA$_2$α activity. (D) cPLA$_2$α could also facilitate ATGL-mediated hydrolysis by hydrolyzing the phospholipid monolayer, thereby increasing membrane fluidity and providing an easier access for ATGL to the neutral lipid core and TAGs.

3.1.5 Conclusion

Lipid droplets are emerging as sources of bioactive lipids. The synthesis of eicosanoids has been traditionally linked with the activity of cPLA$_2$α on phospholipid membranes, leading to the release of AA for eicosanoid generation. Novel evidence suggests that TAG lipids stored in lipid droplets of immune cells represent an important source of AA that may complement the classical pathway of eicosanoid synthesis by ATGL-mediated TAG lipolysis (Dichlberger et al., 2014; Schlager et al., 2015). Our results strongly suggest that lipid droplets in TNBC cells are major sources of AA and other PUFA precursors for the synthesis of various pro-inflammatory eicosanoids and pro-resolving specialized mediators that derive from COX, LOX and CYP enzymatic pathways. We have found that both cPLA$_2$α and ATGL affect lipid droplet turnover in breast cancer cells, leading to changes in lipid mediator generation. Furthermore, we have found that the activity of hGX sPLA$_2$ in TNBC cells leads to the release of various lipid mediators, with a predominant release

of EPA-derived metabolites, but it requires intact lipid droplet metabolism. Targeting lipid droplet turnover may thus represent a novel strategy for reducing inflammation and inflammation-related tumorigenesis.

Chapter 4

Publications

4.1 Lipid Droplets Induced by Secreted Phospholipase A_2 and Unsaturated Fatty Acids Protect Breast Cancer Cells from Nutrient and Lipotoxic Stress

In this part, the paper "Lipid droplets induced by secreted phospholipase A_2 and unsaturated fatty acids protect breast cancer cells from nutrient and lipotoxic stress" by Eva Jarc, Ana Kump, Petra Malavašič, Thomas O. Eichmann, Robert Zimmermann and Toni Petan is presented. The paper was published in *Biochimica et Biophysica Acta – Molecular and Cell Biology of Lipids* in 2018. This paper represents the major research work of the thesis.

Our previous studies showed that the activity of human group X secreted phospholipase A_2 ($sPLA_2$) on triple-negative breast cancer (TNBC) cells leads to lipid droplet accumulation. The breakdown of lipid droplets supports TNBC cell survival during prolonged nutrient starvation, but the mechanism remains unknown (Pucer et al., 2013). In this study, we found that the activity of $sPLA_2$ on TNBC cells leads to the release of unsaturated and polyunsaturated FAs from cellular membranes, and that occurs even in the presence of serum lipoproteins, which are also targets for $sPLA_2$ activity. Importantly, we found that the major TAG lipase ATGL is involved in TAG lipolysis in TNBC cells exposed to prolonged nutrient starvation. Interestingly, its depletion was not sufficient to fully prevent lipid droplet breakdown and significantly reduce the pro-survival effect of lipid droplets, suggesting the involvement of other lipases and/or lipophagy in starvation-induced lipid droplet breakdown in TNBC cells (Jarc, Kump, et al., 2018).

Mechanisms that control FA trafficking and their use by cancer cells are poorly understood. Recent studies suggest that lipid droplets are central regulators of FA metabolism. By transiently storing FAs into lipid droplet core, DGAT-mediated lipid droplet biogenesis protects cells from lipotoxicity of exogenous and endogenous FAs (Chitraju et al., 2017; Listenberger et al., 2003; Nguyen et al., 2017). On the contrary, the precise regulation of ATGL lipolysis is essential for protection against lipid droplet breakdown-mediated lipotoxicity (Aflaki et al., 2011; Fuchs et al., 2012). Polyunsaturated fatty acids (PUFAs), in particular ω-3 PUFAs, have been implicated in cancer therapy due to their potential anti-tumorigenic effects, but their intracellular mobilization and effect on cancer cell survival are not clear (D'Eliseo & Velotti, 2016). The results of this study revealed that the effect of ω-3 PUFAs on breast cancer cells is concentration-dependent, with a pro-tumorigenic effect at low micromolar concentrations and lipotoxic effect at high micromolar concentrations (Jarc, Kump, et al., 2018). Furthermore, we found that starvation-induced breakdown of PUFA-rich lipid droplets, but not lipid droplets

induced by monounsaturated FAs, such as oleate and linoleate, is associated with an ATGL-dependent increase in the production of reactive oxygen species (ROS), leading to oxidative stress-dependent cell death. Importantly, we found that TNBC cells are protected from PUFA lipotoxicity when PUFAs are sequestered in lipid droplets, an effect that was dependent on DGAT1 and ATGL activities on lipid droplet turnover. We demonstrated that interfering with DGAT1 activity augments the effect of PUFA-induced cell damage, while stimulation of lipid droplet biogenesis reduces PUFA lipotoxicity. Furthermore, we showed that blocking ATGL activity reduces PUFA lipotoxicity by preventing PUFA release from lipid droplets.

According to these findings, we have suggested two mechanisms of protection from lipotoxicity mediated by lipid droplets. The first includes the activity of $sPLA_2$, which mitigates PUFA lipotoxicity by stimulating lipid droplet biogenesis and enriching lipid droplets with FAs with a low unsaturation level, which results in the reduction in PUFA-TAG content and reduces the release of PUFA from lipid droplets through "PUFA dilution". The second mechanism includes "PUFA retention" in lipid droplets mediated by the suppression of lipid droplet breakdown in cells depleted of ATGL. We found that ATGL depletion does not affect TAG composition, suggesting that the reduction in PUFA lipotoxicity is based on overall reduction in lipolysis.

Collectively, our results suggest that lipid droplets in TNBC cells act as antioxidants and protect cells from nutrient and oxidative stress. In this paper, I contributed to most of the results with a focus on lipidomic analyses, which were done in the collaboration with Dr. Thomas O. Eichmann and Dr. Robert Zimmermann from the University of Graz, qPCR gene analysis, optimizing ATGL depletion with siRNA and performing several experiments to determine the underlying effects of ATGL depletion on lipid droplet content, cell death and ROS generation. I also performed experiments, studying the role of β-oxidation and DGAT activity on lipid droplets and cell survival. To follow changes in glycerol release and in the amount of TAG, CE and cholesterol in our samples, I performed several biochemical assays. I evaluated most of the results by statistical analysis. Finally, I contributed to the preparation of the manuscript for publication by preparing final figures and writing figure legends, material and methods and actively participating in the final submission of the manuscript. During the revision process, I contributed by performing additional experiments and providing final results for publication.

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Lipid droplets induced by secreted phospholipase A_2 and unsaturated fatty acids protect breast cancer cells from nutrient and lipotoxic stress

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ABSTRACT

Cancer cells driven by the Ras oncogene scavenge unsaturated fatty acids (FAs) from their environment to counter nutrient stress. The human group X secreted phospholipase A_2 (hGX sPLA$_2$) releases FAs from membrane phospholipids, stimulates lipid droplet (LD) biogenesis in Ras-driven triple-negative breast cancer (TNBC) cells and enables their survival during starvation. Here we examined the role of LDs, induced by hGX sPLA$_2$ and unsaturated FAs, in protection of TNBC cells against nutrient stress. We found that hGX sPLA$_2$ releases a mixture of unsaturated FAs, including ω-3 and ω-6 polyunsaturated FAs (PUFAs), from TNBC cells. Starvation-induced breakdown of LDs induced by low micromolar concentrations of unsaturated FAs, including PUFAs, was associated with protection from cell death. Interestingly, adipose triglyceride lipase (ATGL) contributed to LD breakdown during starvation, but it was not required for the pro-survival effects of hGX sPLA$_2$ and unsaturated FAs. High micromolar concentrations of PUFAs, but not OA, induced oxidative stress-dependent cell death in TNBC cells. Inhibition of triacylglycerol (TAG) synthesis suppressed LD biogenesis and potentiated PUFA-induced cell damage. On the contrary, stimulation of LD biogenesis by hGX sPLA$_2$ and suppression of LD breakdown by ATGL depletion reduced PUFA-induced oxidative stress and cell death. Finally, lipidomic analyses revealed that sequestration of PUFAs in LDs by sPLA$_2$-induced TAG remodelling and retention of PUFAs in LDs by inhibition of ATGL-mediated TAG lipolysis protect from PUFA lipotoxicity. LDs are thus antioxidant and pro-survival organelles that guard TNBC cells against nutrient and lipotoxic stress and emerge as attractive targets for novel therapeutic interventions.

1. Introduction

Tumours display metabolic alterations that maximize their ability to survive and proliferate during periods of stress [1]. Apart from the dependence of many cancer types on glucose and glutamine, emerging evidence points to alterations in lipid metabolism pathways that promote tumour growth, including fatty acid (FA) synthesis, β-oxidation, as well as phospho- and neutral lipid metabolism [2–6]. Recent studies have shown that certain cancer cells use opportunistic modes of nutrient acquisition to reduce stress and maintain growth and proliferation in nutrient- and oxygen-poor environments [1,7]. They increase the uptake of exogenous protein and lipid molecules through macropinocytosis to reduce their dependence on *de novo* synthesis [7,8]. Cancer cells exposed to hypoxia or driven by the Ras oncogene upregulate lysophosphatidylcholine internalization to extract FAs and compensate for the deficiency in endogenous unsaturated FAs [9]. Constitutive mammalian target of rapamycin (mTOR) activity during hypoxia also renders cancer cells dependent on exogenous unsaturated

Abbreviations: AA, arachidonic acid; AMPK, AMP-dependent protein kinase; ATGL, adipose triglyceride lipase; cPLA$_2$α, cytosolic group IVA PLA$_2$; CPT1, carnitine *O*-palmitoyltransferase 1; CPT1A, the liver isoform of CPT1; DGAT1, diacylglycerol acyltransferase 1; DHA, docosahexaenoic acid; EPA, eicosapentaenoic acid; FA, fatty acid; FAF, fatty acid-free; HBSS, Hanks' balanced salt solution; hGX sPLA$_2$, human group X sPLA$_2$; LA, linoleic acid; LD, lipid droplet; MMP, mitochondrial membrane potential; NAC, *N*-acetyl-L-cysteine; OA, oleic acid; PC, phosphatidylcholine; PLA$_2$, phospholipase A$_2$; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; SCD-1, stearoyl-CoA desaturase 1; sPLA$_2$, secreted phospholipase A$_2$; SREBP-1, sterol regulatory element-binding protein-1; TAG, triacylglycerol; TNBC, triple-negative breast cancer; TMRM, tetramethylrhodamine, methyl ester; VLCAD, very long-chain acyl-CoA dehydrogenase

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FAs [10]. Breast cancer and glioblastoma cells upregulate FA uptake and increase lipid droplet biogenesis to enable cell survival during hypoxia [11]. Furthermore, mutant Ras-driven lung cancer development is dependent on FA uptake, lipid droplet formation and β-oxidation mediated by acyl-CoA synthetase long chain 3 (ACSL3) [12]. Thus, constitutive activation of growth-promoting pathways in hypoxic and nutrient deprived cancer cells often leads to the dependence on exogenous FAs for cancer cell survival. This critical metabolic vulnerability of cancer cells is a viable therapeutic target and it is important to identify the sources of unsaturated lipids, the mechanisms of acquisition from their microenvironment and the cellular processes that are crucial for their utilization [8].

Lipid droplets (LDs), long regarded as inert storage depots of metabolic fuel and membrane building blocks, have emerged recently as new organelles integrating cellular lipid metabolism and signalling, FA trafficking, protein management and quality control, viral replication and immunity [13]. LDs store neutral lipids, mostly triacylglycerol (TAG) and cholesterol esters, and balance FA uptake, storage and use according to cellular needs. Increased accumulation of neutral lipids in LDs has been demonstrated in several cancers, including breast, brain and colon cancer [4,14]. Interestingly, LD formation is induced in different stressful conditions, including nutrient excess and deprivation, hypoxia and oxidative stress [4]. In most cells exposed to excess exogenous or endogenous FAs, LDs act as transient buffers that reduce FA lipotoxicity [15]. Thus, interfering with TAG synthesis by depletion of diacylglycerol acyltransferase 1 (DGAT1), one of the two DGAT isoforms catalysing the final and dedicated step in TAG synthesis [16], leads to cell death in fibroblasts exposed to exogenous oleate [15]. On the other hand, LDs provide fuel for mitochondrial oxidative metabolism and enable cell survival during nutrient deprivation [17,18]. Bailey et al. [19] have recently shown that LDs can also act as antioxidant organelles that protect *Drosophila* neural stem cells from oxidative damage by storing polyunsaturated FAs (PUFAs) in the form of neutral lipids. They found that DGAT1-mediated LD formation was required for reducing oxidative stress and PUFA peroxidation in these cells [19]. LDs can also protect cancer cells from oxidative damage, since interfering with FA uptake and LD formation in hypoxic cancer cells reduces their ability to sustain oxidative stress [11].

Relatively little is known about the roles of LD lipolysis in the regulation of oxidative stress, lipotoxicity and cancer cell survival during stress [4,20]. Adipose triglyceride lipase (ATGL) catalyses the rate-limiting step in TAG hydrolysis and is the major TAG hydrolase in adipose tissue, heart, liver, and other tissues [21–23]. ATGL depletion leads to growth inhibition in several cancer cell lines [24,25], suggesting a pro-tumourigenic role for the enzyme. Additionally, recent studies suggest that both tumour- and adipose tissue lipolysis, and ATGL in particular, contribute to the adipocyte-induced metabolic reprogramming and aggressiveness of breast, pancreatic and ovarian cancer [26–29]. The enzyme is overexpressed in high grade mammary tumours and its activity in breast cancer cells is important for invasiveness in the presence of adipocytes [29]. However, a tumour suppressor role for ATGL has also been suggested, since its expression is reduced in several human cancers, including breast invasive adenocarcinoma, and it correlates with reduced patient survival [30]. Furthermore, mice lacking ATGL spontaneously develop lung tumours [30], and adipose tissue-specific combined deletion of ATGL and hormone-sensitive lipase (HSL) genes leads to the development of liposarcoma [31]. While its role in cancer is still controversial, the importance of ATGL in organismal and cellular energy metabolism is well accepted. The enzyme has a crucial role in supplying skeletal muscle with FAs from adipose tissue [32]. It enables FA transfer from LDs to mitochondria in cardiomyocytes, hepatocytes, and in starved mouse embryonic fibroblasts [22,33]. Additionally, ATGL-mediated lipolysis promotes signalling pathways leading to elevated mitochondrial and oxidative gene expression thus matching β-oxidation with FA mobilization from LDs in several tissues [22,34,35]. The absence of ATGL-mediated lipolysis protects hepatic cells from FA-induced ER stress [36], while stimulation of lipolysis by ATGL overexpression in cardiomyocytes leads to lipotoxicity and ER stress [37]. On the other hand, ATGL deficiency in macrophages leads to TAG accumulation and cell death associated with ER stress, elevation of reactive oxygen species (ROS), and mitochondrial damage [38]. Clearly, the balance between LD formation and LD breakdown is critical for mitochondrial metabolism and the cellular response to stress, and the role of ATGL in regulating LD lipolysis and (cancer) cell fate may vary depending on the tissue involved and the pathophysiological context.

Phospholipase A_2 (PLA_2) enzymes catalyse the hydrolysis of the *sn*-2 ester bond in glycerophospholipids [39], releasing free FAs and lysophospholipids. Several intracellular PLA_2s, including the Ca^{2+}-dependent cytosolic group IVA PLA_2 (cPLA$_2\alpha$) and the Ca^{2+}-independent group VIA PLA_2 (iPLA$_2\beta$) have been implicated in the regulation of LD formation and TAG synthesis during stress, respectively [40–42]. Secreted phospholipase A_2 (sPLA$_2$) enzymes act in the extracellular environment on healthy and apoptotic cells, microvesicles, lipoproteins and other membranes [43,44]. The group X sPLA$_2$ enzyme is the most potent among mammalian sPLA$_2$s in hydrolysing phosphatidylcholine (PC), the most abundant phospholipid in mammalian cell membranes and lipoproteins. Recent studies in mice have shown that the enzyme releases a mixture of unsaturated FAs, including ω-3 PUFAs [45], and exerts a protective role against inflammation [45], atherosclerosis [46] and obesity [47]. In the murine colon, the group X sPLA$_2$ displays a context-dependent role in modulating both inflammation and tumorigenesis [48]. The enzyme is overexpressed in several cancers in humans, including invasive breast carcinoma [49,50], and it is secreted from immune cells, such as leukocytes, neutrophils and macrophages [46,51,52]. *In vitro*, it stimulates breast and colon cancer cell proliferation by a mechanism that is dependent on its enzymatic activity [18,53]. Recently, we have shown that the human group X (hGX) sPLA$_2$ induces LD formation in Ras-driven MDA-MB-231 triple-negative breast cancer (TNBC) cells and promotes their survival during nutrient stress [18]. It acts through the products of its phospholipolytic activity, most likely by providing free FAs for TAG synthesis and thereby reducing the requirement for *de novo* lipogenesis. Furthermore, it activates AMP-activated protein kinase (AMPK) and upregulates FA oxidation. The breakdown of hGX sPLA$_2$-induced LDs, occurring during prolonged starvation in the absence of the enzyme, was associated with protection from cell death, likely due to the provision of LD-derived free FAs for mitochondrial β-oxidation [17,18,50]. Some, but not all of the effects of hGX sPLA$_2$ on breast cancer cells could be replicated by exogenously added oleic acid (OA), suggesting the involvement of other unsaturated FA products of hGX sPLA$_2$ activity [18]. In fact, the relationships between different unsaturated and polyunsaturated FA species, LD metabolism and cancer cell survival have been poorly investigated.

Since LDs are central regulators of cellular FA trafficking and act as transient repositories for both endogenous and exogenous FAs to reduce their lipotoxicity [15,19,37], we asked whether LDs induced either by sPLA$_2$ or by addition of exogenous unsaturated FAs, differ in their ability to support cancer cell survival during nutrient and lipotoxic stress. Furthermore, we investigated the role of key enzymes in lipid metabolism, such as ATGL and DGAT1, in cancer cell survival during nutrient stress.

2. Materials and methods

2.1. Materials

Breast cancer cells (MDA-MB-231, T-47D) and culture medium (RPMI-1640) were obtained from ATCC (USA), fetal bovine serum (FBS), Hanks' balanced salt solution (HBSS), Dulbecco's phosphate-buffered saline (DPBS), TrypLE Select and Opti-MEM from Life Technologies (USA). Oleic, docosahexaenoic, eicosapentaenoic, linoleic and arachidonic acids were from Cayman Chemical (USA),

Lipofectamine RNAiMAX, BODIPY 493/503 and CM-H_2DCFDA from Thermo Fisher Scientific (USA). Human ATGL-targeting siRNAs and Allstars Negative Control siRNA were from Qiagen (Germany), *N*-acetyl-L-cysteine (NAC) from Calbiochem (USA). Essentially fatty acid-free (EFAF) bovine serum albumin (BSA) (Sigma-Aldrich cat. no. A7511), fatty acid-free (FAF) BSA (Sigma-Aldrich cat. no. A8806), heptadecanoic acid (C17:0), Nile red, tetramethylrhodamine methyl ester (TMRM), 7-aminoactinomycin D (7-AAD), etomoxir sodium salt hydrate, Hoechst stain solution, hydrogen peroxide and A922500 were from Sigma-Aldrich (USA). YO-PRO-1 iodide was from Life Technologies (USA), horseradish peroxidase (HRP)-labelled secondary antibodies from Jackson ImmunoResearch Laboratories (USA), ATGL antibodies (#2138) from Cell Signaling Technology (USA) and β-actin antibodies (NB600-532) were from Novus Biologicals (UK). The recombinant wild-type human group X $sPLA_2$ was prepared as described previously [54,55]. All other chemicals were of at least analytical grade and purchased from Sigma-Aldrich (USA) or Serva (Germany).

2.2. Cell culture and treatments

MDA-MB-231 and T-47D cells were cultured in RPMI-1640 medium supplemented with 10% FBS, and with 0.2 Units/ml of bovine insulin (Sigma-Aldrich, USA) in the case of the T-47D cell line. Adherent cells were detached using TrypLE Select or a non-enzymatic cell dissociation solution (0.3 g/l Na_2EDTA, 0.4 g/l KCl, 0.6 g/l $NaHCO_3$, 1.1 g/l glucose, 8 g/l NaCl). Unless otherwise indicated, cells were seeded in 24-well plates at a concentration of 3×10^4 cells/well or 6×10^4 cells/well and, depending on the experimental goal, three main procedures of cell feeding and/or starvation were performed following cell seeding and attachment for 24 h in complete medium, including 1) growth for 48 h in complete medium, 2) growth for 48 h in complete medium followed by 96–120 h of serum deprivation in RPMI-1640 medium containing 0.02% EFAF-BSA and 3) pre-starvation for 24 h in RPMI-1640 medium containing 0.02% FAF-BSA followed by 96–168 h of serum deprivation in RPMI-1640 medium containing 0.02% EFAF-BSA. Aliquots of stock solutions of FAs in absolute ethanol were stored under argon at −80 °C. Prior to addition to cell culture, FAs were complexed to EFAF-BSA in RPMI-1640 medium at a molar FA:BSA ratio of 4 : 1 (in experiments with serum deprivation) or to 10% FBS in RPMI-1640 by incubating the mixtures for 1 h at room temperature. A922500 was added to cells 2 h prior to, while etomoxir was added at the time of addition of recombinant $sPLA_2$ or FAs, and were present in the medium for the duration of the treatment.

2.3. Silencing of ATGL expression using small interfering RNA (siRNA)

Unless otherwise indicated, MDA-MB-231 cells were reverse transfected at a concentration of 6×10^4 cells/well with 10 nM (when using a single siRNA) or 20 nM (when using a mixture of two siRNAs) total siRNA targeted at ATGL (Qiagen), or with 10 nM or 20 nM Allstars Negative Control siRNA (Qiagen). Transfection complexes were generated using 1 μl/well of Lipofectamine RNAiMAX in 24-well plates, 7.5 μl/well in 6-well plates and 0.5 μl/well in 48-well plates, and Opti-MEM medium according to manufacturer's instructions. In experiments with a 48-h feeding phase followed by 96 h or 120 h of starvation, a second forward transfection with the same siRNA concentrations was performed 72 h after the reverse transfection according to manufacturer's guidelines.

2.4. Real-time quantitative PCR (qPCR)

Cells were seeded in complete medium in 6-well plates at 1.5×10^5 cells/well and treated with 10 nM $sPLA_2$ and/or 10 μM FAs for 48 h. Total RNA was isolated from cell lysates using High Pure RNA Isolation kit (Roche Applied Science, Germany) and quantified using NanoDrop Spectrophotometer (Thermo Scientific, USA). First strand cDNA was generated from 1 μg of RNA using High Capacity cDNA Reverse Transfection kit with RNase inhibitor (Life Technologies, USA) and random primers according to the manufacturer's instructions. qPCR analysis was performed for all genes of interest and two reference genes in each sample using FastStart Universal SYBR Green Master (Roche Applied Science, Germany) on a StepOnePlus Real-Time PCR system (Applied Biosystems, USA). Cycling conditions were set at 95 °C for 10 min, followed by 45 cycles at 95 °C for 10 s and 62 °C for 35 s and followed by a melting curve analysis. PCR efficiencies were at least 80% for all primer pairs and a single melting peak was observed for each primer pair. Relative gene expression was calculated upon normalization to two reference genes, corrected for primer-specific PCR efficiency and considering error propagation as described previously [18].

2.5. Western blot analysis

Cells were seeded in complete medium in 6-well plates at 3×10^5 cells/well and reverse transfected as described above. The cells were treated with 10 nM $sPLA_2$ for 48 h and harvested or, alternatively, forward transfected and starved for 120 h in RPMI-1640 medium containing 0.02% EFAF-BSA. Cell lysates were prepared by scraping adherent cells in Tris-glycine SDS Sample Buffer (2 ×) (Novex by Life Technologies, USA) containing 800 mM DTT (Sigma-Aldrich, USA) with the addition of Halt Protease Inhibitor Cocktail (Thermo Scientific, USA). Lysates were incubated at 95 °C for 10 min and stored on ice. Total protein concentration was determined using Pierce 660 nm Protein Assay (Thermo Scientific, USA). 10 μg of total protein were separated on 10% SDS-PAGE gel and then transferred to a nitrocellulose membrane (Serva, Germany). After transfer, the membrane was blocked for 1 h in 5% non-fat dry milk in TBS/0.1% Tween-20 (TBST) (for ATGL) or in 1% Western Blocking Reagent (WBR) (Roche Applied Science, Germany) in TBS (for β-actin) and incubated overnight at 4 °C in the presence of rabbit anti-human primary antibodies for ATGL (1:1000 dilution) in 5% non-fat dry milk in TBST and rabbit anti-human primary antibodies for β-actin (1:5000 dilution) in 0.5% WBR in TBS. After washing with TBST, membranes were incubated for 1 h with HRP-conjugated secondary antibodies (1:10,000 dilution) in 5% non-fat dry milk in TBST for ATGL or 0.5% WBR in TBS for β-actin. After washing, the signal was visualized using Lumi-Light Western Blotting Substrate (Roche Applied Science, Germany) on a Gel Doc XR system (Bio-Rad, USA).

2.6. Lipidomic analysis of non-esterified FAs in cell supernatants

Non-esterified fatty acid (NEFA) species were extracted from cell supernatants for LC-MS analysis. Cells were seeded in complete medium in 100 mm plates at 2×10^6 cells/plate (MDA-MB-231) or 3.6×10^6 cells/plate (T-47D), left to attach for 24 h, then washed twice with DPBS and the medium was replaced with RPMI-1640 containing 0.02% EFAF-BSA. After 24 h, the cells were incubated with 10 nM $sPLA_2$ in RPMI-1640 medium containing 1% EFAF-BSA for 6 h. Cell supernatants were collected for NEFA extraction and the cells harvested using cell dissociation solution for determination of total protein concentration as described above. NEFAs were extracted using ice-cold acidic Folch solution (1% (v/v) acetic acid in chloroform/methanol 2/1, v/v) with 1 μM of heptadecanoic acid added as an internal standard (iSTD) for extraction recovery determination. Extraction was performed under constant shaking for 30 min at 25 °C. Phase separation was obtained by centrifugation at 2500*g* and 4 °C for 20 min. The organic phase was collected, evaporated using a N_2 stream at 30 °C, and dissolved in 150 μl of methanol/2-propanol/water (30/15/5, v/v/v) for LC-MS analysis. Six external NEFA standards containing a mixture of five FA species (OA, AA, LA, EPA and DHA; each at a final concentration of 125 nM, 250 nM, 500 nM, 1 μM, 2 μM and 4 μM) and containing 1 μM iSTD were extracted as described above and used for final data normalization. Chromatographic separation was modified after

Knittelfelder et al. [56] using an AQUITY-UPLC system (Waters Corporation, USA), equipped with a Kinetex C18 column (2.1 × 50 mm, 1.7 µm; Phenomenex), and a 20-min gradient with 100% solvent A (methanol/water, 1/1, v/v; 10 mM ammonium acetate, 0.1% formic acid). An EVOQ Elite™ triple quadrupole mass spectrometer (Bruker, USA) equipped with an ESI source was used for detection. FA species were analysed by selected reaction monitoring (FA: [M-H]- to [M-H]-, 0 eV). Data acquisition was done by MS Workstation (Bruker, USA). Data were normalised for recovery, extraction and ionization efficacy by calculating analyte/iSTD ratios.

2.7. Lipidomic analyses of triacylglycerol and phospholipid species

Cells were seeded in complete medium in 6-well plates at 3×10^5 cells/well and reverse transfected as described above. The cells were treated with 10 nM $sPLA_2$ and/or 100 µM DHA for 48 h, put on ice and washed twice with ice-cold DPBS, collected by scraping in 300 µl lysis buffer (20 mM Tris-HCl, pH 7.4, 2 mM EDTA, 2 µl Halt Protease Inhibitor Cocktail), centrifuged at 4 °C (1000g, 10 min), the pellet resuspended in 150 µl lysis buffer and sonicated on ice. Total lipids were extracted twice using chloroform/methanol/water (2/1/0.6, v/v/v) containing 500 pmol butylated hydroxytoluene, 1% acetic acid, and 100 pmol of internal standards (iSTD, 17:0–17:0 PC, 17:0–17:0 PE, 17:0–17:0–17:0 TAG, 17:0 LPC, Avanti Polar Lipids) per sample. Extraction was performed under constant shaking for 60 min at room temperature (RT). After centrifugation at 1000g for 15 min at RT the lower organic phase was collected, 2.5 ml chloroform added to the remaining aqueous phase and the second extraction performed as described above. Combined organic phases of the double extraction were dried under a stream of nitrogen and dissolved in 150 µl 2-propanol/chloroform/methanol (7/2/1, v/v/v) for UPLC-qTOF analysis. Chromatographic separation was modified after Knittelfelder et al. [56] using an AQUITY-UPLC system (Waters Corporation), equipped with an ACQUITY BEH C18 column (2.1 × 50 mm, 1.7 µm; Waters Corporation). A SYNAPT™G1 qTOF HD mass spectrometer (Waters Corporation) equipped with an ESI source was used for detection. Data acquisition was done by the MassLynx 4.1 software (Waters Corporation) and lipid classes were analysed with the "Lipid Data Analyzer 1.6.2" software [57]. Data were normalised for recovery and extraction- and ionization efficacy using iSTDs.

2.8. Nile red staining of lipid droplets

Cellular neutral lipids were quantified by flow cytometry as described previously [18]. Briefly, the cells were harvested, centrifuged at 300 × g for 10 min and the pellet resuspended in 500 µl of 1 µg/ml Nile Red solution in DPBS. After 10 min incubation in the dark, the cells were analysed by flow cytometry on a FACSCalibur system equipped with a 488-nm Ar-ion laser using the CellQuest software (Becton Dickinson, USA) and the FL-1 (530/30) filter on at least 2×10^4 events per sample.

2.9. TMRM/YO-PRO-1 cell death assay

Cell death was determined by measuring mitochondrial and plasma membrane integrity using TMRM/YO-PRO-1 double staining as described previously [18,58]. Briefly, floating and adherent cells were collected and centrifuged at 300g for 10 min, the pellet resuspended in 100 µl 150 nM TMRM in DPBS and incubated for 15 min in the dark at 25 °C. YO-PRO-1 was added to a final concentration of 50 nM and cells incubated for an additional 10 min. The cell suspension was diluted with 200 µl DPBS containing 0.1% FAF-BSA and analysed by flow cytometry. TMRM and YO-PRO-1 fluorescence signals were collected using FL-1 (530/30) and FL-3 (650LP) filters, respectively. YO-PRO-1 positive and TMRM negative cells were considered dead. At least 2×10^4 events were analysed per sample.

2.10. Mitochondrial membrane potential assay

Changes in mitochondrial membrane potential were determined using a modified TMRM-based assay described previously [59]. Floating and adherent cells were harvested and centrifuged at 300 × g for 10 min, the pellet resuspended in 25 nM TMRM in DPBS and incubated for 15 min in the dark at room temperature. To exclude dead cells from the analysis, YO-PRO-1 was added to a final concentration of 50 nM and cells incubated for an additional 10 min. The cell suspension was diluted with 200 µl DPBS containing 0.1% FAF-BSA and analysed by flow cytometry using FL-1 (530/30) and FL-3 (650LP) filters. At least 2×10^4 events were analysed per sample.

2.11. Reactive oxygen species and cell death assay with CM-H_2DCFDA and 7-AAD

Adherent cells were stained with 1 µM CM-H_2DCFDA for 30 min in HBSS at 37 °C, in a humidified atmosphere containing 5% CO_2, with a subsequent recovery period of 2 h in RPMI-1640 phenol red-free medium (Life Technologies, USA) containing 10% FBS (for fed cells) or 0.02% EFAF-BSA (for starved cells). The cells were harvested using cell dissociation solution, resuspended in HBSS, 7-AAD was added to a final concentration of 5 µM and the cells incubated at room temperature for an additional 10 min. At least 2×10^4 cells per sample were analysed by flow cytometry using the FL-1 (530/30) and FL-3 (650LP) filters for CM-H_2DCFDA and 7-AAD fluorescence, respectively.

2.12. Glycerol assay

Cellular lipolytic activity was assessed by measuring glycerol release in cell supernatants. Cells were seeded in complete medium in 48-well plates at 3×10^4 cells/well and reverse transfected as described above. After 24 h, the cells were incubated with 10 nM $sPLA_2$ in complete medium for 48 h. Cell supernatants were collected and glycerol concentration determined using the Glycerol Cell-Based Assay Kit (Cayman Chemical, USA) according to manufacturer's instructions.

2.13. Cholesterol and triglyceride assays

Cellular triglyceride, cholesterol and cholesteryl ester contents were determined using the PicoProbe Triglyceride Quantification Assay Kit (Abcam, USA) and the Cholesterol/Cholesteryl Ester Quantification Assay Kit (Abcam, USA), respectively. Cells were seeded in complete medium in 6-well plates at 3×10^5 cells/well. After 24 h, the cells were incubated with 10 nM $sPLA_2$, 100 µM OA or 100 µM DHA in complete medium for 48 h. Cell lysates were prepared and used for lipid quantification according to manufacturer's instructions.

2.14. Epifluorescence microscopy

Cytosolic LDs were visualized using BODIPY 493/503 neutral lipid staining. Cells were seeded on glass bottom culture plates at 3×10^4 or 6×10^4 cells/well. Twenty-four hours later, medium was replaced with complete culture medium containing 10 nM $sPLA_2$ and/or different concentrations of FAs. After 48 h, cells were washed twice with DPBS and stained with 1 µg/ml BODIPY 493/503 in RPMI-1640 medium for 10 min, washed with DPBS, incubated with Hoechst stain solution for 30 min, and recovered for 30 min in RPMI-1640 medium in a CO_2 incubator before live imaging on a Zeiss Axio Observer Z1 inverted microscope (Carl Zeiss, Germany). Images were processed using ZEN software (Carl Zeiss, Germany).

2.15. Statistical analysis

Statistical analysis was performed using Prism 7.0 (GraphPad Software, USA). Data are presented as means ± SEM. Statistical

significance was determined using Student's *t*-test, 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.

3. Results

3.1. Exogenous hGX sPLA$_2$ releases unsaturated FAs, including ω-3 and ω-6 PUFAs, from adherent breast cancer cells

We have previously shown that hGX sPLA$_2$ releases radiolabelled OA from adherent MDA-MB-231 cells [18]. Interestingly, OA mimicked most of the effects of hGX sPLA$_2$ on MDA-MB-231, but not on T-47D breast cancer cells [18]. In this study, we first determined the profile of FA species released by hGX sPLA$_2$ from MDA-MB-231 and T-47D cells in the absence of serum-derived lipoproteins and other lipids, which are known targets of hGX sPLA$_2$ activity [60]. Lipidomic analysis of cell supernatants from MDA-MB-231 cells grown in the presence of FA-free BSA and exposed to exogenous hGX sPLA$_2$ for 6 h revealed that hGX sPLA$_2$-mediated hydrolysis of cell membrane phospholipids leads to a significant increase in the total amount of free FAs in the supernatant (Supp. Fig. 1A). The enzyme released numerous unsaturated FAs, with significant increases in the amounts of several PUFA species (Fig. 1A and Supp. Table 1). With the exception of 14:0, no changes in the amounts of saturated FAs were observed. Quantitative lipidomic analysis (Fig. 1B and Supp. Table 2), using OA (18:1, ω-9), linoleic acid (LA; 18:2, ω-6), arachidonic acid (AA; 20:4, ω-6), eicosapentaenoic (EPA; 20:5, ω-3) and docosahexaenoic acid (DHA; 22:6, ω-3) as external standards, revealed that OA and LA are the most abundant species released by the enzyme (the molar ratio of OA : LA : AA : EPA : DHA was 41 : 50 : 2.7 : 0.8 : 5.5). This is in line with the high abundance of OA and LA esterified at the *sn*-2 position of glycerophospholipids in MDA-MB-231 and T-47D cells [61]. hGX sPLA$_2$ displayed potent enzymatic activity also on T-47D cells (Supp. Fig. 1B), releasing a mixture of unsaturated FA products (Supp. Fig. 1C and Supp. Table 1). Supernatants of untreated T-47D cells contained higher amounts of PUFAs than those of MDA-MB-231 cells, suggesting that PUFAs are more abundant in T-47D cells (Fig. 1C and Supp. Table 2). Accordingly, the total pmol amount of PUFAs released by hGX sPLA$_2$ from T-47D cells was higher than that released from MDA-MB-231 cells (Fig. 1B and C and Supp. Table 2). Thus, hGX sPLA$_2$ is efficient in hydrolysing cell membranes of both MDA-MB-231 and T-47D cells leading to the release of a mixture of unsaturated FAs containing significant amounts of ω-3 and ω-6 PUFAs.

Exogenous hGX sPLA$_2$ induces LD biogenesis in breast cancer cells both in rapidly proliferating cells, grown in the presence of serum, and in starving, quiescent cells, grown in the absence of serum [18]. Since the enzyme has potent activity on both mammalian cell membranes and lipoprotein particles, it is possible that lipoproteins are the predominant target of hGX sPLA$_2$ activity in *in vitro* cellular assays performed in complete growth media. To confirm that the enzyme acts on breast cancer cells even in the presence of serum, we performed lipidomic analyses of lysates and supernatants of MDA-MB-231 cells grown in complete media and treated with 50 nM of exogenous hGX sPLA$_2$ for 3, 6 and 24 h. We found that the enzyme hydrolysed most of the extracellular PC species in the cell supernatant (data not shown), but there were also reductions in the amounts of several cellular PC and phosphatidylinositol (PI) species, evident already after 3 h (data not shown) and most prominent after 6 h of treatment (Fig. 1D and E). Furthermore, we observed an increase in total lysophosphatidylcholine (LPC) in cell lysates and in cell supernatants (Fig. 1F), with similar changes in the profile of individual LPC species in both cases (Supp. Fig. 1D and E). There was a significant reduction in total PI in cells treated with hGX sPLA$_2$, while the apparent decrease of total PC amount was not statistically significant (Fig. 1G). However, hGX sPLA$_2$ induced a significant reduction in the amounts of the most abundant PC species 32:1, 34:1, 36:1 and 36:2 (Fig. 1D and Supp. Table 3), and, importantly, of several PUFA-containing PI species, including the dominant 38:3 and 38:4 (Fig. 1E and Supp. Table 4). Notably, the total amount of serum-derived PI per mg protein in supernatants of untreated cells was > 2 orders of magnitude lower than that of cellular PI (data not shown), suggesting that lipoprotein PI is not an important substrate for the sPLA$_2$ under these conditions. These results suggest that, in typical *in vitro* cellular assays performed in the presence of 10% FBS in the media, hGX sPLA$_2$ potently releases unsaturated FAs and lysophospholipids from both serum phospholipids and from breast cancer cell membranes. Moreover, while lipoprotein and cellular PC hydrolysis likely provide the bulk of unsaturated FAs released by enzyme, cellular PI phospholipids emerge as important sources of PUFAs in breast cancer cells treated with hGX sPLA$_2$.

3.2. Unsaturated FAs induce LD biogenesis and prevent cell death in starved breast cancer cells

We next examined whether OA and different PUFAs differ in their ability to induce LD formation and protect TNBC cells from serum deprivation-induced cell death. We found that low micromolar concentrations (10 μM) of exogenously added OA, LA, DHA, EPA and AA induce LD formation in MDA-MB-231 cells grown in nutrient-rich conditions (Fig. 2A and B) and that the addition of hGX sPLA$_2$ in combination with FA loading further augments LD accumulation (Fig. 2A). Pre-treatment of cells with hGX sPLA$_2$ in nutrient-rich conditions increased resistance to starvation-induced cell death (Fig. 2C). A similar effect was observed when cells were pre-treated with unsaturated FAs (Fig. 2C), suggesting that starvation-induced LD breakdown is associated with cell survival. Notably, hGX sPLA$_2$ treatment in combination with individual FAs significantly augmented the amount of neutral lipids remaining after the starvation phase (Supp. Fig. 2A). Furthermore, a strong pro-survival effect of unsaturated FAs was observed also in cells pre-starved for 24 h and treated with FAs and sPLA$_2$ during the following 96 h of serum withdrawal (Fig. 2D). In these conditions, OA was significantly more potent than PUFAs in inducing LD accumulation (Supp. Fig. 2B), but all unsaturated FAs reduced the percentage of apoptotic cells in the population by at least 2-fold and hGX sPLA$_2$ augmented the pro-survival effects of individual FAs (Fig. 2D). Thus, low micromolar concentrations of unsaturated FAs induce LD formation and protect MDA-MB-231 cells from starvation-induced cell death and co-treatment with exogenous hGX sPLA$_2$ further augments their effects.

We have previously shown that hGX sPLA$_2$-induced LD formation and prevention of cell death in MDA-MB-231 cells is associated with upregulation of genes involved in β-oxidation and suppression of those responsible for *de novo* FA synthesis [18]. We next asked whether individual unsaturated FAs differ in their ability to alter the expression of selected genes involved in lipid metabolism. We found that hGX sPLA$_2$ and low micromolar concentrations of unsaturated FAs induce similar changes in gene expression in proliferating MDA-MB-231 cells (Fig. 2E and Supp. Fig. 2C): an elevated expression of the *PLIN2* gene, encoding the LD-coating protein perilipin 2 (PLIN2), upregulation of *CPT1A*, encoding the rate-limiting β-oxidation enzyme carnitine palmitoyltransferase 1A (CPT1A), and a significant downregulation of two major lipogenic genes, the transcription factor sterol regulatory element-binding protein-1 (SREBP-1; encoded by *SREBF1*) and the FA desaturase SCD1 (encoded by *SCD*). Interestingly, the expression of ATGL (encoded by the *PNPLA2* gene) was not affected by hGX sPLA$_2$, but it was slightly reduced in cells treated with OA, LA and AA. There were no statistically significant changes in the expression of *DGAT1* (encoding diacylglycerol acyltransferase 1, DGAT1), *ACADVL* (encoding the β-oxidation enzyme very long-chain acyl-CoA dehydrogenase, VLCAD), *FASN* (encoding fatty acid synthase, FAS), nor in the expression of the *PPARA* and *PPARG* genes, encoding the transcription factors peroxisome proliferator-activated receptors α and γ, PPARα and PPARγ, respectively (Fig. 2E and Supp. Fig. 2C). Collectively, these results are consistent

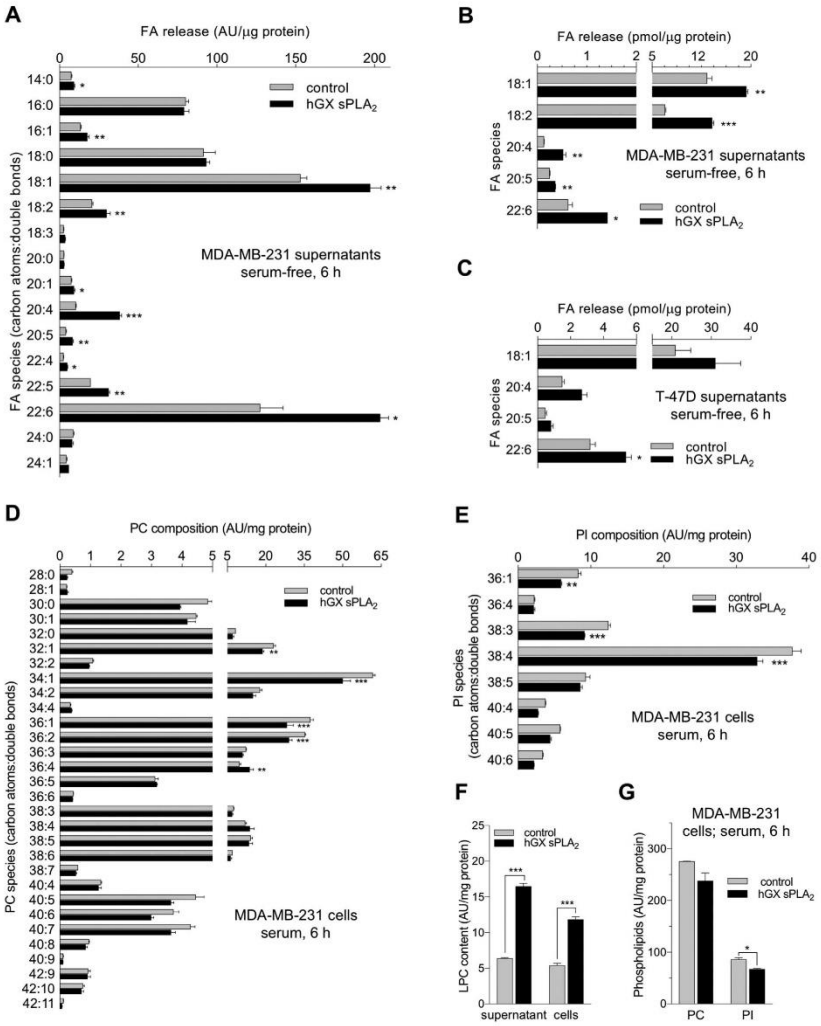

Fig. 1. hGX $sPLA_2$ releases unsaturated FAs, including ω-3 and ω-6 PUFAs, from breast cancer cells. MDA-MB-231 (A, B) and T-47D (C) cells were serum-starved for 24 h and incubated with 10 nM hGX $sPLA_2$ in the presence of 1% EFAF-BSA for 6 h; non-esterified FAs were extracted from supernatants and analysed by LC-MS. (A) The relative changes in the amounts of FA species released from MDA-MB-231 cells treated with hGX $sPLA_2$ were corrected for extraction recovery and normalised to total cellular protein. (B, C) Quantification was performed using standard FA solutions containing OA (18:1), LA (18:2), AA (20:4), EPA (20:5) and DHA (22:6). (D–G) MDA-MB-231 cells were grown in complete media (10% FBS in RPMI-1640) and treated with 50 nM hGX $sPLA_2$ for 6 h. Lipids were extracted separately from cell lysates and cell supernatants. Lysophospholipids and phospholipids were analysed by LC-MS and values normalised to total protein. Values on the graphs are means ± SEM of two (C) or three (A, B, D–G) independent experiments; statistically significant differences in mean values are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; *t*-test (A–C); two-way ANOVA with Bonferroni adjustment (D–G)); AU, arbitrary units.

with the notion of a similar action of hGX $sPLA_2$ and unsaturated FAs. Thus, unsaturated FAs, the products of $sPLA_2$ enzymatic activity, induce changes in lipid metabolism, thereby mediating its effects on TNBC cell survival.

3.3. The pro-survival effects of hGX $sPLA_2$ and unsaturated FAs are not dependent on ATGL-mediated lipolysis

LDs, induced by low micromolar concentrations of unsaturated FAs or by hGX $sPLA_2$, provide a survival advantage to MDA-MB-231 cells when exposed to nutrient stress. LD-derived FAs have been shown to fuel β-oxidation and enable cell survival in different cell types during

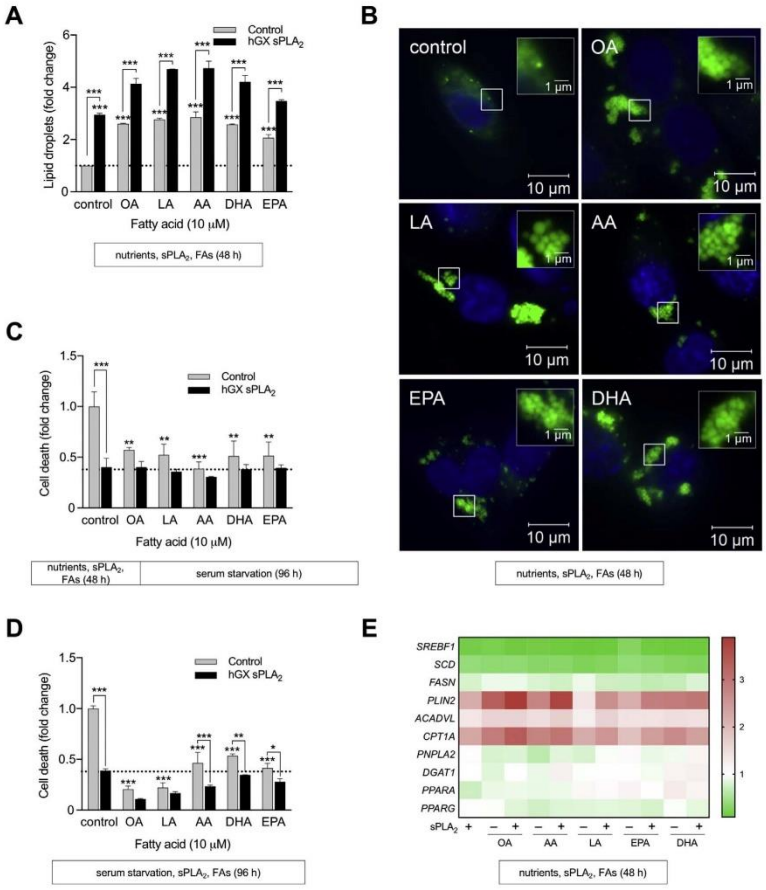

Fig. 2. Unsaturated FAs induce LD formation and protect TNBC cells from starvation-induced cell death. (A–C, E) Proliferating cells grown in complete medium were treated with 10 nM hGX $sPLA_2$ and/or 10 μM exogenous FAs for 48 h (A, B, E) and starved for additional 96 h (C) in the absence of serum, hGX $sPLA_2$ and FAs. (D) Quiescent cells were treated with 10 nM hGX $sPLA_2$ and/or 10 μM of FAs for 96 h in the absence of serum. (B) Cells were stained with BODIPY 493/503 and Hoechst stain to visualize LDs (green) and nuclei (blue), respectively. The images shown are representative of two experiments. Neutral lipid content was quantified by Nile Red staining (A) and cell death by the TMRM/YO-PRO-1 assay and flow cytometry (C, D). The relative gene expression levels of genes involved in lipid metabolism were determined by qPCR (E). Changes in gene expression are presented in the heat map as fold-change values relative to untreated cells (red, upregulation; green, downregulation). Values on the graphs are means ± SEM of at least two experiments; statistically significant differences in mean values are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni adjustment).

starvation [17,33,62]. ATGL, the rate-limiting enzyme in TAG lipolysis [6,21], enables FA transfer from LDs to mitochondria in acutely starved fibroblasts [33], suggesting that it is involved in supporting cell survival during starvation. We have previously found that β-oxidation is important for the ability of hGX $sPLA_2$-induced LDs to support cell survival [18,50], but a role for LD lipolysis has not been investigated. We thus asked whether suppression of LD lipolysis by knocking-down the expression of ATGL would reduce the ability of $sPLA_2$ and unsaturated FAs to promote cell survival during starvation. siRNA-based ATGL knock-down efficiently suppressed ATGL protein levels, both in cells exposed to nutrient-rich conditions and in those exposed to subsequent prolonged starvation (Fig. 3A and B). ATGL depletion resulted in an increase in LD accumulation (Fig. 3C) and a reduction in the amount of glycerol released into the cell culture supernatant (Fig. 3D), indicating that ATGL is involved in TAG lipolysis and LD breakdown in MDA-MB-231 cells. Surprisingly, hGX $sPLA_2$ increased glycerol release both in wild-type and in ATGL-deficient cells, suggesting that the $sPLA_2$ induces lipolysis and that this occurs independently of ATGL (Fig. 3D). Furthermore, we found that ATGL depletion, though significantly increasing the total amount of neutral lipids, did not block LD breakdown during the starvation period in $sPLA_2$-treated cells (Fig. 3E). Accordingly, although the increase in LD accumulation due to ATGL depletion was still significant after 120 h of starvation in both control and $sPLA_2$-treated cells (Fig. 3C and F), there was only a modest rise in cell death (Fig. 3G). This suggests that ATGL-mediated lipolysis contributes to, but it is not essential for the pro-survival effect of hGX $sPLA_2$. Even when

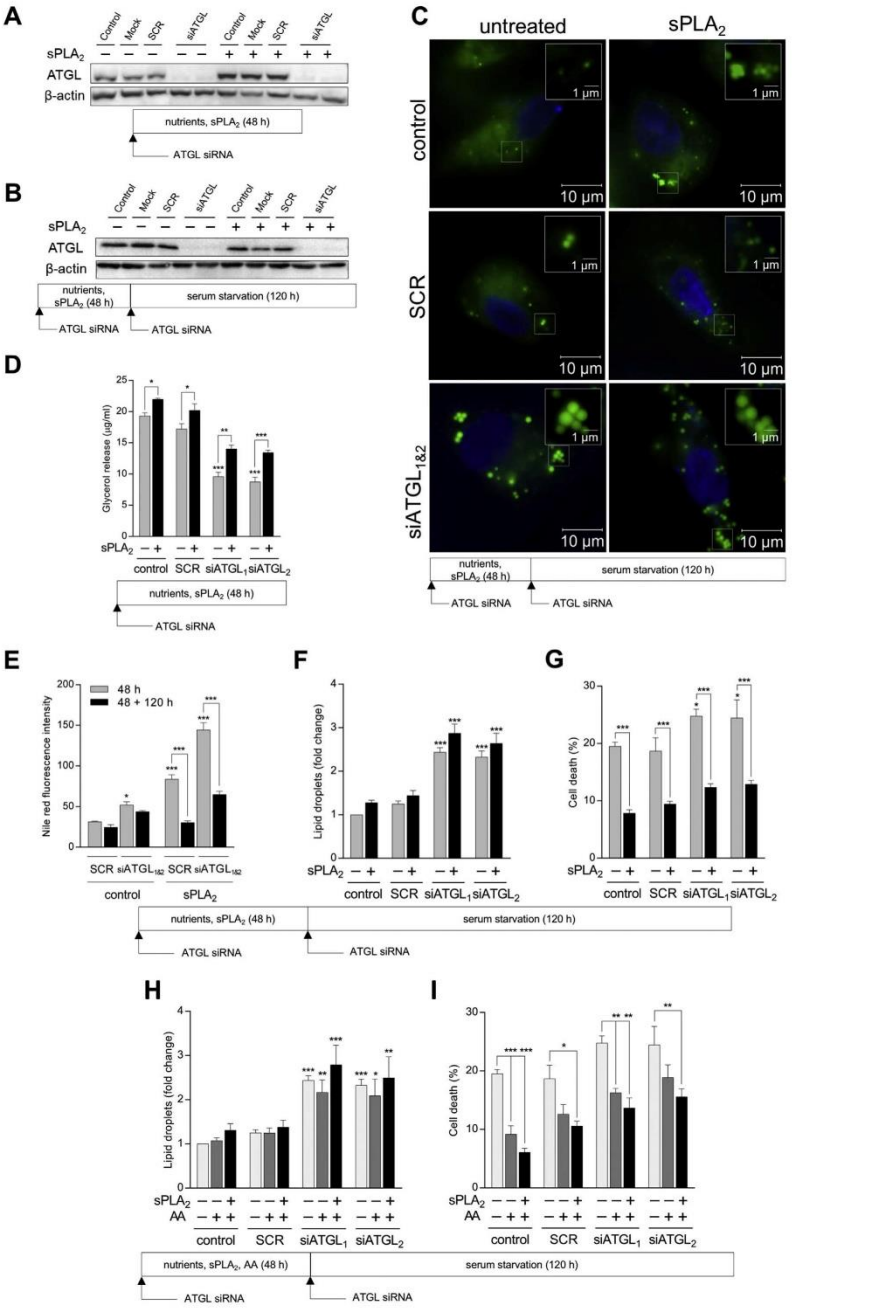

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Fig. 3. ATGL contributes to LD breakdown during starvation, but it is not necessary for the pro-survival effects of hGX $sPLA_2$ and exogenous unsaturated FAs. (A–I) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNAs (siATGL) or non-targeting scrambled siRNA (SCR), treated with 10 nM hGX $sPLA_2$ and/or 10 μM AA (H, I) for 48 h in complete medium and either harvested for analysis (A, D, E) or forward transfected for the second time and serum-starved for 120 h (B, C, E–I). (A, B) Cell lysates were analysed for the presence of ATGL protein and β-actin by western blotting. (C) LDs and nuclei were visualized using BODIPY 493/503 and Hoechst stain, respectively. (D) Glycerol levels were determined in cell supernatants as described in Methods. Neutral lipid content (E, F, H) and cell death (G, I) were quantified by flow cytometry using Nile Red and TMRM/YO-PRO-1 staining, 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 (G) or Bonferroni adjustment (D, E, F, H, I)).

cells were exposed to harsher conditions of nutrient deprivation (prolonged starvation in HBSS or PBS), ATGL depletion did not significantly affect cell survival (data not shown). Similarly, although ATGL knockdown led to net increase in LD content at the end of the starvation period in cells pre-treated with OA and AA, it did not impair their ability to support cell survival during starvation (Fig. 3H and I and Supp. Fig. 3A and B). These results suggest that ATGL is not critically important for the survival of TNBC cells containing LDs induced by exogenous hGX $sPLA_2$ and unsaturated FAs.

We have previously found that LD accumulation and β-oxidation are important for the pro-survival effects of hGX $sPLA_2$ [18]. Studies in cardiomyocytes suggest that both endogenous and exogenous FAs must be first incorporated into LDs and then released through ATGL-mediated TAG lipolysis in order to activate PPARα signalling pathways and stimulate mitochondrial biogenesis and oxidative metabolism [34]. Additionally, ATGL is important for channelling FAs from LDs to mitochondria during acute starvation in mouse embryonic fibroblasts [33]. However, lipolysis was not essential for the survival of MDA-MB-231 cells exposed to prolonged starvation in low glucose media [63]. To attest the importance of the lipolysis-β-oxidation axis in supporting cell survival in serum-starved MDA-MB-231 cells, we used the CPT1A inhibitor etomoxir in addition to ATGL depletion in cells containing $sPLA_2$-induced LDs. We found that etomoxir, added to cells during the starvation-induced LD breakdown phase, reduced the amount of neutral lipids remaining after the starvation in ATGL depleted and $sPLA_2$-treated cells (Fig. 4A). It also significantly augmented the negative effect of ATGL depletion on cell survival and suppressed the pro-survival activity of hGX $sPLA_2$ in both control and ATGL depleted cells (Fig. 4B). Etomoxir reduced mitochondrial membrane potential (MMP) in control, hGX $sPLA_2$-treated and ATGL-depleted cells (Supp. Fig. 4), indicating that it does inhibit mitochondrial oxidative metabolism in our experimental conditions. Simultaneous inhibition of both lipolysis and FA oxidation is more effective in reducing cancer cell survival during serum starvation than inhibiting either pathway alone. Collectively, these results suggest that while both ATGL-mediated TAG lipolysis and β-oxidation contribute to cell survival during serum deprivation, only β-oxidation is necessary for the pro-survival activity of hGX $sPLA_2$-induced LDs.

3.4. *hGX $sPLA_2$ augments PUFA-induced LD formation and reduces PUFA lipotoxicity*

PUFAs have been shown to induce cytotoxic effects in different cancer cells *in vitro* and *in vivo* [64], including MDA-MB-231 breast cancer cells [65]. In order to investigate the relationship between PUFA-induced LD formation and lipotoxicity in TNBC cells, we first exposed cells to high micromolar concentrations of unsaturated FAs and hGX $sPLA_2$ and determined their effects on LD biogenesis and cell death in nutrient-rich conditions. Exogenous addition of 50 and 100 μM FAs to proliferating MDA-MB-231 cells led to notable 2- to 7-fold increases in the levels of neutral lipids (Fig. 5A). Biochemical analyses of cellular lipids revealed that the observed hGX $sPLA_2$-induced change in LD content is primarily associated with an elevation of cellular TAG synthesis (Fig. 5B), since the enzyme reduced total cholesterol levels (Fig. 5C) and there were no significant changes in cholesteryl ester content (Fig. 5C). Cells treated with 100 μM OA and DHA displayed a significant increase in the amount of cholesteryl esters, but the corresponding elevation of TAG content was more pronounced (Fig. 5B). Incubating cells with hGX $sPLA_2$ in addition to individual FAs led in most cases to further augmentation of LD formation. DHA, EPA and AA displayed significant cytotoxicity, but LA and OA were not toxic (Fig. 5D). Remarkably, hGX $sPLA_2$ significantly suppressed the lipotoxic effects of PUFAs, markedly reducing cell death in cells treated with AA, EPA and DHA. There was no correlation between LD content induced by a particular FA and its lipotoxicity, but hGX $sPLA_2$ consistently potentiated LD formation and reduced FA-induced lipotoxicity.

We next examined whether exposing FA-loaded cells to prolonged serum deprivation would alter the lipotoxic potency of PUFAs or impair the ability of hGX $sPLA_2$ to protect from lipotoxicity. Interestingly, lipid loading of MDA-MB-231 cells with 50 or 100 μM of OA and LA, or 50 μM EPA and DHA, was associated with protection from starvation-induced cell death (Fig. 5E). Only cells exposed to 100 μM DHA, EPA and AA displayed a reduced ability to withstand serum starvation. Treating cells with hGX $sPLA_2$ in addition to the individual FAs

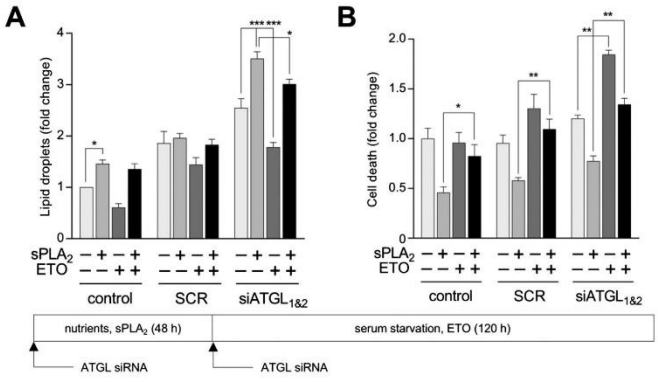

Fig. 4. β-oxidation is important for cell survival during starvation. MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNAs (siATGL) or non-targeting scrambled siRNA (SCR), treated with 10 nM hGX $sPLA_2$ for 48 h in complete medium, forward transfected for the second time and serum-starved for 120 h in the presence or absence of the CPT1A inhibitor etomoxir (50 μM). Cell death was determined using the TMRM/YO-PRO-1 assay (B) and LD content using Nile Red staining (A). Values on the graphs are means ± SEM of at least three experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni adjustment).

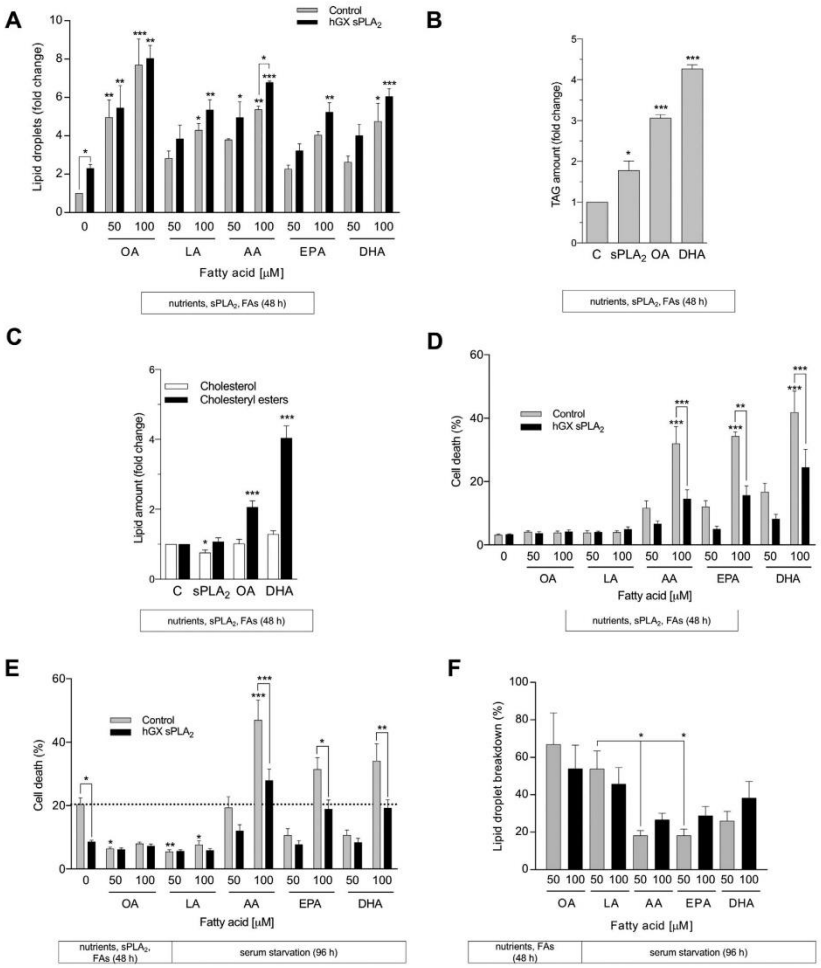

Fig. 5. hGX $sPLA_2$ potentiates PUFA-induced LD formation and suppresses PUFA lipotoxicity in proliferating and in starved cells. (A, D) MDA-MB-231 cells were grown in complete medium and treated with 10 nM hGX $sPLA_2$ and/or 50–100 μM FAs for 48 h. (B, C) Cells were grown in complete medium and treated with 10 nM hGX $sPLA_2$ or 100 μM FAs for 48 h. (E) Cells were grown in complete medium for 48 h in the presence of 10 nM hGX $sPLA_2$ and/or 50–100 μM FAs and serum-starved for 96 h. (F) Cells were grown in complete medium for 48 h in the presence of 50–100 μM FAs and either harvested for analysis or serum-starved for 96 h and then analysed. The percentage of LD breakdown during the 96-h starvation period was calculated from neutral lipid measurements at the beginning and the end of the starvation. Cholesterol, cholesteryl ester and triglyceride amounts were determined using biochemical assays and the data normalised to total cellular protein (B, C). Cell death was determined by the TMRM/YO-PRO-1 assay (D, E) and LD content by Nile Red staining and flow cytometry (A, F). Values on the graphs are means ± SEM of at least three experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; one-way (B, F) or two-way (A, C–E) ANOVA with Bonferroni adjustment).

augmented LD content remaining after starvation (Supp. Fig. 5A) and, importantly, the toxic effects of DHA, EPA and AA were suppressed (Fig. 5E). By comparing LD content before and after the 96-h starvation period, we confirmed that substantial LD breakdown occurred, albeit the magnitude varied depending on the FA species used for lipid loading (Fig. 5F). Interestingly, in the case of the non-toxic FAs, OA and LA, cells displayed the most significant relative decrease in neutral lipid amount (44–66%), while LDs induced by the toxic PUFAs were broken down to a significantly lower degree (18–38%). These results suggest that LDs containing OA and LA are more readily degraded than those containing the potentially toxic PUFAs, EPA, DHA and AA. Furthermore, starvation-induced breakdown of LDs induced by EPA, DHA and AA, but not by OA and LA, was associated with an increase in MMP, whereas hGX $sPLA_2$ suppressed PUFA-induced MMP elevation (Supp. Fig. 5B). This suggests that the breakdown of LDs induced by co-supplementation with hGX $sPLA_2$ is associated with a reduction in the amount of PUFAs that reach mitochondria during the starvation. Thus, unsaturated FAs exert differential, species- and concentration-

dependent effects on MDA-MB-231 cell survival. In general, OA and LA are not toxic and reduce starvation-induced cell death, while EPA, DHA and AA may be both cytoprotective or cytotoxic depending on the concentrations used. Lipid loading with hGX $sPLA_2$ in addition to individual FAs augments LD biogenesis and reduces PUFA toxicity during prolonged serum deprivation.

We next asked if serum deprivation prior to the addition of exogenous FAs sensitizes MDA-MB-231 cells to FA-induced damage and reduces the ability of $sPLA_2$ to protect from lipotoxicity. OA was more effective than PUFAs in inducing LD accumulation in quiescent, serum-starved cells (Supp. Fig. 5C) and displayed a pro-survival activity even at high concentrations (Supp. Fig. 5D). Serum starvation sensitized cells to LA and reversed its pro-survival activity to pro-apoptotic at a concentration of 50 μM. Similarly, DHA, AA and EPA were toxic at concentrations above 30–40 μM (Supp. Fig. 5D). Importantly, the addition of hGX $sPLA_2$ augmented FA-induced LD formation, it efficiently suppressed the toxicity of LA, EPA, DHA and AA and it even further potentiated the strong anti-apoptotic effect of OA (Supp. Fig. 5C and D). Clearly, quiescent, nutrient-deprived cells are more sensitive to PUFA-induced lipotoxicity than cells exposed to serum deprivation following prior LD biogenesis induced by the same amounts of PUFAs in nutrient-rich conditions. Thus, serum starvation sensitizes MDA-MB-231 cells to PUFA-induced damage, but does not abolish the ability of hGX $sPLA_2$ to augment FA-induced LD formation and to protect from PUFA lipotoxicity.

We have recently found that in contrast to hGX $sPLA_2$, exogenous OA does not reduce starvation-induced cell death in the ER-positive and weakly tumourigenic T-47D breast cancer cells, despite the relatively high levels of LDs induced by this FA [18]. In contrast to the Ras-driven MDA-MB-231 cells, T-47D cells possess an activating mutation in phosphatidylinositol 3-kinase (PI3K) leading to sustained activity of the PI3K/Akt pathway [66]. To determine if the effects of unsaturated FAs on T-47D cell survival differ from those observed on TNBC cells, we exposed T-47D cells to exogenous FAs and hGX $sPLA_2$ and measured cell death after prolonged starvation. Surprisingly, hGX $sPLA_2$ and 50–100 μM concentrations of PUFAs reduced cell death during prolonged starvation, but OA was cytotoxic (Supp. Fig. 5E). This suggests that the effects of hGX $sPLA_2$ on T-47D cells are mediated predominantly by PUFAs and that different breast cancer cells have different abilities to use unsaturated FAs for cell survival. Ras-driven MDA-MB-231 cells apparently possess metabolic alterations that enable optimal use of both mono- and polyunsaturated FAs to fight against nutrient stress.

3.5. Breakdown of DHA-rich LDs is associated with the induction of oxidative stress

PUFA lipotoxicity has been associated with the induction of oxidative stress [67]. To determine if the observed DHA toxicity in MDA-MB-231 cells is a consequence of an increase in ROS, we treated cells with DHA, the glutathione precursor NAC and hGX $sPLA_2$. The use of an optimized double staining flow cytometry assay with the ROS-sensitive probe CM-H_2DCFDA and the DNA-binding dye 7-AAD enabled a simultaneous quantification of cell death and selective determination of ROS levels in viable cells by excluding dead cells from the analysis (Fig. 6A). Exogenously-added DHA induced a significant increase in oxidative stress and cell death in MDA-MB-231 cells, grown in nutrient-rich conditions (Fig. 6A–C). Both NAC and hGX $sPLA_2$ reduced DHA-induced cell death and levels of ROS, confirming that oxidative stress contributes to DHA lipotoxicity and that hGX $sPLA_2$ suppresses DHA lipotoxicity by reducing oxidative stress. Control experiments with H_2O_2-treated MDA-MB-231 cells confirmed that NAC acts as a potent antioxidant (Supp. Fig. 6A and B). Furthermore, prolonged starvation of cells loaded with DHA resulted in a marked elevation of ROS and cell death (Fig. 6D and E). When hGX $sPLA_2$ was added along with DHA in the pre-incubation mixture in nutrient-rich conditions, or when NAC was present during the starvation phase, both cell death and the levels of ROS were reduced (Fig. 6D and E). These results suggest that starvation-induced breakdown of DHA-induced LDs is associated with cell death due to oxidative stress and that the latter is reduced when cells are loaded with DHA in the presence of hGX $sPLA_2$.

Given that OA and LA were not toxic to proliferating cells grown in the presence of serum (Fig. 5D), that the breakdown of OA- and LA-induced LDs was cytoprotective during serum deprivation (Fig. 5E), and that these FAs are the most abundant FA products of hGX $sPLA_2$ activity (Fig. 1B), we assumed that OA and LA would also reduce DHA-induced cell damage. Indeed, co-treatment of proliferating MDA-MB-231 cells with DHA and different concentrations of OA resulted in a reduction of DHA-induced cell death and oxidative stress (Supp. Fig. 6C and D). LA displayed a similar potency in reducing DHA lipotoxicity (Supp. Fig. 6E). These results suggest that OA and LA, within the mixture of products released by hGX $sPLA_2$, play a major role in the protective action of the enzyme against lipotoxic PUFA-induced cell damage.

3.6. LD biogenesis and LD lipolysis reciprocally regulate DHA lipotoxicity

The results presented above indicate that starvation-induced breakdown of PUFA-rich LDs leads to oxidative damage and cell death, suggesting that lipolysis is detrimental to TNBC cells under these conditions. Recent studies in cardiomyocytes and *Drosophila* glial cells have shown that augmenting storage of lipotoxic FAs in LDs reduces their toxicity and that both prevention of LD formation and overstimulated lipolysis may lead to lipotoxic cell damage [19,37]. We thus asked whether suppressing lipolysis by depletion of ATGL reduces DHA-induced cell damage and possibly alters the ability of hGX $sPLA_2$ to protect from lipotoxicity. ATGL depleted cells incubated for 48 h with 100 μM DHA showed a marked increase in LD accumulation (Fig. 7A and B) and a decrease in cell death and oxidative stress in comparison with cells transfected with control siRNA and treated with DHA (Fig. 7C and D). ATGL depletion also potentiated the ability of hGX $sPLA_2$ to stimulate LD accumulation and reduce lipotoxicity and oxidative stress in DHA-treated cells. This is in accordance with the idea that the effects of hGX $sPLA_2$ are dependent on its capacity to stimulate LD formation, thereby channelling exogenous DHA into LDs. Thus, promotion of neutral lipid storage, by hGX $sPLA_2$ phospholipid hydrolysis or by suppression of ATGL-mediated lipolysis, reduces oxidative stress and cell death induced by exogenous DHA.

We next hypothesized that inhibiting TAG synthesis would likely have the opposite effect on DHA toxicity than that observed upon reducing TAG lipolysis. To test this idea, we inhibited DGAT1-mediated TAG synthesis using A922500 [68]. Incubating TNBC cells with A922500 led to a reduction in DHA-induced LD accumulation and potentiation of DHA-induced cell death (Fig. 8A and B). Importantly, DGAT1 inhibition reduced the ability of hGX $sPLA_2$ to stimulate LD accumulation and reduce cell death in DHA-treated cells, suggesting that this activity of hGX $sPLA_2$ is dependent on TAG synthesis and storage in LDs (Fig. 8A and B). Thus, stimulating LD biogenesis by hGX $sPLA_2$ or inhibiting lipolysis by ATGL depletion reduces DHA lipotoxicity. In contrast, interfering with TAG synthesis augments DHA-induced cell damage, suggesting that LDs protect breast cancer cells from PUFA-induced oxidative stress and lipotoxicity.

3.7. hGX $sPLA_2$ reduces the fraction of highly unsaturated PUFA-TAG species stored in LDs, while ATGL depletion does not alter PUFA-TAG composition

The results shown above suggest that hGX $sPLA_2$ and ATGL depletion reduce PUFA lipotoxicity by stimulating their esterification into TAGs and reducing their release from TAGs, respectively. To confirm this hypothesis, we extracted cellular TAGs from DHA- and $sPLA_2$-treated cells and determined their composition by LC-MS. We found that DHA-induced LDs contain elevated amounts of very long-chain and

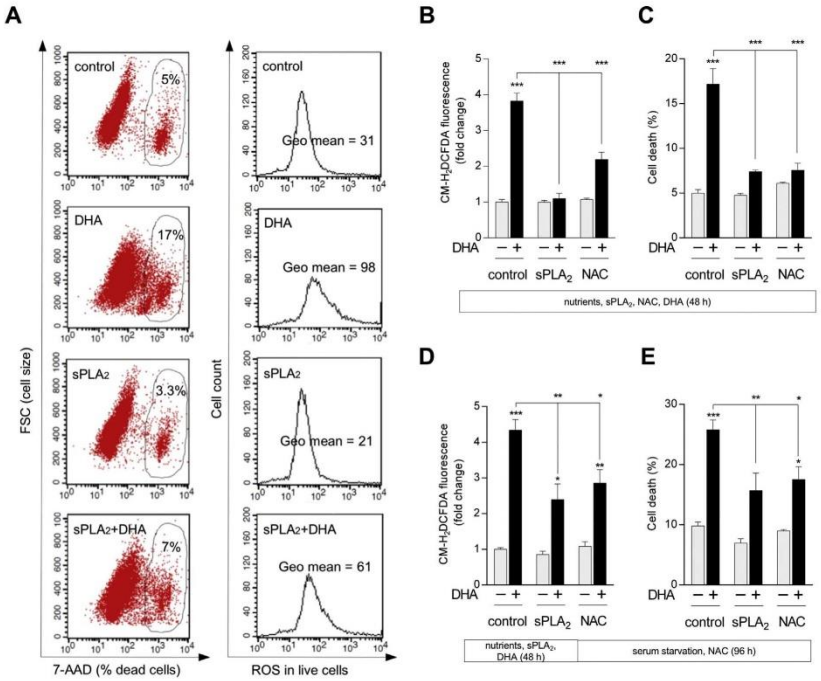

Fig. 6. Breakdown of DHA-induced LDs is associated with elevated lipotoxicity and oxidative stress, which are both suppressed by hGX $sPLA_2$. (A, B, C) MDA-MB-231 cells were treated with combinations of 10 nM hGX $sPLA_2$, 100 μM DHA and 5 mM NAC in complete medium for 48 h. (D, E) Cells treated with 10 nM hGX $sPLA_2$ and 100 μM DHA for 48 h in complete medium were serum-starved for 96 h in the presence of 5 mM NAC. Cellular ROS and cell death were determined using CM-H_2DCFDA and 7-AAD double staining. Dot plots and histograms (A) were taken from a representative experimental analysis and depict the percentage of dead cells (7-AAD positive) and the geometric mean value of CM-H_2DCFDA fluorescence in the remaining live cell population (7-AAD negative). Values on the graphs are means ± SEM of at least three independent experiments and results that are statistically significant over control are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni adjustment).

highly unsaturated TAG species (PUFA-TAGs) (Fig. 9A). In particular, there was a striking increase in the proportion of PUFA-TAGs containing > 7 double bonds (such as 58:8, 58:9, 58:12, 60:10, 60:12, 62:12, 62:13 and 66:18), which are minor species amounting to only 3% in control cells, but are dominant in DHA-treated cells comprising 75% of all TAG species (Fig. 9A and C). In contrast to our expectations, hGX $sPLA_2$ did not increase, but rather reduced the average degree of TAG unsaturation in DHA-treated cells. In fact, we found that hGX $sPLA_2$ elevates the proportion of PUFA-TAGs with < 3 and with 3–7 double bonds (including 54:2, 56:6, 56:7 and 58:7) and reduces PUFA-TAG species with > 7 double bonds (such as 58:12, 60:13, 62:14 and 66:18), thus effectively lowering the fraction of DHA-induced highly unsaturated TAG species to 60% (Fig. 9A and C). Furthermore, DHA caused a significant increase in the fraction of long-chain and highly unsaturated PC species (including 38:6, 40:6 and 40:7) and a decrease in PC species with lower levels of unsaturation (34:1, 36:1 and 36:2). hGX $sPLA_2$ antagonized its effects, significantly elevating PC species with low unsaturation (in particular 34:1, 36:1 and 36:2) and reducing highly unsaturated PC species, such as PC 38:6, thus lowering the fraction of highly unsaturated PC species from 32% to 26% (Fig. 9B and D). Therefore, hGX $sPLA_2$ mitigates the effects of exogenous DHA on MDA-MB-231 cells by enriching LDs with PUFA-TAGs with low and medium degree of unsaturation and significantly reducing the proportion of highly unsaturated fatty acyl chains in the PC and TAG profile.

On the other hand, ATGL depletion resulted in minor changes in the TAG and PC profiles of MDA-MB-231 cells. In DHA-treated cells, there was a slight increase in the average proportion of TAG species with low degree of unsaturation (< 3 double bonds) (Fig. 9E) and no changes in the average unsaturation level of PC (Fig. 9F), despite evident, but minor alterations in the quantity of several individual TAG and PC species (Supp. Fig. 7A and B). Similarly, ATGL depletion did not significantly alter the TAG profile of cells treated with both DHA and hGX $sPLA_2$, although it did result in a minor increase of average PC unsaturation (Fig. 9E and F, and Supp. Fig. 7C). Therefore, ATGL depletion does not significantly affect the TAG and PC composition of DHA- and/or $sPLA_2$-treated MDA-MB-231 cells, suggesting that the reduction in DHA lipotoxicity upon ATGL depletion is based on reduction in lipolysis.

4. Discussion

Cancer cells are often exposed to metabolic and oxidative stress, which may arise from both nutrient deprivation and nutrient excess, and their ability to alleviate stress is crucial for tumour progression [8]. Unsaturated FAs have been shown to reduce the stress associated with hypoxia, nutrient deprivation and oncogene activation [1,7–9,11]. Yet, the sources of exogenous and endogenous FAs, the mechanisms controlling FA uptake and trafficking, and their use by cancer cells have not been fully described. Results of this study suggest that LDs are central regulators of unsaturated FA trafficking and coordinate FA metabolism

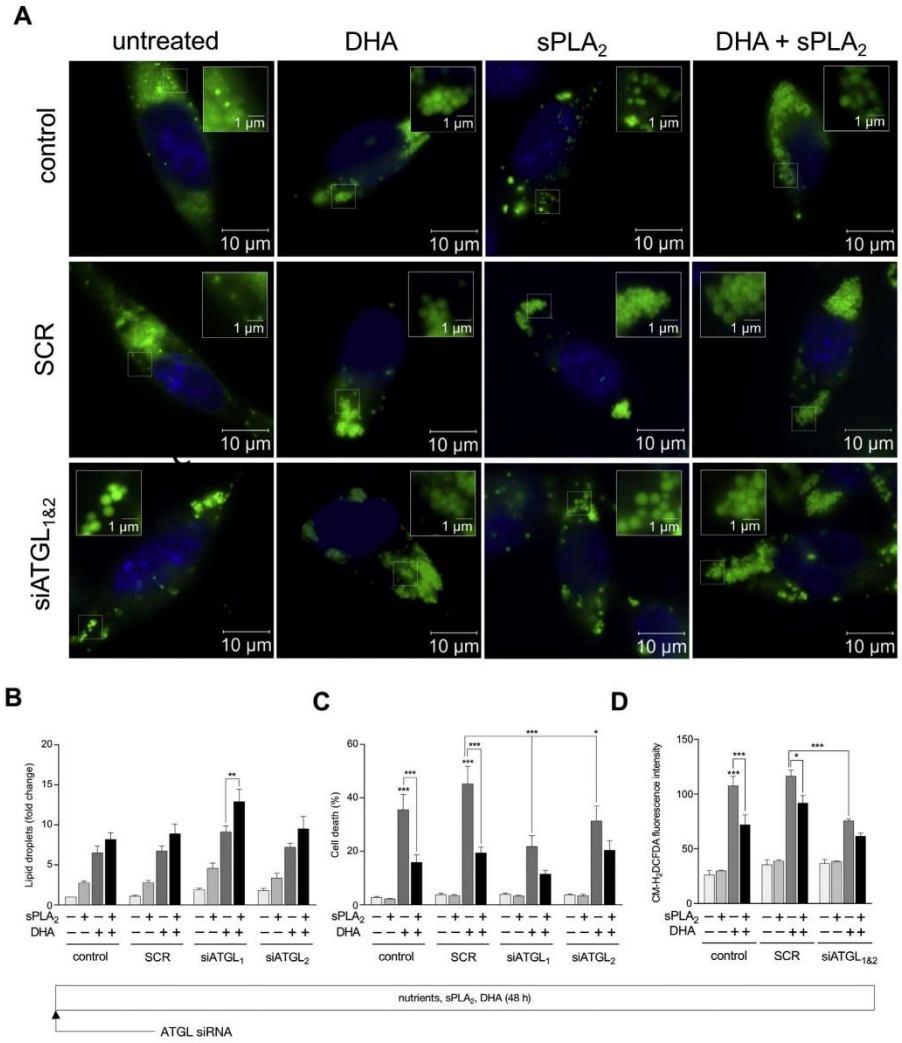

Fig. 7. Suppressing ATGL-mediated lipolysis reduces DHA-induced oxidative stress and lipotoxicity. (A–D) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNAs (siATGL) or non-targeting scrambled siRNA (SCR) and treated with 10 nM hGX $sPLA_2$ and/or 100 µM DHA for 48 h in complete medium. (A) Cells were stained with BODIPY 493/503 and Hoechst stain for visualizing LDs (green) and nuclei (blue), respectively. The images shown are representative of two experiments. Neutral lipids, cell death and ROS were quantified by flow cytometry using Nile red (B), TMRM/YO-PRO-1 (C) and CM-H_2DCFDA (D) staining, respectively. Values on the graphs are means ± SEM of at least three experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; two-way ANOVA with Bonferroni adjustment).

and cell survival during nutrient and lipotoxic stress in TNBC cells. By transiently storing unsaturated FAs in the form of neutral lipids, TNBC cells are not only protected from the acute insult of PUFA-induced oxidative stress, but may also use these PUFAs as energy fuel to enable cell survival during nutrient deprivation. Sequestration of PUFAs in LDs by $sPLA_2$-induced TAG remodelling or retention of PUFAs in LDs by inhibition of ATGL-mediated TAG lipolysis protects TNBC cells from PUFA-induced lipotoxicity (Fig. 10). This segregation of PUFAs in cytoplasmic LDs is also associated with reduced oxidative stress, thus revealing that LDs exert an antioxidative effect, thereby protecting the highly tumourigenic and invasive TNBC cells from oxidative damage by storing (poly)unsaturated FAs esterified in TAGs. Our results suggest that impairment of LD biogenesis and stimulation of lipolysis offer therapeutic strategies for reducing the resistance of TNBC cells to both

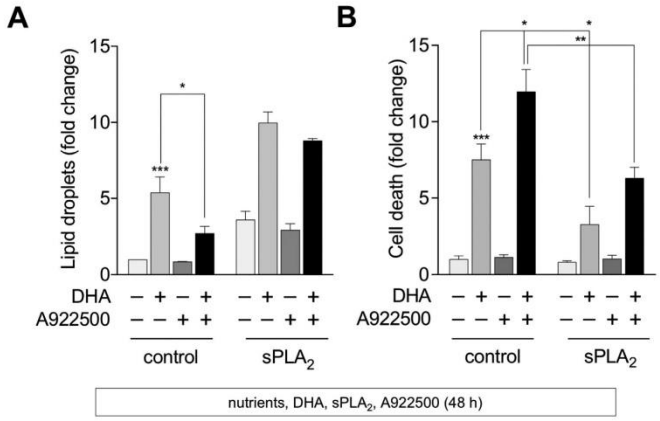

Fig. 8. Inhibition of TAG synthesis augments DHA-induced lipotoxicity. (A, B) MDA-MB-231 cells were grown in complete medium and treated with 10 nM hGX $sPLA_2$ and/or the DGAT1 inhibitor A922500 (1 μM). Neutral lipids and cell death were quantified by flow cytometry using Nile Red (A) and TMRM/YO-PRO-1 staining (B), 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 Bonferroni adjustment).

nutrient and lipotoxic stress.

4.1. *hGX $sPLA_2$ acts through its mono- and polyunsaturated FA products of membrane hydrolysis*

We show here that hGX $sPLA_2$ liberates a mixture of unsaturated FAs, including mono- and polyunsaturated FAs, from breast cancer cell membrane phospholipids. Our lipidomic analyses are in line with previous studies showing that murine and human group X $sPLA_2$ activity on cells and plasma lipoproteins *in vitro* leads to the release of a mixture of lysophospholipids and unsaturated FAs, including PUFAs [44,53,60,69,70]. Importantly, the products of its *in vivo* activity also include ω-3 and ω-6 PUFAs [45]. The ability of the enzyme to release unsaturated FAs from MDA-MB-231 and T-47D breast cancer cells grown in the absence of serum (Fig. 1A–C) suggests a direct phospholipolytic action on the plasma membrane of intact cells. Importantly, we found that, even in the presence of serum, which contains high amounts of lipoproteins, exogenous hGX $sPLA_2$ acts on both serum lipoproteins and cell membranes to release unsaturated FAs and lysophospholipids. The enzyme released high amounts of LPC from both sources, indicating that it acts on both serum-derived and cellular PC, which likely provides the bulk of unsaturated FAs. However, our results strongly suggest that plasma membrane PI is an important substrate for hGX $sPLA_2$ and, importantly, a major source of PUFAs.

We have previously shown that enzymatic activity is necessary for the effects of hGX $sPLA_2$ on LD metabolism and cell survival in TNBC cells and that OA may be one of the major products responsible for its actions [18]. However, exogenous OA could not replicate the pro-survival activity of hGX $sPLA_2$ on T-47D cells, despite inducing relatively high levels of LDs, suggesting the involvement of other $sPLA_2$ hydrolytic products. Indeed, the results of the present study demonstrate that all of the tested mono- and polyunsaturated FA products of hGX $sPLA_2$ activity induce LD formation and support the survival of starved MDA-MB-231 cells. Furthermore, exogenous addition of PUFAs or $sPLA_2$ protected T-47D and MDA-MB-231 cells from starvation-induced cell death, suggesting an important role for $sPLA_2$-released PUFAs in the effects of the enzyme on cell survival. This observation corroborates the idea that all FA products of $sPLA_2$ hydrolysis may contribute to its actions.

hGX $sPLA_2$ may be secreted from cancer or various stromal cells and act on different target membranes in the extracellular milieu [50]. Its ability to liberate a variety of unsaturated FAs, including the essential PUFAs, but not saturated FAs, from cancer or neighbouring stromal cells may be particularly beneficial for hypoxic and nutrient deprived tumour regions where both *de novo* FA synthesis and access to serum lipids are severely compromised [7]. The hydrolytic activity of $sPLA_2$ on cell membranes and lipoproteins also results in the release of lysophospholipids, whose roles in cancer are emerging [2]. It is possible that lysophospholipids also contribute to the effects of $sPLA_2$ on cancer cells, either by providing an additional source of unsaturated FAs or by other mechanisms [2,7,50]. Although the ability of the group X $sPLA_2$ to affect LD metabolism has yet to be demonstrated *in vivo*, we suggest that the mechanisms described in our work are of pathophysiological relevance, based on a) a number of studies demonstrating the ability of group X $sPLA_2$ to affect different aspects of cellular and organismal lipid metabolism [44,47,71], b) its potent enzymatic activity on mammalian plasma membranes and lipoproteins [44,53,60,69,70], c) its ability to release unsaturated FAs, including PUFAs, and affect inflammation and tumourigenesis *in vivo* [45,48], c) its expression in immune cells and overexpression in tumours of patients with invasive breast carcinoma and other cancers [49,50] and d) the increasing recognition of the importance of unsaturated FA uptake, FA oxidation, LD accumulation and lipolysis as critical pro-tumourigenic pathways in various cancers [3,5–7,9,11,26,28]. Our results identify hGX $sPLA_2$ as a modulator of mono- and polyunsaturated FA trafficking and LD metabolism that augments the resistance of Ras-driven TNBC cells to nutrient and oxidative stress. However, the mechanisms described here may extend to other cancer types and may be relevant for the general cellular stress response in different tissues and pathophysiological settings, in particular those associated with aberrant lipid accumulation.

4.2. *Exogenous and $sPLA_2$-derived unsaturated FAs induce LD formation and protect breast cancer cells from nutrient stress*

One of the most important findings of this work is that all tested unsaturated FAs, including ω-3 and ω-6 PUFAs, used at low micromolar concentrations, protect TNBC cells from starvation-induced cell death and only high micromolar concentrations of PUFAs, but not OA, induce lipotoxic damage to the cells. This was surprising in light of the generally accepted notion of an anti-cancer activity of ω-3 PUFAs, which induce cell death in a wide range of tumour cells, including breast cancer cells [64,65]. However, most studies, including many studying the effects of PUFAs on MDA-MB-231 cells [65,72–74] used only high micromolar concentrations of FAs and typically did not test different conditions of nutrient availability or studied cell survival during starvation. Although it is difficult to estimate the local, physiological

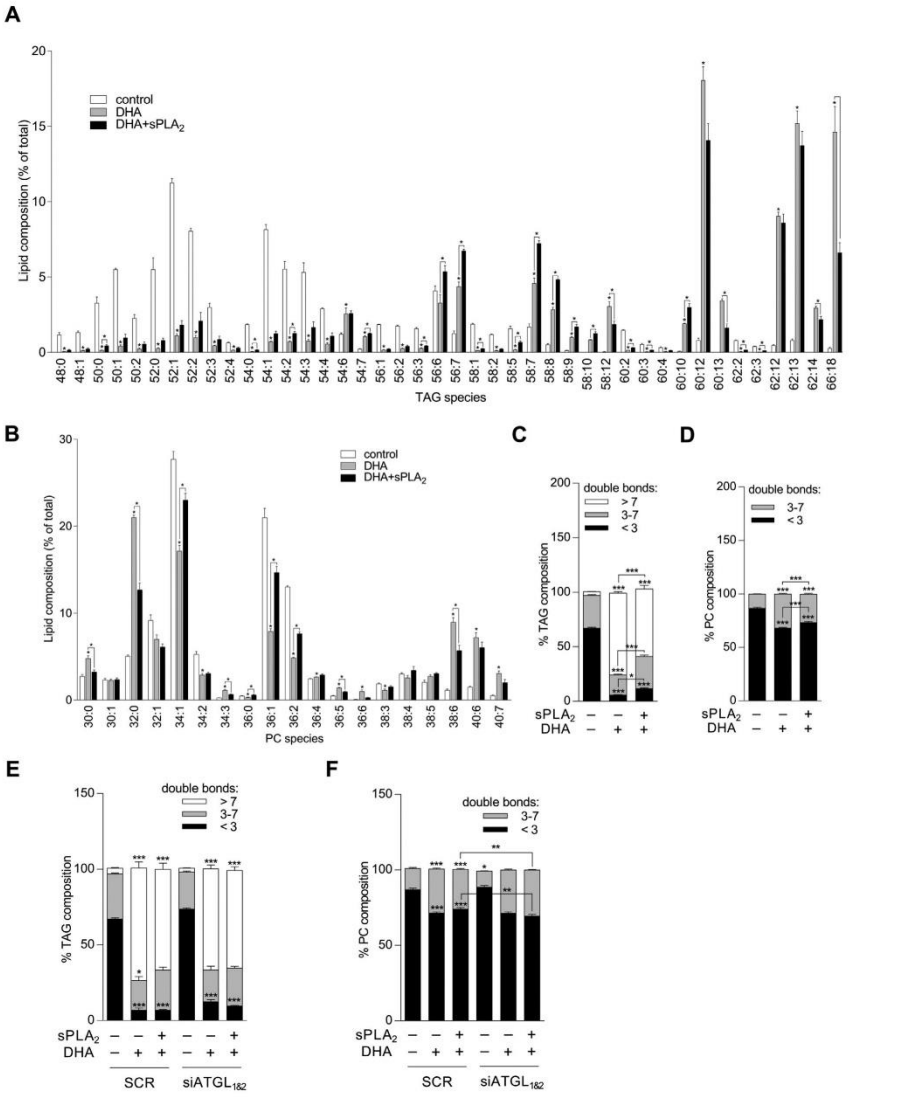

Fig. 9. hGX $sPLA_2$ reduces the proportion of highly unsaturated PUFA-TAGs in DHA-treated cells. (A–D) MDA-MB-231 cells were grown in complete medium and treated with 10 nM hGX $sPLA_2$ and/or 100 μM DHA for 48 h. (E, F) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) or non-targeting scrambled siRNA (SCR) and treated with 10 nM hGX $sPLA_2$ and/or 100 μM DHA for 48 h in complete medium. Cells were lysed and lipids extracted for UPLC/qToF-MS analysis. Values on the graphs are means ± SEM of three experiments and results that are statistically significant are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; (A, B) unpaired *t*-test; (C–F) two-way ANOVA with Bonferroni adjustment).

concentrations of exogenous FAs available in the tumour microenvironment or to correlate those with *in vitro* experimental settings [7], recent lipidomic studies have provided some clues. The total free (non-esterified) FA concentration in human plasma is approximately 200 μM after overnight fasting, but the concentrations of individual FA species differ significantly [75]. OA is the most abundant FA in human plasma (80 μM), followed by LA (15 μM), AA (3 μM), DHA (1 μM) and EPA (0.4 μM). OA is also one of the most abundant FAs incorporated into membrane phospholipids, including those of MDA-MB-231 cells [61]. Thus, the low micromolar and cytoprotective concentrations of

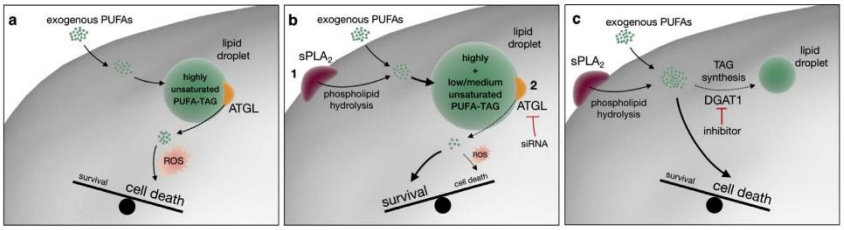

Fig. 10. Antioxidant roles of hGX $sPLA_2$ and LDs in breast cancer cells exposed to a surplus of exogenous PUFAs. Ras-driven TNBC cells exposed to excess PUFAs accumulate LDs containing highly unsaturated PUFA-TAG species. (a) Starvation-induced breakdown of these LDs is associated with ATGL-dependent increase in oxidative stress and cell death. (b) PUFA toxicity may be reduced by two complementary mechanisms that lower PUFA-induced oxidative stress: (1) hGX $sPLA_2$ membrane phospholipid hydrolysis leads predominantly to the release of unsaturated FAs with low unsaturation levels, such as OA and LA, and stimulates their incorporation into TAGs, thus reducing the fraction of highly unsaturated PUFA-TAGs stored in LDs; hGX $sPLA_2$ also induces PC remodelling and reduces the average unsaturation level of membrane phospholipids; (2) suppression of TAG lipolysis by ATGL depletion, which reduces PUFA release from LDs. (c) In contrast, suppression of TAG synthesis by inhibiting DGAT1 augments PUFA-induced cell damage and reduces the ability of hGX $sPLA_2$ to alleviate PUFA lipotoxicity.

PUFAs used in the present study are likely near or slightly above their plasma levels, while the high micromolar, cytotoxic concentrations of EPA, DHA and AA used here (100 μM) are roughly 30–250-fold higher than their respective concentrations in plasma. Therefore, starvation-induced breakdown of LDs induced by physiologically relevant concentrations of unsaturated FAs and hGX $sPLA_2$ is associated with the prevention of cell death in aggressive Ras-driven breast cancer cells. It remains to be established whether this is also true for other cancer cells with activating Ras mutations, but given their propensity for opportunistic modes of nutrient acquisition and metabolic plasticity such a scenario seems highly likely [1,7–9]. Finally, our results suggest that the dual, concentration-dependent effects of ω-3 and ω-6 PUFAs described here must be taken into account when designing and interpreting experiments or clinical trials in various chemopreventive or therapeutic settings.

4.3. ATGL-mediated TAG lipolysis is not necessary for cell survival during prolonged serum deprivation

Our results suggest that ATGL-mediated TAG lipolysis contributes to, but is not essential for cell survival during prolonged serum starvation of Ras-driven MDA-MB-231 TNBC cells. ATGL deficiency clearly reduced TAG lipolysis and LD breakdown and compromised cell survival, but it did not result in massive cell death during starvation. Intriguingly, although ATGL depletion significantly increased the total amount of neutral lipids remaining after starvation in cells pre-treated with $sPLA_2$ or unsaturated FAs, it did not abolish their pro-survival activity. These findings together with the facts that 1) hGX $sPLA_2$ retained its ability to induce glycerol release in ATGL depleted cells and that 2) ATGL depletion did not significantly reduce the fraction of $sPLA_2$-induced LDs broken down during the starvation period (Fig. 3E), strongly suggest the involvement of other lipases and/or other processes (*e.g.* lipophagy/autophagy) in serum starvation-induced LD breakdown and cell survival in TNBC cells. Indeed, the role of lipolysis, autophagy and lipophagy in cell survival during starvation may depend on the type of nutrients being restricted, the length of starvation (acute, prolonged) and on the particular cell type [13,33,62,63,76]. Furthermore, it is conceivable that the relative contribution of autophagy/lipophagy *vs.* lipolysis may be altered depending on the availability of exogenous lipids and the amount and composition of pre-existing LDs at the onset of starvation. Notably, in acutely starved mouse embryonic fibroblasts (*e.g.* hours in HBSS) autophagic digestion of cellular membranes provides FAs for DGAT1-dependent LD synthesis [33,76]. Although ATGL is indispensable for the delivery of FAs from LDs to mitochondria under these conditions [33], the primary role of LDs may be the prevention of lipotoxicity of autophagy-derived FAs rather than delivery of LD-derived FAs to mitochondria for cell survival [76]. On the other hand, during milder, but prolonged starvation (*e.g.* days of serum deprivation) lipophagy and other forms of autophagy may also be induced and become dominant [13,63,77]. Intriguingly, a recent study in MDA-MB-231 cells has shown that during prolonged low glucose starvation in the absence of exogenous lipids, autophagy of bulk membrane phospholipids may be critical for supplying FAs to mitochondria and cell survival [63]. Finally, our results are in agreement with a recent study showing that ATGL-mediated LD breakdown in breast cancer cells exposed to adipose-derived FAs is important for cancer invasiveness, but not for cell growth or survival [29]. The authors suggest that ATGL is critical for the ability of cancer cells to progressively release FAs from LDs and use them when needed to sustain the invasiveness of circulating tumour cells. Clearly, the relative importance of autophagy, lipophagy and TAG lipolysis for the pro-survival activity of unsaturated FAs and $sPLA_2$ in TNBC breast cancer cells exposed to nutrient stress remains to be established in further studies.

Interestingly, suppression of both β-oxidation and ATGL-mediated lipolysis was more detrimental to the cells than inhibiting either process alone. Of note, a low, non-toxic concentration of etomoxir was used (50 μM), which is sufficient to reduce the effects of $sPLA_2$, both on LD formation and cell survival [18], but presumably does not fully block β-oxidation during prolonged serum starvation. In fact, it has been shown that a 5–10-fold higher concentration of etomoxir is necessary to cause LD build-up and cell death in starved MDA-MB-231 cells [18]. A partial inhibition of β-oxidation in ATGL-depleted cells may lead to a compensatory increase in LD breakdown by other lipases [17,33], or by lipophagy [13]. This may explain the reduction of LD content caused by etomoxir both in $sPLA_2$-treated and in ATGL-depleted cells. It is also possible that CPT1A activity is in fact necessary for the ability of $sPLA_2$ to induce LD formation, as suggested previously [18]. Indeed, reducing CPT1A activity or mitochondrial fusion has been shown to reduce FA uptake or activate a mechanism of FA expulsion from cells to prevent FA lipotoxicity [33,78].

4.4. Lipid droplets are antioxidant and pro-survival organelles

Cells protect themselves from FA lipotoxicity by upregulating β-oxidation and neutral lipid storage in LDs [15,79]. Directing FAs to LDs protects against lipid-induced cell damage in different cells and tissues. For example, reducing TAG synthesis by DGAT1 depletion leads to cell death in fibroblasts exposed to exogenous OA, while sequestration of palmitate into LDs by co-supplementation with OA protects from palmitate-induced cell death [15]. DGAT1-dependent LD biogenesis also reduces the lipotoxicity of excess endogenous FAs, liberated by

starvation-induced autophagic degradation of membranous organelles [76]. In the same study, DGAT1-depletion led to a concomitant reduction in TAGs and cholesteryl esters, suggesting a tight association between the biogenesis of TAG-rich LDs and cholesterol ester synthesis and storage [76]. Elevated cholesteryl ester accumulation has been associated with the aggressiveness of several cancers, including prostate and breast cancer [80,81], and high acyl-CoA:cholesterol acyltransferase 1 (ACAT1) expression has been linked to increased MDA-MB-231 cell proliferation in the presence of exogenous lipids [82]. We found that hGX $sPLA_2$ reduces total cholesterol levels, which is in line with its negative effect on lipogenesis, in particular the reduction in SREBP-1 and hydroxymethylglutaryl-coenzyme A reductase (HMGCR) gene expression (Fig. 2E and [18]). However, in accordance with its inability to alter ACAT1 (*SOAT1*) gene expression [18], hGX $sPLA_2$ did not affect cholesteryl ester content, suggesting that its effects on TNBC cells mostly depend on changes in TAG storage. However, high concentrations of OA and DHA induced a significant increase in cholesteryl ester accumulation. Thus, we cannot exclude a possible contribution of cholesteryl ester metabolism to the stress response of TNBC cells exposed to high levels of exogenous unsaturated FAs.

The results of the current study indicate that sequestration of exogenous PUFAs into TAGs stored in LDs of MDA-MB-231 breast cancer cells protects from oxidative stress and cell death. Indeed, knock-down of ATGL expression and exogenous $sPLA_2$ treatment suppressed PUFA toxicity and were associated with increased LD content, whereas, on the contrary, an increased sensitivity to PUFAs was observed upon suppression of TAG synthesis by inhibition of DGAT1. Furthermore, the protection from PUFA toxicity by hGX $sPLA_2$ was associated with extensive TAG remodelling: an enrichment of LDs with PUFA-TAG species with low to medium unsaturation levels and a reduction in the fraction of the most highly unsaturated PUFA-TAG species. In effect, this results in dilution of the most oxidation-prone PUFA-TAGs within LDs and reduces their availability for lipolytic release. This could explain the ability of hGX $sPLA_2$ to suppress the rise in mitochondrial membrane potential and oxidative stress associated with the breakdown of PUFA-rich LDs during starvation. Additionally, lipid loading with DHA in combination with OA or LA was associated with a reduction in lipotoxicity, suggesting that the effects of hGX $sPLA_2$ are mediated by these abundant FAs with the lowest degree of unsaturation among its products of membrane hydrolysis. Indeed, the enrichment of LDs with PUFA-TAG species with low and medium unsaturation caused by hGX $sPLA_2$ in DHA-treated cells strongly suggests that $sPLA_2$-released OA and LA are extensively incorporated into LDs. Interestingly, OA- and LA-rich LDs were more readily broken down during starvation in comparison with PUFA-rich LDs, suggesting that MDA-MB-231 cells likely refrain from using peroxidation-prone PUFAs for β-oxidation and cell survival during starvation. It is thus possible that hGX $sPLA_2$-induced TAG remodelling both segregates cell-damaging PUFAs in LDs and simultaneously increases the availability of harmless FAs with lower degree of unsaturation for β-oxidation. Finally, hGX $sPLA_2$ also suppressed the substantial DHA-induced increase in acyl chain unsaturation in membrane PC species, suggesting that membrane phospholipid remodelling also contributes to its ability to protect from PUFA lipotoxicity. In accordance, increasing the relative degree of membrane (poly)unsaturation by inhibition of *de novo* lipogenesis sensitizes cancer cells to lipid peroxidation and oxidative stress-induced cell death [83].

The fact that ATGL depletion reduces DHA-induced oxidative stress and cell death suggests that TAG lipolysis contributes to PUFA lipotoxicity. ATGL depletion augmented DHA-induced LD accumulation, but it did not alter the TAG profiles of control, DHA- or $sPLA_2$-treated cells. This is in line with the lack of significant TAG acyl chain specificity reported for the enzyme [84]. Thus, the reduced PUFA toxicity in ATGL-deficient cells is most likely a consequence of an overall reduction in TAG lipolysis and therefore cellular unesterified FA content [85]. These results are in accordance with recent studies showing that excessive TAG lipolysis may cause cell damage, presumably by increasing the pool of cytosolic FAs and stimulating β-oxidation and ROS production [86,87]. Namely, stimulation of lipolysis by overexpression of ATGL leads to ER stress in cardiomyocytes treated with the otherwise non-toxic OA [37]. Furthermore, inhibition of ATGL-mediated lipolysis by perilipin 5 suppresses hepatic lipotoxic injury [88] and protects hearts against oxidative stress induced by excessive β-oxidation and FA peroxidation [86,89]. In summary, our results demonstrate that while ATGL supports, but is not essential for cell survival during starvation, it also contributes to lipotoxicity when TNBC cells are challenged with high concentrations of lipotoxic FAs. Thus, the level of expression and activity of ATGL in cancer cells may directly affect cell fate, particularly in stressful conditions, and a reduction in ATGL expression may be beneficial for tumour progression, as reported recently [30]. Finally, our results are in line with the recently reported antioxidant function of LDs in *Drosophila* glial cells [19] in showing that sequestration of PUFAs into LDs protects from oxidative damage and reduces cell death in cancer cells exposed to excess exogenous PUFAs.

5. Conclusion

Cancer cells often depend on exogenous unsaturated FAs for survival during stressful conditions. Our results suggest that LDs coordinate unsaturated FA uptake, storage and use with cell survival mechanisms during nutrient and lipotoxic stress in TNBC cells. LDs transiently store unsaturated FAs, including ω-3 and ω-6 PUFAs, thus providing protection against lipotoxicity and nutrient deprivation by releasing them gradually when needed. Furthermore, we have found that TNBC cells are protected from PUFA lipotoxicity when PUFAs are sequestered in LDs either by $sPLA_2$-induced TAG synthesis and remodelling or by inhibition of ATGL-mediated TAG lipolysis. The capacity of cells to balance unsaturated FA sequestration and release from LDs is thus important for their ability to balance FA-induced cell survival and lipotoxicity. By managing the storage and release of (poly) unsaturated FAs, LDs play an important antioxidant role and protect TNBC cells from stress associated with both nutrient excess and nutrient deprivation. Inhibition of TAG synthesis and LD formation and promotion of lipolysis are thus potential therapeutic strategies in TNBC and likely in other aggressive Ras-driven cancers.

Transparency document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary data

Supplementary data to this article containing Supplementary Tables 1–4 and Supplementary Figures 1–7 can be found online at https://doi.org/10.1016/j.bbalip.2017.12.006.

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4.2 Lipidomic Data on Lipid Droplet Triglyceride Remodeling Associated with Protection of Breast Cancer Cells from Lipotoxic Stress

This paper was prepared to present the full lipidomic data from the research article entitled "Lipid droplets induced by secreted phospholipase A_2 and unsaturated fatty acids protect breast cancer cells from nutrient and lipotoxic stress". Authors of this paper are Eva Jarc, Thomas O. Eichmann, Robert Zimmermann and Toni Petan. The paper was published in the journal *Data in brief* in 2018.

We have previously reported that elevated lipid droplet accumulation is characteristic of aggressive breast cancer cells and support cancer cell survival during nutrient and lipotoxic stress (Jarc, Kump, et al., 2018). We found that changes in lipid composition of membrane phospholipids and lipid droplets mediated by $sPLA_2$ activity and/or ATGL depletion are associated with protection from the lipotoxicity of PUFAs. Here, we provide complete lipidomic datasets acquired by Ultra-Performance Liquid Chromatography/Quadrupole Time-of-Flight Mass Spectrometry (UPLC/qTOF-MS). We analyzed the lipid composition of TAG and phosphatidylcholine (PC) species extracted from MDA-MB-231 cells treated with the exogenous ω-3 PUFA docosahexaenoic acid (DHA) and $sPLA_2$ and depleted in ATGL. The data describes changes in lipid composition following inhibition of TAG lipolysis and lipid droplet breakdown by ATGL depletion, and changes in lipid composition caused by $sPLA_2$ activity on breast cancer cells (Jarc, Eichmann, Zimmermann, & Petan, 2018).

We found that the protection from PUFA lipotoxicity by $sPLA_2$ is associated with extensive TAG remodeling: an enrichment of lipid droplets with PUFA-TAG species with low to medium unsaturation levels and a reduction in the fraction of the most highly unsaturated PUFA-TAG species (Jarc, Kump, et al., 2018). In the dataset of TAG profile of DHA-treated cells we found a striking increase in the proportion of PUFA-TAG species containing > 7 double bonds, such as 58:12, 60:13, 62:11, 62:14 and 66:18. We also found that treating TNBC cells with DHA affects PC composition by increasing the amount of PC species with > 5 double bonds, such as 36:6, 38:6, 40:6 and 40:7. Treating TNBC cells with $sPLA_2$ alongside DHA led to a significant reduction in PUFA-TAG and PUFA-PC content. We found that $sPLA_2$ elevates the proportion of PUFA-TAGs with < 3 and 3–7 double bonds, such as 54:2, 56:6, 56:7 and 58:7, and reduces the amount of PUFA-TAGs with > 7 double bonds. It also elevates the proportion of PC species with low unsaturation, in particular 34:1, 36:1 and 36:2, and reduces the fraction of highly unsaturated PCs, such as 36:6, 38:6, 40:6 and 40:7 (Jarc, Eichmann, et al., 2018).

On the other hand, we found that the protection from PUFA lipotoxicity by ATGL depletion is not associated with extensive TAG remodeling (Jarc, Kump, et al., 2018). We observed only minor alterations in TAG and PC species in ATGL depleted cells treated with DHA and/or $sPLA_2$ (Jarc, Eichmann, et al., 2018). This suggests that the reduction of DHA toxicity in ATGL depleted cells is based on the reduction in the release of DHA from lipid droplets.

I contributed to this work by analyzing lipidomic data and preparing final datasets for publication. I also wrote and prepared this manuscript for publication.

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Data article

Lipidomic data on lipid droplet triglyceride remodelling associated with protection of breast cancer cells from lipotoxic stress

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ABSTRACT

The data presented here is related to the research article entitled "Lipid droplets induced by secreted phospholipase A_2 and unsaturated fatty acids protect breast cancer cells from nutrient and lipotoxic stress" by E. Jarc et al., Biochim. Biophys. Acta 1863 (2018) 247–265. Elevated uptake of unsaturated fatty acids and lipid droplet accumulation are characteristic of aggressive cancer cells and have been associated with the cellular stress response. The present study provides lipidomic data on the triacylglycerol (TAG) and phosphatidylcholine (PC) composition of MDA-MB-231 breast cancer cells exposed to docosahexaenoic acid (DHA; 22:6, ω-3). Datasets provide information on the changes in lipid composition induced by depletion of adipose triglyceride lipase (ATGL) and by exogenous addition of secreted phospholipase A_2 ($sPLA_2$) in DHA-treated cells. The presented alterations in lipid composition, mediated by targeting lipid droplet biogenesis and lipolysis, are associated with protection from lipotoxicity and allow further

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investigation into the role of lipid droplets in the resistance of cancer cells to lipotoxic stress.

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Specifications Table

Subject area	*Biochemistry and Molecular Biology*
More specific subject area	*Lipidomics*
Type of data	*Tables*
How data was acquired	*Ultra-performance liquid chromatography-quadrupole time-of-flight mass spectrometry (UPLC/qTOF-MS)*
Data format	*Analysed*
Experimental factors	*Analysis of triacylglycerol and phosphatidylcholine species extracted from MDA-MB-231 cells treated with exogenous docosahexaenoic acid, human group X secreted phospholipase A_2 and depleted in adipose triglyceride lipase*
Experimental features	*Lipidomic analyses of cellular lipids by UPLC/qTOF-MS*
Data source location	*Not applicable*
Data accessibility	*Provided with this article*

Value of the data

- This lipidomic data provides information on the lipid composition of Ras-driven aggressive MDA-MB-231 breast cancer cells exposed to nutrient stress [1].
- It identifies changes in membrane and lipid droplet (LD) composition induced by alterations in LD biogenesis and lipolysis and associated with protection from the lipotoxicity of polyunsaturated fatty acids.
- The data describes triacylglycerol (TAG) and phosphatidylcholine (PC) remodelling mediated by human group X secreted phospholipase A_2 ($sPLA_2$) in docosahexaenoic acid (DHA)-treated and adipose triglyceride lipase (ATGL)-depleted breast cancer cells.
- The data describes changes in lipid composition following inhibition of TAG lipolysis and LD breakdown by ATGL depletion in DHA-treated breast cancer cells.
- The data will foster further examination of the role of LDs in the resistance of cancer cells to nutrient and lipotoxic stress.

1. Data

In order to determine the changes in TAG composition of LDs and in the profile of cellular PC associated with $sPLA_2$-induced protection of breast cancer cells from DHA lipotoxicity, we extracted lipids from MDA-MB-231 cells treated with 100 μM DHA and a combination of 100 μM DHA and 10 nM $sPLA_2$ and performed lipidomic analyses [1]. The TAG and PC profiles determined are presented in Tables 1 and 2, respectively.

In order to determine the changes in TAG composition of LDs and in the profile of cellular PC in ATGL-depleted breast cancer cells, MDA-MB-231 cells were transfected with ATGL-targeting siRNA or non-targeting scrambled siRNA (SCR). In addition, cells were treated with 10 nM $sPLA_2$ and 100 μM DHA. Lipids were extracted and lipidomic analyses performed. The data presented includes the

Table 1
TAG profiles of control, DHA- and $sPLA_2$-treated MDA-MB-231 breast cancer cells.

TAG composition (% of total)[a]			
TAG species[b]	Untreated[c]	DHA	DHA+$sPLA_2$
46:0	0.36 ± 0.03	n.d.	0.05 ± 0.02
48:0	1.2 ± 0.1	0.07 ± 0.01	0.16 ± 0.03
48:1	1.3 ± 0.1	0.12 ± 0.01	0.2 ± 0.1
50:0	3.3 ± 0.4	0.14 ± 0.02	0.4 ± 0.1
50:1	5.5 ± 0.1	0.4 ± 0.1	1.0 ± 0.2
50:2	2.3 ± 0.2	0.2 ± 0.1	0.6 ± 0.1
52:0	6.0 ± 1.0	0.26 ± 0.04	0.8 ± 0.1
52:1	11.3 ± 0.3	1.1 ± 0.1	1.8 ± 0.3
52:2	8.1 ± 0.2	1.0 ± 0.1	2.0 ± 1.0
52:3	3.0 ± 0.3	0.44 ± 0.03	0.9 ± 0.2
52:4	0.64 ± 0.03	0.15 ± 0.01	0.3 ± 0.1
54:0	1.8 ± 0.1	0.06 ± 0.01	0.17 ± 0.01
54:1	8.2 ± 0.3	0.72 ± 0.02	1.3 ± 0.2
54:2	6.0 ± 1.0	0.7 ± 0.1	1.3 ± 0.1
54:3	5.0 ± 1.0	0.8 ± 0.1	1.7 ± 0.4
54:4	2.9 ± 0.1	0.6 ± 0.1	1.1 ± 0.2
54:5	2.4 ± 0.1	n.d.	n.d.
54:6	1.2 ± 0.1	2.6 ± 0.4	2.6 ± 0.2
54:7	0.22 ± 0.03	1.1 ± 0.1	1.26 ± 0.03
56:0	1.0 ± 0.1	n.d.	0.10 ± 0.01
56:1	1.85 ± 0.02	0.16 ± 0.03	0.23 ± 0.03
56:2	1.8 ± 0.1	0.26 ± 0.02	0.4 ± 0.1
56:3	1.6 ± 0.1	0.28 ± 0.02	0.43 ± 0.03
56:6	4.1 ± 0.3	3.0 ± 1.0	5.4 ± 0.4
56:7	1.2 ± 0.2	4.4 ± 0.3	6.7 ± 0.1
58:1	1.9 ± 0.1	0.120 ± 0.004	0.25 ± 0.01
58:2	1.19 ± 0.02	0.12 ± 0.02	0.23 ± 0.03
58:3	0.6 ± 0.1	n.d.	0.17 ± 0.02
58:5	1.6 ± 0.2	0.17 ± 0.02	0.7 ± 0.1
58:6	2.5 ± 0.2	n.d.	n.d.
58:7	1.7 ± 0.2	4.6 ± 0.4	7.2 ± 0.2
58:8	0.5 ± 0.1	2.8 ± 0.2	4.8 ± 0.1
58:9	0.12 ± 0.02	1.00 ± 0.03	1.7 ± 0.1
58:10	n.d.	0.83 ± 0.02	1.3 ± 0.1
58:11	0.02 ± 0.01	0.91 ± 0.04	1.1 ± 0.1
58:12	0.03 ± 0.02	3.1 ± 0.3	1.9 ± 0.2
60:1	2.21 ± 0.04	n.d.	0.31 ± 0.04
60:2	1.47 ± 0.03	0.146 ± 0.002	0.31 ± 0.02
60:3	0.5 ± 0.1	0.04 ± 0.01	0.16 ± 0.01
60:4	0.32 ± 0.04	0.05 ± 0.02	0.12 ± 0.02
60:10	0.07 ± 0.01	1.91 ± 0.02	3.0 ± 0.2
60:12	0.8 ± 0.1	18.0 ± 1.0	14.1 ± 1.1
60:13	n.d.	3.4 ± 0.1	1.6 ± 0.3
62:1	0.9 ± 0.1	n.d.	n.d.
62:2	0.79 ± 0.03	0.05 ± 0.01	0.163 ± 0.003
62:3	0.40 ± 0.02	0.027 ± 0.002	0.112 ± 0.004
62:11	n.d.	1.1 ± 0.2	1.5 ± 0.2
62:12	0.5 ± 0.1	9.0 ± 0.3	9.0 ± 1.0
62:13	0.8 ± 0.1	15.0 ± 1.0	14.0 ± 1.0
62:14	n.d.	2.9 ± 0.1	2.2 ± 0.2
66:18	0.3 ± 0.1	15.0 ± 2.0	7.0 ± 1.0

[a] TAG composition data is presented as mean percentages of each species per total detected species and normalised to total cellular protein.

[b] TAG species are described based on the number of carbon atoms and double bonds.

[c] MDA-MB-231 cells were treated with 100 μM DHA and 10 nM $sPLA_2$ in complete medium for 48 h and cell lysates were collected for UPLC/MS analysis. Values are means ± SEM of three independent experiments; n.d., not determined.

Table 2
PC profiles of control, DHA- and $sPLA_2$-treated MDA-MB-231 breast cancer cells.

PC composition (% of total)[a]			
PC species[b]	Untreated[c]	DHA	DHA + $sPLA_2$
30:0	2.7 ± 0.2	4.8 ± 0.3	3.2 ± 0.2
30:1	2.3 ± 0.2	2.3 ± 0.1	2.4 ± 0.2
32:0	5.0 ± 0.1	21.0 ± 0.3	13.0 ± 1.0
32:1	9.0 ± 1.0	7.0 ± 1.0	6.1 ± 0.3
34:1	28.0 ± 1.0	17.0 ± 1.0	23.0 ± 1.0
34:2	5.3 ± 0.3	2.9 ± 0.2	3.1 ± 0.1
34:3	0.26 ± 0.01	1.1 ± 0.1	0.6 ± 0.1
36:0	0.47 ± 0.04	0.31 ± 0.02	0.6 ± 0.1
36:1	21.0 ± 1.0	7.9 ± 0.3	15.0 ± 1.0
36:2	13.0 ± 0.1	4.9 ± 0.1	7.6 ± 0.2
36:4	2.46 ± 0.04	2.65 ± 0.01	2.9 ± 0.1
36:5	0.48 ± 0.04	1.4 ± 0.1	0.9 ± 0.1
36:6	0.07 ± 0.01	1.0 ± 0.2	0.29 ± 0.01
38:3	1.9 ± 0.1	1.1 ± 0.1	1.5 ± 0.1
38:4	3.0 ± 0.1	2.6 ± 0.3	3.4 ± 0.4
38:5	2.0 ± 0.3	2.7 ± 0.2	3.1 ± 0.1
38:6	1.1 ± 0.1	9.0 ± 1.0	6.0 ± 1.0
40:6	1.5 ± 0.1	7.0 ± 1.0	6.0 ± 1.0
40:7	0.5 ± 0.1	3.1 ± 0.3	2.0 ± 0.3

[a] PC composition data is presented as mean percentages of each species per total detected species and normalised to total cellular protein.
[b] PC species are described based on the number of carbon atoms and double bonds.
[c] MDA-MB-231 cells were treated with 100 μM DHA and 10 nM $sPLA_2$ in complete medium for 48 h and cell lysates were collected for UPLC/MS analysis. Values are means ± SEM of three independent experiments; n.d., not determined.

detected TAG (Table 3) and PC species (Table 4) in control (SCR) and ATGL-depleted MDA-MB-231 breast cancer cells (siATGL), either untreated or treated with exogenous DHA, $sPLA_2$ or both.

2. Experimental design, materials and methods

2.1. Materials

Breast cancer cells (MDA-MB-231) and culture medium (RPMI-1640) were obtained from ATCC (USA), fetal bovine serum (FBS), Dulbecco's phosphate-buffered saline (DPBS), TrypLE Select and Opti-MEM from Life Technologies (USA). Docosahexaenoic acid (DHA) was from Cayman Chemical (USA), Lipofectamine RNAiMAX from Thermo Fisher Scientific (USA). Human ATGL-targeting siRNAs and Allstars Negative Control siRNA were from Qiagen (Germany). The recombinant wild-type human group X $sPLA_2$ was prepared as described previously [2–4]. All other chemicals were of at least analytical grade and purchased from Sigma-Aldrich (USA) or Serva (Germany).

2.2. Cell culture and treatment

MDA-MB-231 cells were cultured in RPMI-1640 medium in the presence of 10% FBS. Adherent cells were detached using TrypLE Select. Aliquots of stock solutions of DHA in absolute ethanol were stored under argon at −80 °C. Prior to addition to cell culture, DHA was incubated in complete medium for 1 h at room temperature. Cells were seeded in complete medium in 6-well plates at 3×10^5 cells/well. ATGL silencing was performed by reverse transfection at the time of seeding using a 20 nM mixture of two validated siRNAs targeted at ATGL (Qiagen) or 20 nM Allstars Negative Control siRNA (Qiagen). Transfection complexes were generated using 7.5 μl/well of Lipofectamine RNAiMAX and Opti-MEM

Table 3
TAG profiles of MDA-MB-231 breast cancer cells depleted in ATGL and treated with exogenous DHA, $sPLA_2$ or both.

	TAG composition (% of total)[a]					
TAG species[b]	SCR[c]	siATGL	SCR+DHA	siATGL+DHA	SCR+DHA+$sPLA_2$	siATGL+DHA+$sPLA_2$
46:0	0.30 ± 0.03	0.42 ± 0.02	0.031 ± 0.003	0.05 ± 0.01	0.03 ± 0.01	0.03 ± 0.01
48:0	1.3 ± 0.1	1.9 ± 0.1	0.11 ± 0.02	0.16 ± 0.01	0.08 ± 0.01	0.14 ± 0.01
48:1	1.19 ± 0.02	1.5 ± 0.1	0.16 ± 0.02	0.26 ± 0.04	0.15 ± 0.03	0.17 ± 0.03
50:0	3.8 ± 0.3	5.41 ± 0.03	0.3 ± 0.1	0.5 ± 0.1	0.2 ± 0.1	0.5 ± 0.1
50:1	5.1 ± 0.2	6.8 ± 0.1	0.5 ± 0.1	1.1 ± 0.2	0.6 ± 0.1	0.7 ± 0.1
50:2	1.94 ± 0.04	2.4 ± 0.2	0.3 ± 0.1	0.6 ± 0.1	0.3 ± 0.1	0.3 ± 0.1
52:0	6.3 ± 0.4	7.9 ± 0.2	0.5 ± 0.1	0.9 ± 0.1	0.4 ± 0.1	0.75 ± 0.02
52:1	11.0 ± 1.0	12.0 ± 1.0	1.1 ± 0.2	2.2 ± 0.3	1.1 ± 0.2	1.6 ± 0.1
52:2	8.0 ± 1.0	8.9 ± 0.4	1.4 ± 0.1	2.1 ± 0.3	1.3 ± 0.2	1.4 ± 0.2
52:3	2.3 ± 0.2	2.7 ± 0.2	0.6 ± 0.1	0.7 ± 0.1	0.5 ± 0.1	0.4 ± 0.1
52:4	0.68 ± 0.03	0.7 ± 0.1	0.20 ± 0.03	0.21 ± 0.02	0.21 ± 0.03	0.19 ± 0.02
54:0	1.7 ± 0.2	1.75 ± 0.03	0.09 ± 0.01	0.2 ± 0.1	0.07 ± 0.01	0.1327 ± 0.0002
54:1	8.1 ± 0.4	8.0 ± 1.0	0.7 ± 0.1	1.3 ± 0.2	0.7 ± 0.1	1.2 ± 0.2
54:2	5.5 ± 0.3	5.4 ± 0.2	0.6 ± 0.1	1.3 ± 0.2	0.9 ± 0.1	1.02 ± 0.01
54:3	5.0 ± 1.0	5.0 ± 1.0	1.1 ± 0.1	1.5 ± 0.2	1.2 ± 0.1	0.98 ± 0.01
54:4	2.5 ± 0.2	2.3 ± 0.1	0.6 ± 0.1	0.7 ± 0.1	0.8 ± 0.1	0.7 ± 0.1
54:5	2.1 ± 0.04	1.7 ± 0.1	n.d.	n.d.	n.d.	n.d.
54:6	1.4 ± 0.2	1.11 ± 0.04	2.5 ± 0.4	2.9 ± 0.3	2.4 ± 0.4	2.3 ± 0.2
54:7	0.21 ± 0.03	0.16 ± 0.02	1.0 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.1
56:0	1.0 ± 0.1	1.10 ± 0.02	0.06 ± 0.01	0.11 ± 0.04	n.d.	0.11 ± 0.02
56:1	2.0 ± 0.1	1.6 ± 0.1	0.14 ± 0.01	0.25 ± 0.03	0.12 ± 0.02	0.169 ± 0.004
56:2	1.9 ± 0.1	1.7 ± 0.1	0.23 ± 0.02	0.40 ± 0.1	0.23 ± 0.03	0.25 ± 0.02
56:3	1.50 ± 0.03	1.28 ± 0.03	0.20 ± 0.03	0.34 ± 0.04	0.32 ± 0.04	0.30 ± 0.01
56:6	3.9 ± 0.4	2.4 ± 0.04	4.0 ± 1.0	3.6 ± 0.4	5.0 ± 1.0	4.7 ± 0.3
56:7	1.7 ± 0.3	1.1 ± 0.1	5.0 ± 1.0	4.6 ± 0.3	6.9 ± 0.4	6.3 ± 0.4
58:1	1.91 ± 0.02	1.68 ± 0.04	0.11 ± 0.02	0.21 ± 0.04	0.13 ± 0.02	0.22 ± 0.04
58:2	1.2 ± 0.1	1.02 ± 0.01	0.12 ± 0.01	0.20 ± 0.02	0.12 ± 0.02	0.13 ± 0.01
58:3	0.62 ± 0.04	0.51 ± 0.03	0.06 ± 0.01	0.11 ± 0.01	0.11 ± 0.02	0.14 ± 0.04
58:5	1.7 ± 0.2	0.8 ± 0.1	0.3 ± 0.1	0.26 ± 0.02	0.7 ± 0.2	n.d.
58:6	3.0 ± 0.4	1.9 ± 0.3	n.d.	n.d.	n.d.	n.d.
58:7	2.1 ± 0.3	1.7 ± 0.4	4.9 ± 0.4	4.9 ± 0.2	7.0 ± 1.0	6.9 ± 0.3
58:8	0.9 ± 0.1	0.6 ± 0.2	3.4 ± 0.4	3.4 ± 0.2	5.2 ± 0.2	4.9 ± 0.1
58:9	0.2 ± 0.1	0.2 ± 0.1	1.0 ± 0.1	0.92 ± 0.01	1.8 ± 0.1	1.7 ± 0.1
58:10	n.d.	0.07 ± 0.03	0.82 ± 0.01	0.78 ± 0.02	1.267 ± 0.002	1.23 ± 0.01
58:11	n.d.	n.d.	0.9 ± 0.1	0.88 ± 0.03	1.1 ± 0.1	1.2 ± 0.1
58:12	n.d.	0.03 ± 0.01	2.7 ± 0.4	2.9 ± 0.3	2.0 ± 0.4	2.1 ± 0.1
60:1	2.3 ± 0.1	1.85 ± 0.02	0.13 ± 0.04	0.22 ± 0.03	0.15 ± 0.03	0.26 ± 0.03
60:2	1.50 ± 0.02	1.21 ± 0.02	0.14 ± 0.01	0.20 ± 0.02	0.16 ± 0.02	0.21 ± 0.04
60:3	0.60 ± 0.02	0.47 ± 0.02	0.05 ± 0.01	0.10 ± 0.02	0.09 ± 0.01	0.10 ± 0.02
60:4	0.4 ± 0.1	0.3 ± 0.10	n.d.	0.05 ± 0.01	0.09 ± 0.02	0.08 ± 0.01
60:10	0.1 ± 0.1	0.11 ± 0.04	1.9 ± 0.1	1.76 ± 0.01	3.1 ± 0.2	3.2 ± 0.1
60:12	1.1 ± 0.4	0.45 ± 0.03	18.7 ± 0.3	18.0 ± 1.0	15.0 ± 1.0	16.0 ± 0.4
60:13	0.1 ± 0.1	0.062 ± 0.004	3.0 ± 0.4	3.1 ± 0.1	2.0 ± 1.0	2.5 ± 0.1
62:1	1.0 ± 0.1	0.76 ± 0.03	n.d.	0.08 ± 0.01	n.d.	0.10 ± 0.01
62:2	0.9 ± 0.1	0.620 ± 0.003	0.06 ± 0.02	0.08 ± 0.01	0.08 ± 0.02	0.12 ± 0.01
62:3	0.43 ± 0.02	0.31 ± 0.01	0.04 ± 0.01	0.050 ± 0.004	0.07 ± 0.02	0.07 ± 0.01
62:11	n.d.	0.04 ± 0.01	1.0 ± 0.1	n.d.	1.0 ± 0.1	1.2 ± 0.1
62:12	0.32 ± 0.04	0.3 ± 0.1	8.7 ± 0.2	6.6 ± 0.4	8.2 ± 0.1	7.0 ± 1.0
62:13	0.5 ± 0.2	0.43 ± 0.01	17.3 ± 1.3	14.6 ± 1.1	15.59 ± 0.02	15.0 ± 1.0
62:14	n.d.	n.d.	2.7 ± 0.4	2.6 ± 0.2	2.2 ± 0.4	2.4 ± 0.2
66:18	0.18 ± 0.04	0.18 ± 0.01	12.3 ± 2.2	11.2 ± 1.4	7.0 ± 2.0	7.0 ± 1.0

[a] The lipidomic data is presented as mean percentages of each species per total detected species and normalised to total cellular protein.

[b] TAG species are presented according to the number of carbon atoms and double bonds.

[c] MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) or non-targeting scrambled siRNA (SCR) and treated with 100 μM DHA and 10 nM $sPLA_2$ in complete medium for 48 h. Cell lysates were collected for UPLC/MS analysis. Values are means ± SEM of three independent experiments; n.d., not determined.

Table 4
PC profiles of MDA-MB-231 breast cancer cells depleted in ATGL and treated with exogenous DHA, $sPLA_2$ or both.

	PC composition (% of total)[a]					
PC species[b]	SCR[c]	siATGL	SCR+DHA	siATGL+DHA	SCR+DHA+$sPLA_2$	siATGL+DHA+$sPLA_2$
30:0	2.6 ± 0.3	2.7 ± 0.2	4.3 ± 0.1	5.7 ± 0.2	2.8 ± 0.1	4.7 ± 0.4
30:1	1.9 ± 0.3	2.2 ± 0.2	1.88 ± 0.04	2.44 ± 0.02	1.8 ± 0.1	2.3 ± 0.1
32:0	5.3 ± 0.2	5.3 ± 0.2	21.0 ± 1.0	25.0 ± 1.0	12.0 ± 1.0	12.2 ± 1.1
32:1	9.0 ± 1.0	9.5 ± 0.4	6.6 ± 0.2	7.3 ± 0.2	5.8 ± 0.1	6.3 ± 0.2
34:1	28.0 ± 1.0	28.8 ± 1.4	19.0 ± 1.0	18.0 ± 1.0	25.0 ± 1.0	21.2 ± 0.2
34:2	5.4 ± 0.2	4.9 ± 0.4	3.1 ± 0.1	2.9 ± 0.1	3.2 ± 0.1	2.6 ± 0.2
34:3	0.26 ± 0.02	0.28 ± 0.01	1.12 ± 0.01	1.2 ± 0.1	0.58 ± 0.03	0.6 ± 0.1
36:0	0.38 ± 0.01	0.5 ± 0.1	0.321 ± 0.001	0.29 ± 0.02	0.51 ± 0.03	0.5 ± 0.1
36:1	20.0 ± 1.0	21.8 ± 1.4	9.0 ± 0.1	6.0 ± 1.0	15.0 ± 1.0	13.6 ± 1.1
36:2	13.4 ± 0.3	12.8 ± 0.3	5.8 ± 0.1	4.3 ± 0.3	9.0 ± 1.0	6.3 ± 0.3
36:4	2.4 ± 0.1	2.4 ± 0.1	2.6 ± 0.1	2.8 ± 0.1	2.9 ± 0.2	2.8 ± 0.1
36:5	0.5 ± 0.1	0.26 ± 0.02	1.31 ± 0.03	1.8 ± 0.1	0.9 ± 0.1	1.52 ± 0.02
36:6	0.11 ± 0.02	0.04 ± 0.01	0.8 ± 0.1	0.8 ± 0.1	0.4 ± 0.1	0.39 ± 0.02
38:3	1.8 ± 0.1	1.6 ± 0.1	1.05 ± 0.01	0.8 ± 0.1	1.5 ± 0.1	1.33 ± 0.03
38:4	2.8 ± 0.4	2.4 ± 0.2	2.5 ± 0.1	1.9 ± 0.1	3.1 ± 0.2	3.0 ± 0.1
38:5	2.0 ± 1.0	1.3 ± 0.1	2.4 ± 0.4	1.9 ± 0.2	3.2 ± 0.2	3.1 ± 0.3
38:6	1.7 ± 0.3	0.8 ± 0.1	8.0 ± 1.0	9.0 ± 1.0	6.0 ± 1.0	7.9 ± 0.3
40:6	1.5 ± 0.2	1.2 ± 0.1	6.0 ± 1.0	6.0 ± 1.0	5. 0 ± 1.0	7.8 ± 0.3
40:7	1.0 ± 0.1	0.31 ± 0.01	3.0 ± 0.1	2.3 ± 0.2	2.5 ± 0.2	2.0 ± 0.1

[a] The lipidomic data is presented as mean percentages of each species per total detected species and normalised to total cellular protein.
[b] PC species are presented according to the number of carbon atoms and double bonds.
[c] MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) or non-targeting scrambled siRNA (SCR) and treated with 100 μM DHA and 10 nM $sPLA_2$ in complete medium for 48 h. Cell lysates were collected for UPLC/MS analysis. Values on the graphs are means ± SEM of three independent experiments; n.d., not determined.

medium. Reverse transfection was performed according to manufacturer's instructions. Cells were left to attach for 24 h and treated with 10 nM $sPLA_2$ and 100 μM DHA for the following 48 h.

2.3. *Lipidomic analyses of triacylglycerol and phospholipid species*

Culture plates were put on ice, the cells washed twice with ice-cold DPBS, collected by scraping in 300 μl lysis buffer (20 mM Tris–HCl, pH 7.4, 2 mM EDTA, 2 μl Halt Protease Inhibitor Cocktail, Thermo Scientific, USA), centrifuged at 4 °C (1000 *g*, 10 min), the pellet resuspended in 150 μl lysis buffer and sonicated on ice. Aliquots of 10 μl were taken for total protein determination using the Pierce 660 nm Protein Assay (Thermo Scientific, USA). Total lipids were extracted twice using chloroform/methanol/water (2/1/0.6, v/v/v) containing 500 pmol butylated hydroxytoluene, 1% acetic acid, and 100 pmol of internal standards (ISTD, 17:0-17:0 PC, 17:0-17:0 PE, 17:0-17:0-17:0 TAG, 17:0 LPC, Avanti Polar Lipids) per sample. Extraction was performed under constant shaking for 60 min at room temperature (RT). After centrifugation at 1000 × *g* for 15 min at RT the lower organic phase was collected, 2.5 ml chloroform added to the remaining aqueous phase and the second extraction performed as described above. Combined organic phases of the double-extraction were dried under a stream of nitrogen and dissolved in 150 μl 2-propanol/chloroform/methanol (7/2/1, v/v/v) for UPLC-qTOF analysis. Chromatographic separation was modified after Knittelfelder et al. [5] using an AQUITY-UPLC system (Waters Corporation), equipped with an ACQUITY BEH C18 column (2.1 × 50 mm, 1.7 μm; Waters Corporation). A SYNAPT™G1 qTOF HD mass spectrometer (Waters Corporation) equipped with an ESI source was used for detection. Data acquisition was done by the MassLynx 4.1 software (Waters Corporation) and lipid classes were analysed with the "Lipid Data Analyzer 1.6.2" software [6]. Data were normalised for recovery and extraction- and ionization efficacy using ISTDs.

2.4. Data handling

The acquired lipidomic data was normalised to total cellular protein. The values are presented as mean percentages of each species per total detected species in each sample with standard errors of the mean (SEM) of three independent experiments.

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Transparency document. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.dib.2018.03.033.

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4.3 Lipid Droplet Formation in HeLa Cervical Cancer Cells Depends on Cell Density and the Concentration of Exogenous Unsaturated Fatty Acids

The short research article "Lipid droplet formation in HeLa cervical cancer cells depends on cell density and the concentration of exogenous unsaturated fatty acids" by Ema Guštin, Eva Jarc, Ana Kump and Toni Petan was published in *Acta Chimica Slovenica* in 2017. Ema Guštin and Eva Jarc equally contributed to this work.

The role of different FA species in supporting lipid droplet accumulation and cell survival in different cancer cells in different environmental conditions is not clear. In general, lipid droplets act as transient buffers that protect from FA lipotoxicity by storing excess and lipotoxic FAs in TAG (Jarc & Petan, 2019b; Petan et al., 2018). Saturated FAs are particularly toxic to the cell and induce cell death, while monounsaturated FAs, such as oleate (ω-9; 18:1), are generally regarded as cytoprotective (Nolan & Larter, 2009). A number of studies have reported that polyunsaturated fatty acids (PUFAs) have distinct and contrasting effects in cancer, with ω-6 PUFAs, such as arachidonic acid (ω-6; 20:4; AA), mostly displaying pro-tumorigenic effects, and ω-3 PUFAs, such as docosahexaenoic (DHA) and eicosapentaenoic (EPA) acids, showing anti-tumorigenic effects (Chénais & Blanckaert, 2012; D'Eliseo & Velotti, 2016). We have shown that unsaturated FAs, including both ω-6 and ω-3 PUFAs, induce lipid droplet biogenesis and prevent starvation-induced death of TNBC cells (Jarc, Kump, et al., 2018). However, the effect of ω-3 PUFAs on cell survival was concentration-dependent. PUFAs used at low micromolar concentration display a pro-tumorigenic effect, while higher concentrations become lipotoxic in breast cancer cells.

In this study, we hypothesized that different exogenous unsaturated FAs vary in their abilities to induce lipid droplet synthesis and prevent starvation-induced cell death in HeLa cervical cancer cells. We found that unsaturated FAs have dual effects on lipid droplet accumulation in HeLa cells. Treating cells with low micromolar concentrations of monounsaturated FAs and PUFAs led to a reduction in neutral lipid content, while high micromolar concentrations stimulated lipid droplet formation (Guštin, Jarc, Kump, & Petan, 2017). Furthermore, we found that lipid droplet content in HeLa cells increases proportionally with cell density, while in contrast, lipid droplet content in MDA-MB-231 cells decreases with cell density. The increase in lipid droplet content in HeLa cells may indicate that the cells gradually increase the rate of exogenous lipid uptake and/or rate of de novo lipid synthesis, leading to lipid droplet formation to support fast proliferation rates. Finally, oleate-induced lipid droplet accumulation was previously associated with a protection from starvation-induced cell death in MDA-MB-231 cells (Pucer et al., 2013). Here, we observed that only high micromolar concentrations of oleate led to lipid droplet accumulation in HeLa cells. Surprisingly, we found that oleate-induced lipid droplet accumulation does not reduce starvation-induced cell death, but it rather promotes it at higher concentrations of the FA (Guštin et al., 2017). This suggests that starved HeLa cells cannot use exogenous oleate to support cell survival during starvation.

I contributed to this work by preparing and writing the draft of the manuscript for publication.

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Scientific paper

Lipid Droplet Formation in HeLa Cervical Cancer Cells Depends on Cell Density and the Concentration of Exogenous Unsaturated Fatty Acids

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Abstract

Cytosolic lipid droplets (LDs) store excess fatty acids (FAs) in the form of neutral lipids and prevent starvation-induced cancer cell death. Here we studied the ability of mono- and polyunsaturated FAs to affect LD formation and survival in HeLa cervical cancer cells. We found that the LD content in HeLa cells increases with cell density, but it decreases in MDA-MB-231 breast cancer cells. Exogenously-added unsaturated FAs, including oleic (OA), linoleic (LA), arachidonic (AA), eicosapentaenoic (EPA) and docosahexaenoic acid (DHA) displayed a similar ability to alter LD formation in HeLa cells. There was a dual, concentration-dependent effect on neutral lipid accumulation: low micromolar concentrations of LA, AA and DHA reduced, while all FAs induced LD formation at higher concentrations. In serum starved HeLa cells, OA stimulated LD formation, but, contrary to expectations, it promoted cell death. Our results reveal a link between cell population density and LD formation in HeLa cells and show that unsaturated FAs may both suppress or stimulate LD formation. This dynamic regulation of LD content must be accounted for when studying the effects of lipids and lipid metabolism-targeting drugs on LD metabolism in HeLa cells.

Keywords: lipid droplets, lipid metabolism, cell density, HeLa cervical cancer cells, unsaturated fatty acids, apoptosis

1. Introduction

Tumours have developed different molecular mechanisms that enable their uncontrolled proliferation.[1] Oncogene activation and loss of tumour suppressors lead to both proliferative and metabolic reprogramming of cancer cells. The most common metabolic adaptations in cancer are increased glucose uptake, high rate of anaerobic glycolysis and increased glutamine consumption, but recent studies have revealed a dependence on elevated *de novo* lipid synthesis, internalization of exogenous lipids, mitochondrial fatty acid (FA) oxidation and lipid mediator-induced proliferative signalling.[1–4] Lipids and their metabolites are currently under intense investigation in cancer-related studies, where new aspects of their role in cancer signalling, survival and apoptosis are beginning to emerge.

Lipid droplets (LDs) are now widely recognized as dynamic cytosolic organelles, present in all eukaryotic cells, with prominent roles reaching beyond that of inert energy storage depots.[5] LDs are composed of a tightly packed core of neutral lipids, triacylglycerol (TAG) and sterol esters, surrounded by a phospholipid monolayer embedded with proteins and enzymes. At the cellular level, besides providing energy and building blocks for biosyntheses, they have important roles in protein quality control, viral replication and cell signalling, and are implicated in physiological processes ranging from immunity to neural development.[6] Strong evidence suggests that LDs also control the synthesis of an array of lipid signal-

ling molecules and that changes in LD metabolism influence the risk of developing metabolic diseases and cancer.[7,8]

Increased accumulation of LDs has been observed in cancer cells *in vivo* and *in vitro* and LDs have been proposed as markers of cancer aggressiveness.[2,4,9-12] The mechanisms of their involvement in cancer are currently under intense investigation. LDs are primary cellular sources of FAs for mitochondrial β-oxidation, which has been shown to enable cancer cell survival during various stress conditions.[13-15] We have previously shown that secreted phospholipase A_2-induced release of unsaturated FAs induces TAG synthesis and LD formation in aggressive breast cancer cells, thus stimulating proliferation and preventing cell death during serum starvation.[16,17] The protective effects of LDs are associated with upregulation of β-oxidation enzymes and may be abolished with etomoxir, a potent inhibitor of β-oxidation. Intriguingly, LD formation is also induced during complete cell starvation, i.e. in the absence of glucose, amino acids and exogenous sources of lipid, when LDs become critical for providing FAs for β-oxidation that enables the survival of different cells, including HeLa cervical cancer cells.[18,19]

The role of different unsaturated FA species in supporting LD accumulation and cell survival in different cancer cell types in different environmental conditions is not clear. In general, LDs prevent FA lipotoxicity by acting as transient buffers for excess endogenous and exogenous FAs. Saturated FAs are particularly toxic to the cell and induce ER-stress and apoptosis, while monounsaturated FAs, such as oleic acid (OA; 18:1, ω-9), are generally regarded as cytoprotective.[20] The supplementation of unsaturated FAs may even avert lipotoxicity by syphoning saturated FAs to inert TAGs stored in LDs.[21] A number of studies have demonstrated that polyunsaturated FAs (PUFAs) have distinct and contrasting effects in cancer, with ω-6 PUFAs, such as arachidonic acid (AA; 20:4, ω-6), mostly displaying pro-tumorigenic effects and ω-3 PUFAs, such as eicosapentaenoic (EPA; 20:5, ω-3) and docosahexaenoic acid (DHA; 22:6, ω-3), showing anti-tumorigenic, anti-inflammatory and pro-apoptotic effects in cancer cells.[22,23] However, the association between LD metabolism and individual FA species in the context of cancer cell survival and metabolism is unknown. Here we studied the ability of different exogenous unsaturated FAs, namely OA, linoleic acid (LA; 18:2, ω-6), AA, EPA and DHA to induce LD formation and affect cell death in HeLa cervical cancer cells.

2. Experimental

2. 1. Materials

HeLa cervical adenocarcinoma cells (HeLa H2 clone) were obtained from John V. Moran, Howard Hughes Medical Institute, USA. MDA-MB-231 breast adenocarcinoma cells and RPMI-1640 culture medium were from ATCC (USA). DMEM, foetal bovine serum (FBS) and Dulbecco's phosphate buffered saline (DPBS) were from Gibco, USA. Nile Red, tetramethylrhodamine, methyl ester (TMRM), LA, EPA, DHA, AA, fatty acid-free bovine serum albumin (FAF-BSA) were from Sigma-Aldrich (USA), YO-PRO-1 iodide, TrypLE Select were from Life Technologies (USA), while OA and DMSO were from Merck (Germany).

2. 2. Cell Lines and Culture Conditions

HeLa and MDA-MB-231 cells were cultured in DMEM and RPMI-1640 medium, respectively, supplemented with 10% FBS. In experiments with serum-deprived cells, FBS was replaced by 0.02% FAF-BSA to render cells quiescent. Exogenously added FAs were complexed with 0.5% FAF-BSA or 10% FBS in culture medium for 1 h at room temperature before addition to cell culture.

2. 3. Flow Cytometry Analysis of Cellular LD Content

LD analysis was performed as described previously.[16] Briefly, cells were seeded in complete medium in 24-well culture plates, left to attach for 24 h, and treated with different concentrations of FAs for 48 h. Prior to treatment, FAs were complexed as described above. Cells were harvested, the pellet resuspended in 500 µl of 1 µg/ml Nile Red solution in DPBS, incubated in the dark for 10 min and analysed by flow cytometry on a BD FACSCalibur system (BD Biosciences, USA). Logarithmic fluorescence signals were collected using the FL1 filter (530/30) from at least 2×10^4 events per sample.

2. 4. TMRM/YO-PRO-1 Apoptosis Assay

Cell death was determined by measuring mitochondrial and plasma membrane integrity using the TMRM/YO-PRO-1 assay and flow cytometry as described prevoiously.[17] Cells were seeded in complete medium in 24-well plates at a concentration of 3×10^4 cells/well. 24 h later, the medium was replaced by serum-free DMEM containing 0.02% FAF-BSA and the cells pre-starved for 24 h. Medium was replaced, cells treated with different concentrations of FAs (pre-complexed with DMEM containing 0.5% FAF-BSA) and incubated for 168 h. Cells were harvested, the pellet resuspended in 100 µl of 150 nM TMRM in DPBS and incubated in the dark for 15 min. Finally, 1 µl of 20 µM YO-PRO-1 in DMSO was added and cells incubated for additional 10 min. TMRM and YO-PRO-1 signals from 5×10^4 cells per sample were measured by flow cytometry using the FL1 (530/30) and FL3 (650LP) filters. TMRM negative and YO-PRO-1 positive cells were considered apoptotic.

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2. 5. Statistical Analysis

Data are presented as means ± SEM. Graphpad Prism (GraphPad Software, USA) was used for statistical analysis, using one-way ANOVA with Bonferroni adjustment for multiple comparisons. P values lower than 0.05 were considered statistically significant.

3. Results and Discussion

In the course of our flow cytometry assay optimization for the detection of LDs in HeLa cells, we observed significant changes in their LD content depending on the number of cells seeded. In order to determine whether cell density affects LD accumulation, HeLa cells were seeded at different densities, grown for 48 h in complete medium and the average LD amount in the cell population was determined by flow cytometry. We found that the LD content in HeLa cells increased proportionally with cell density (Fig. 1a). On the contrary, the average LD amount in MDA-MB-231 breast cancer cells, a cell line with high propensity for LD formation from exogenous FAs,[16] decreased with cell density (Fig. 1b). Namely, rapidly proliferating cancer cells grown in complete medium are highly metabolically active and they take up and metabolize glucose and amino acids to support biosynthesis, cell growth and proliferation.[1] Lipids may be synthesized *de novo* or are taken up from exogenous sources, and are necessary for the maintenance and synthesis of cell membranes and thus a prerequisite for cell proliferation.[4] Therefore, we expected that LD content will decrease with increasing cell density, since there is an increasing number of cells competing for the fixed supply of exogenous lipids found in serum. The increase in LD amount in HeLa cells suggests that with increasing cell density, and in the absence of contact inhibition, HeLa cells either gradually increase the rate of exogenous lipid uptake or significantly upregulate *de novo* lipid synthesis. In both cases, LDs may be necessary for supporting cell growth and proliferation processes characteristic of HeLa cells. In support of our finding, a similar relationship between LD amount and cell density has been reported for C6 rat glioma cells in culture,[24] and, interestingly, an increase in the amount and size of LDs is characteristic of adipogenic differentiation, which may occur also in cancer cells, including cancer stem cell subpopulations of HeLa cells.[25,26] Although the role of LDs in cell growth and proliferation is not clear, it may be easily envisaged that LDs act as master regulators of cellular lipid homeostasis, finely tuning the balance between lipid requirements and supply during cell growth. Additionally, LDs may be necessary to alleviate ER-stress that occurs in cancer cells undergoing oncogene-driven, uncontrolled proliferation.[27,28]

We next asked whether different exogenous unsaturated FA species vary in their abilities to induce LD accumulation in HeLa cells. We found that all tested FAs displayed a similar ability to modulate LD accumulation in HeLa cells (Fig. 2a–e). As expected, high micromolar concentrations of FAs, which are regularly used to induce LD formation in various cell types, stimulated LD accumulation in HeLa cells. This is in accordance with the conserved mechanism of prevention of FA lipotoxicity by their incorporation into TAG and LD formation.[20,21] Surprisingly, a slight decrease in neutral lipid levels was observed when cells were exposed to low concentrations of FAs (10 μM and below; Fig. 2a–e). A statistically significant reduction for LA, AA and DHA, which reduced LD amounts by 20, 15 and 19%, respectively, was indeed confirmed in a separate experiment using

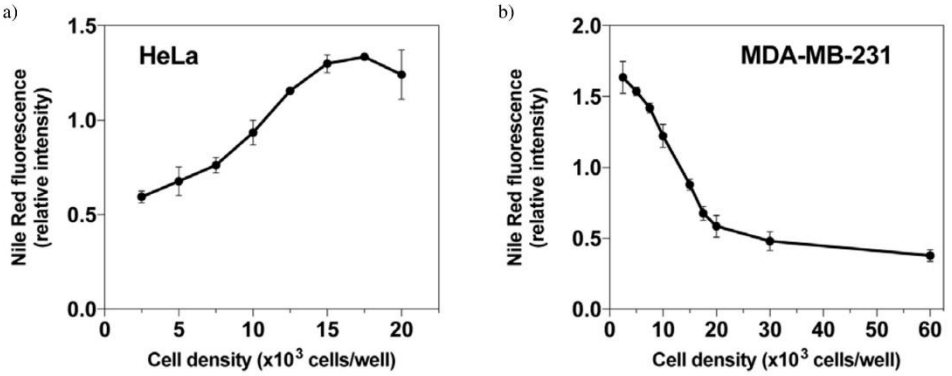

Figure 1. LD content in HeLa cells increases with cell density. (a) HeLa and (b) MDA-MB-231 cells were seeded in 24-well plates at the stated cell densities in complete medium. After 24 h the medium was replaced and cells were cultivated for another 48 h in complete medium. Cells were collected, stained with Nile Red and LD content was determined by flow cytometry. The resulting values are means ± SEM of at least two experiments performed in duplicate.

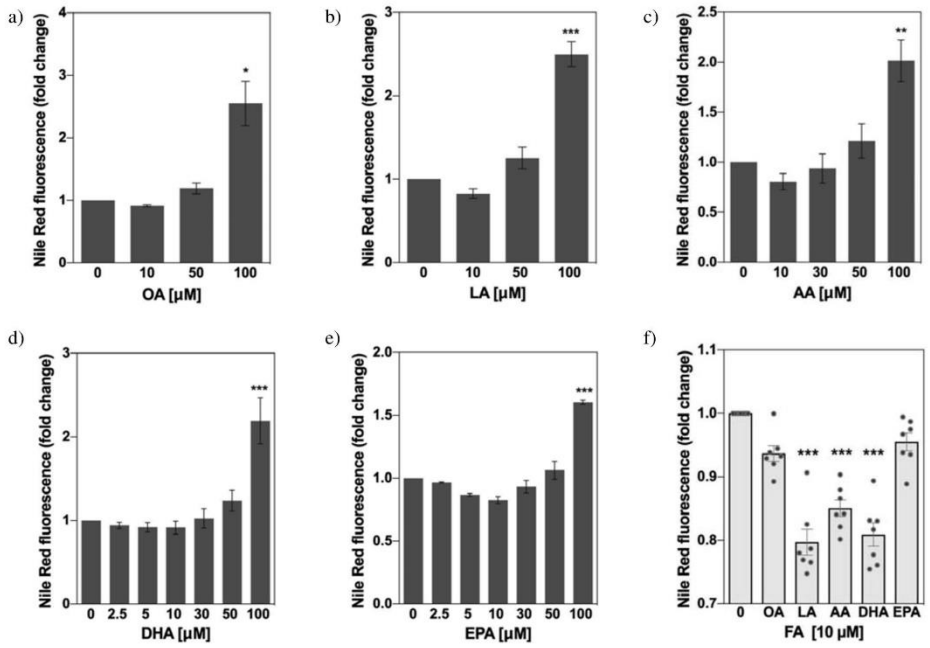

Figure 2. Unsaturated FAs have dual effects on LD accumulation in HeLa cells. (a) HeLa cells were grown for 48 h in complete media in the presence of a range of concentrations of OA (a), LA (b), AA (c), DHA (d) and EPA (e) or in the presence of 10 μM of each FA (f). LD content was determined by flow cytometry. The resulting values are means ± SEM of at least two (Fig. 2a–e) or seven (Fig. 2f) experiments performed in duplicate. Results that are statistically significant over control samples are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA with Bonferroni adjustment).

only 10 μM FAs (Fig. 2f). The negative effect on LD accumulation may be a consequence of stimulated LD breakdown or suppressed LD formation, and is reminiscent to the anti-adipogenic effect of unsaturated FAs, in particular ω-3 PUFAs, in differentiating adipocytes.[29] The relevance of this finding for cancer cell biology remains to be established.

We have shown recently that OA induces LD formation in MDA-MB-231, MCF-7 and T-47D breast cancer cells, but it suppresses starvation-induced cell death only in MDA-MB-231 cells.[16] To find out whether OA promotes LD accumulation and suppresses the death of HeLa cells, we treated starving HeLa cells with a range of concentrations of OA. Interestingly, we observed that OA induced significant LD accumulation only at the highest concentration used and it did not reduce, but it rather promoted cell death at higher concentrations (Fig. 3). Thus, in contrast to the highly invasive MDA-MB-231 cells,[16] and despite the well-known cytoprotective effect of OA,[20] our results suggest that serum-deprived HeLa cells cannot use exogenous OA to support cell survival. It is possible that starved HeLa cells are either not efficient in packaging exogenous OA into TAGs for transient storage in LDs or their LD breakdown pathways are overly active, thus leading to lipotoxicity of the unesterified OA.[3,31]

4. Conclusions

We show that LD content in HeLa cells is proportional with cell culture density, that unsaturated FAs have differential effects on LD accumulation, and that OA cannot support the survival of starved HeLa cells. These interesting novel features of LD metabolism in HeLa cells may be useful for future studies providing fresh insights into cancer LD metabolism, in particular the relationship between cancer cell proliferation and lipid metabolism.

5. Acknowledgements

We are grateful to John V. Moran for providing the HeLa cell line. This work was supported by the 1000-15-

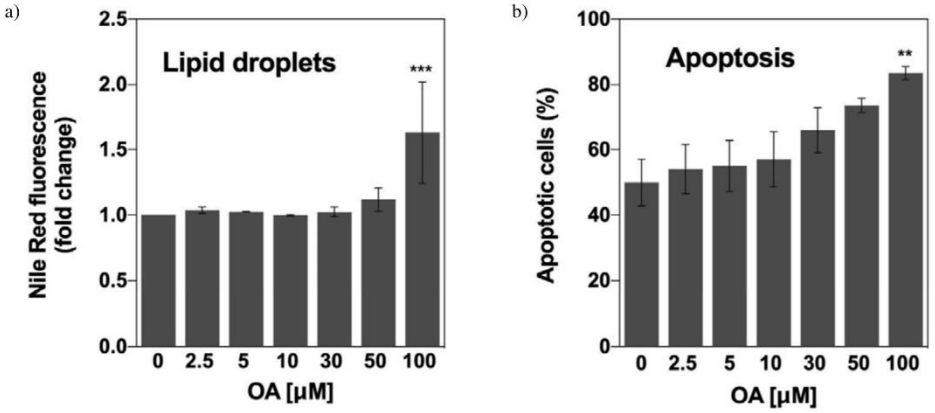

Figure 3. OA-induced LD accumulation does not provide a survival advantage for serum-deprived HeLa cells. HeLa cells were seeded in complete medium, left to attach for 24 h and pre-starved for 24 h in serum-deprived medium. Cells were then incubated with different concentrations of FAs for 168 h in serum-deprived medium. (a) LD content and (b) apoptosis were determined by flow cytometry. The resulting values are means ± SEM of at least two experiments performed in duplicate. Results that are statistically significant over control samples are indicated (*, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; one-way ANOVA with Bonferroni adjustment).

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Conflicts of interest

None of the authors declare any conflict of interest.

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Povzetek

Citosolne lipidne kapljice shranjujejo prese ne ma obne kisline (MK) v obliki nevtralnih lipidov in prepre ujejo smrt rakavih celic tekom stradanja. V tem delu smo preu ili vplive mono- in polinenasi enih MK na tvorbo lipidnih kapljic in pre ivetje celic raka materni nega vratu HeLa. Pokazali smo, da vsebnost lipidnih kapljic v celicah HeLa nara a z gostoto celi ne kulture, v celicah raka dojke MDA-MB-231 pa pada. Eksogeno dodane nenasi ene MK, oleinska (OK), linolna (LK), arahidonska (AK), eikozapentaenojska (EPK) in dokozaheksaenojska kislina (DHK) so imele podobne vplive na tvorbo lipidnih kapljic v celicah HeLa. Opazili smo dvojni, od koncentracije odvisen u inek na kopi enje nevtralnih lipidov: nizke mikromolarne koncentracije LK, AK in DHK so zni ale koli ino lipidnih kapljic, medtem ko so pri vi jih mikromolarnih koncentracijah vse MK inducirale njihovo tvorbo. OK je spodbudila tvorbo lipidnih kapljic v stradanih celicah HeLa, a je, nasprotno od pri akovanj, spodbudila tudi celi no smrt. Na i rezultati razkrivajo povezavo med gostoto celic v populaciji in tvorbo lipidnih kapljic pri celicah HeLa in ka ejo, da lahko nenasi ene MK tako zavirajo kot tudi stimulirajo tvorbo lipidnih kapljic. To dinami no regulacijo vsebnosti lipidnih kapljic je potrebno upo tevati pri tudijah u inkov lipidov in u inkovin, ki ciljajo lipidni metabolizem, na metabolizem lipidnih kapljic v celicah HeLa.

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Chapter 5

Conclusions

This study was focused on the principal mechanisms that link lipid droplets with cancer cell survival during metabolic stress. We addressed the roles of lipid droplets in the protection of TNBC cells from nutrient and lipotoxic stress, and their role as platforms for the production of lipid mediators in cancer. Furthermore, we established that DGAT and ATGL are major enzymes in lipid droplet turnover in TNBC cells and determined some of their effects on metabolism and signaling that affect cancer cell function and survival during stress. We also addressed lipid droplet formation in HeLa cervical cancer cells and determined the effects of exogenous unsaturated FAs on lipid droplet metabolism and cancer cell survival.

We came to the following conclusions, which are presented here according to the order of scientific papers presented in the thesis:

1. **Lipid droplets are sources of precursors for lipid mediator synthesis in TNBC cells** – We have found that lipid droplet breakdown in starving TNBC cells provides precursors for the synthesis of pro-inflammatory eicosanoids and pro-resolving specialized lipid mediators. We have shown that ATGL activity is essential for lipid mediator synthesis in TNBC cells. Blocking ATGL-mediated lipolysis leads to a significant reduction in the synthesis of a broad range of lipid mediators. Furthermore, our results show that sPLA$_2$-induced lipid droplets drive lipid mediator synthesis during starvation. This process is dependent on ATGL-mediated lipolysis and it leads to the release of COX-, LOX- and CYP-derived lipid mediators. In particular, it promotes the release of 12/15-LOX-generated EPA and DHA metabolites. Finally, we have found that ATGL and cPLA$_2$α play complementary roles in lipid droplet-mediated lipid mediator synthesis. Together, these results suggest that lipid droplets are sources of bioactive lipids in TNBC cells and may contribute to the protection of cancer cells against stress.
2. **Lipid droplets coordinate unsaturated FA uptake, storage and use with cell survival mechanisms during nutrient and lipotoxic stress in TNBC cells** – We have shown that lipid droplets transiently store unsaturated FAs, including ω-6 and ω-3 PUFAs, thus providing a protection against lipotoxicity and nutrient starvation by releasing FAs when needed. We have shown that ATGL is involved in lipid droplet breakdown in TNBC cells, but is not essential for the pro-survival effect of lipid droplets during serum starvation. In conditions of lipid overload, we have shown that TNBC cells are protected from PUFA lipotoxicity when they are sequestered in TAGs in lipid droplets either by ATGL inhibition or sPLA$_2$-induced TAG synthesis and remodeling. In addition, we have demonstrated that inhibition of DGAT1 activity suppresses lipid droplet biogenesis and potentiates PUFA lipotoxicity and cell death in TNBC cells. Collectively, our results suggest that lipid droplets are antioxidant and pro-survival organelles that protect TNBC cells from nutrient and lipotoxic stress.
3. **Lipid droplet triglyceride remodeling is associated with protection of breast cancer cells from lipotoxic stress** – We have shown that treating TNBC

cells with exogenous PUFAs leads to their incorporation in lipid droplets, thereby increasing the level of highly unsaturated TAG species, e.g. those that contain more than 7 double bonds in their structure (PUFA-TAG). We have also shown that the addition of exogenous PUFAs results in significantly increased levels of membrane phosphatidylcholine (PC) (poly)unsaturation (PUFA-PC). Importantly, we have shown that treating TNBC cells with $sPLA_2$ leads to a reduction in PUFA-TAG content by elevating the total amount of neutral lipids and the proportion of TAG with low unsaturation levels, indicating PUFA dilution in the TAG pool. Furthermore, we have shown that ATGL depletion does not lead to extensive TAG and/or PC remodeling, suggesting that its effects are dependent on overall inhibition of lipolysis.

4. **Lipid droplet formation in HeLa cervical cancer cells depends on cell density and exogenous unsaturated fatty acids** – We have found that lipid droplet content in HeLa cells increases proportionally with cell density, while in contrast, lipid droplet content in TNBC cells decreases with cell density. We have shown that exogenous unsaturated FAs exhibit a dual effect on lipid droplet metabolism in HeLa cells: they reduce lipid droplet accumulation at low micromolar concentrations and increase it at high micromolar concentrations. Finally, we have shown that the otherwise cytoprotective and lipid droplet-inducing oleate does not provide a survival advantage in starving HeLa cells. These interesting findings warrant further experimental work in order to explain the mechanisms and establish the relevance of FA and lipid droplet metabolism in HeLa and other cancer cells.

The major contribution of this work is the revealing of lipid droplet metabolism, and in particular the DGAT and ATGL enzymes, as potential therapeutic targets for the development of novel strategies in the fight against some of the most aggressive types of cancer.

Appendix A.

Review Paper "Lipid Droplets in Cancer: Guardians of Fat in a Stressful World"

The paper “Lipid Droplets in Cancer: Guardians of Fat in a Stressful World” by Toni Petan, Eva Jarc and Maida Jusović was published in *Molecules* in 2018. We discuss here the roles of lipid droplets in stressed cells, focusing in particular on cancer. Lipid droplets accumulate in various tumors and in cells exposed to nutrient and oxidative stress, suggesting a close relationship between lipid droplets and the cellular stress response. Here, we focus on the emerging mechanisms of stress-induced lipid droplet biogenesis, the roles of lipid droplets during stress, and the complex bidirectional relationship between lipid droplets and autophagy in cancer and beyond. I contributed to this work by performing extensive literature research and writing sections of the manuscript draft.

[Petan, T., Jarc, E., & Jusović, M. (2018). Lipid droplets in cancer: guardians of fat in a stressful world. *Molecules, 23* (8): E1941, doi: 10.3390/molecules23081941.]

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Review

Lipid Droplets in Cancer: Guardians of Fat in a Stressful World

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Abstract: Cancer cells possess remarkable abilities to adapt to adverse environmental conditions. Their survival during severe nutrient and oxidative stress depends on their capacity to acquire extracellular lipids and the plasticity of their mechanisms for intracellular lipid synthesis, mobilisation, and recycling. Lipid droplets, cytosolic fat storage organelles present in most cells from yeast to men, are emerging as major regulators of lipid metabolism, trafficking, and signalling in various cells and tissues exposed to stress. Their biogenesis is induced by nutrient and oxidative stress and they accumulate in various cancers. Lipid droplets act as switches that coordinate lipid trafficking and consumption for different purposes in the cell, such as energy production, protection against oxidative stress or membrane biogenesis during rapid cell growth. They sequester toxic lipids, such as fatty acids, cholesterol and ceramides, thereby preventing lipotoxic cell damage and engage in a complex relationship with autophagy. Here, we focus on the emerging mechanisms of stress-induced lipid droplet biogenesis; their roles during nutrient, lipotoxic, and oxidative stress; and the relationship between lipid droplets and autophagy. The recently discovered principles of lipid droplet biology can improve our understanding of the mechanisms that govern cancer cell adaptability and resilience to stress.

Keywords: lipid droplets; fatty acids; autophagy; lipophagy; cancer; oxidative stress; nutrient stress; lipotoxicity; lipase; phospholipase

1. Introduction

Cancer cells are often faced with an inconsistent and limited availability of nutrients due to poor vascularization [1]. They possess powerful oncogene-driven mechanisms of metabolic reprogramming that enable cell survival and growth even in the harshest environmental conditions [2,3]. Their remarkable adaptability to adverse conditions is critical for their propensity to migrate and invade neighbouring and distant tissues [1]. Cancer cells exposed to severe stress display an impaired ability to synthesize their own fatty acids (FAs) and their survival depends on lipids from the microenvironment and on those that can be recommissioned from their own structural and storage pools [3,4]. Cancer cells having greater capabilities to acquire extracellular lipids and efficient mechanisms for intracellular lipid mobilisation and recycling will thus have a significantly higher chance for survival. Indeed, some of the most aggressive cancer types driven by the Ras-oncogene use opportunistic modes of FA acquisition, possess an increased capacity for FA accumulation in intracellular lipid droplets, and upregulate mitochondrial FA oxidation to resist nutrient and oxidative stress [5–10]. Lipid droplets are dynamic organelles that play a pivotal role in the management of lipid uptake, distribution and consumption. Lipid droplet accumulation has been observed in many cancers and is elevated in cancer cells exposed to hypoxia or nutrient starvation [8,11–15]. Emerging studies suggest that these organelles suppress

nutrient and oxidative stress and contribute to cancer cell survival and growth [7–9,15–19], suggesting that lipid droplet metabolism is an attractive target for reducing the resilience of cancer cells to stress.

Lipid droplets store neutral lipids, mostly triacylglycerol (TAG) and sterol esters, and balance lipid uptake, storage and use according to cellular needs [20]. They have long been regarded as inert fat depots, but numerous roles beyond energy storage are emerging, including regulation of lipid trafficking, protein management and quality control, and viral replication [18,19,21,22]. Most cells respond to lipid overload by synthesizing lipid droplets to dampen the potentially damaging surge of lipids, but, intriguingly, their biogenesis is also induced during various types of stress, including complete deprivation of nutrients and lipids (Figure 1). It is becoming clear that a common feature of the cellular states that promote lipid droplet production are imbalances in energy metabolism and redox homeostasis. These may be instigated by different stressors, such as complete or partial nutrient deprivation, nutrient overload, oxygen deprivation, treatments with pro-oxidants, ceramides, chemotherapeutic agents, and inducers of endoplasmic reticulum (ER) stress [7–9,16,23–27] (Figure 1). These conditions lead to the activation of stress-response pathways and result in different cellular defects that have been shown to induce lipid droplet biogenesis: oxidative stress [8,24,26,28,29], ER stress [30], activation of autophagy [31–34], inflammation [13,35], mitochondrial dysfunction, and cell death [36–38]. The fact that cells undergo a seemingly futile cycle of lipid droplet biogenesis and breakdown during such challenging conditions suggests that the benefits of lipid droplets must outweigh the expense of their biogenesis. Indeed, recent studies reveal the pleiotropic nature of lipid droplets and their broad impact on cellular homeostasis during stress. It is now clear that some of the major roles of lipid droplets are associated with the management of cellular stress (Figure 2).

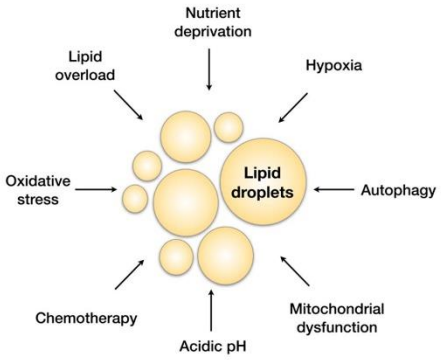

Figure 1. Lipid droplet biogenesis is induced during stress. Environmental factors and cellular states that are characterised by imbalances in cellular energy metabolism and redox homeostasis, and are typical of tumour-like stress, induce the biogenesis of lipid droplets, including lipid overload, complete or partial nutrient and oxygen deprivation, oxidative stress, high autophagic flux, acidic environment, mitochondrial damage and apoptotic cell death, and treatment with chemotherapeutics.

First, lipid droplets are central anti-lipotoxic organelles that control FA, diacylglycerol (DAG), cholesterol and ceramide lipotoxicity by sequestering these potentially toxic lipids into inert triglycerides, cholesterol esters and acylceramides [9,27,39–41] (see Sections 4 and 5). The biogenesis of lipid droplets is protective because it reduces direct cell damage caused by these lipids and reduces their availability for conversion into other toxic lipid species. Importantly, recent studies have shown that lipid droplets not only protect cells from exogenous lipid overload, but that they also guard against a surge of endogenous lipids, such as that occurring during high autophagic flux [31,32]. Lipid droplets also sequester oxidation-prone polyunsaturated FAs in their protective core, thus restricting

lipid peroxidation reactions and maintaining optimal membrane saturation and organelle homeostasis during hypoxia, oxidative stress, lipid overload, and apoptotic cell death [9,24,37]. Lipid droplets are thus crucial for managing lipid imbalances arising due to different stress conditions and are critical for diverting potentially toxic lipids from a bioactive or a structural pool to a storage pool within the protective neutral lipid droplet core.

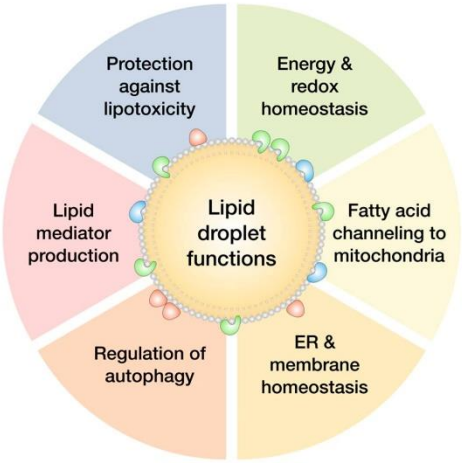

Figure 2. Lipid droplet functions during stress. Lipid droplets can assume different roles in stressed cells: (1) reduction of lipotoxic cell damage by sequestration of potentially toxic endogenously- or exogenously-derived lipids, such as saturated and polyunsaturated fatty acids (FAs), ceramides and cholesterol, and storing them in the form of inert triacylglycerols (TAGs), acylceramides, and cholesterol esters; (2) maintenance of energy and redox homeostasis by providing substrates for energy production, stimulating NADPH synthesis, and regulating oxidative metabolism via lipolysis-mediated signalling; (3) regulation of FA trafficking and distribution, e.g., enabling optimal transfer of FAs to mitochondria for efficient FA oxidation; (4) maintenance of endoplasmic reticulum (ER) and membrane homeostasis, protein quality control, and protection against ER stress; (5) regulation of autophagy; and (6) production of bioactive lipid mediators, including pro- and anti-inflammatory signalling molecules derived from polyunsaturated FAs, such as eicosanoids and specialized pro-resolving mediators.

Second, lipid droplets provide FAs not only for energy production during both nutrient and oxygen deprivation, but also for the maintenance of redox homeostasis (see Sections 6 and 7). In cancer cells exposed to hypoxia, FA uptake is upregulated and lipid droplets are formed to protect cells from reactive oxygen species (ROS) by supporting NADPH synthesis [8]. They also provide temporary storage for FAs that may be used as needed during the rapidly changing conditions in the microenvironment, such as hypoxia/reoxygenation and feeding/starvation [7–9,17]. Lipid droplets thus act as switches that coordinate FA trafficking and consumption either as fuel for energy production, for the maintenance of redox homeostasis or for membrane biogenesis during rapid cell growth [19,42,43].

Third, lipid droplets dynamically interact with mitochondria in response to nutrient cues and provide a network of conduits for efficient channelling of FAs, thereby enabling a homogenous distribution of FAs into tubulated mitochondria and maximizing the efficiency of FA oxidation during starvation (see Section 6) [25,41]. However, recent studies have only begun to reveal the complexity of the relationship between mitochondria and lipid droplets. Intriguingly, a subset of peridroplet

mitochondria with an elevated TCA cycle capacity but a reduced FA oxidation, in fact, provides ATP for the synthesis of TAG and supports lipid droplet biogenesis [44].

Fourth, lipid droplets are crucial for the maintenance of membrane and ER homeostasis. They contribute to membrane and protein quality control and are involved in the removal of damaged lipids, aggregated and unfolded proteins, thereby reducing ER stress [16,19,26,40,45–47]. Emerging studies suggest that alterations in lipid droplet breakdown through lipolysis and autophagy have a profound effect on the management of membrane and ER homeostasis, oxidative stress, mitochondrial function, and stress signalling (see Sections 5, 7 and 8) [48–57]. The breakdown of lipid droplets leads to the release of hundreds of lipid species with different characteristics and cellular roles, including FAs, DAGs, ceramides, and cholesterol. An excessive lipid droplet turnover may cause cell damage, and the balance between lipid droplet biogenesis and breakdown must be tightly controlled by cells exposed to stress.

Fifth, lipid droplets are engaged in a complex relationship with autophagy (see Section 8 and Figure 5). On the one hand, lipid droplets have been shown to provide lipids for the formation of autophagosomal membranes, manage ER stress, or stimulate signalling processes that promote autophagy [58–61]. On the other hand, autophagy drives lipid droplet biogenesis by providing lipids recycled from membranous organelles [25,31,34], but it may also participate in their breakdown through lipophagy, a lipid droplet-selective form of autophagy [62].

Finally, the breakdown of lipid droplets provides FAs and other lipids that act as signalling molecules by themselves—e.g., by interacting with transcription factors such as peroxisome proliferator-activated receptors (PPARs) and sterol-regulatory element binding proteins (SREBPs)—or they are converted into bioactive lipid mediators, such as eicosanoids, that act as paracrine and autocrine messengers affecting inflammatory signalling, metabolism, proliferation, migration, and metastasis [14,63–66]. This emerging aspect of lipid droplet biology will not be covered in this review, but we would like to refer the reader to several excellent recent reviews [13,18,67–69].

In this review, we focus on recent advances describing the involvement of lipid droplets in the protection against nutrient, lipotoxic, and oxidative stresses. We discuss the emerging mechanisms of stress-induced lipid droplet biogenesis, the roles of lipid droplets during stress, and the complex bidirectional relationship between lipid droplets and autophagy. Because studies on the role of lipid droplets in cancer are relatively scarce, we provide a broader overview of lipid droplet function in cellular stress in various tissues and pathophysiological settings. Many of these recently discovered principles of lipid droplet biology can improve our understanding of the mechanisms that govern cancer cell resistance to stress.

2. Lipid Droplets Basics

Lipid droplets have a unique structure among organelles with a central core of neutral lipids surrounded by a phospholipid monolayer and numerous peripheral and embedded proteins [70–72]. Their neutral lipid core is composed mostly of TAGs and sterol esters, but they also store acylceramides and fat-soluble vitamins [18,39]. These are cytosolic organelles, derived from the ER, but have also been observed in the nucleus and may be produced at the inner nuclear membrane in yeast cells [73,74]. The most widely accepted mechanism of lipid droplet biogenesis posits that lipid droplets are formed de novo following the synthesis of TAGs and sterol esters between the two leaflets of the ER membrane [70,75,76]. Diacylglycerol acyltransferases 1 and 2 (DGAT1 and DGAT2) catalyse the last step in the TAG biosynthesis pathway, while cholesterol ester (CE) synthesis depends on acyl-CoA cholesterol acyltransferases 1 and 2 (ACAT1 and ACAT2). When sufficient lipids accumulate within the growing lipid lens in the ER bilayer, a nascent lipid droplet buds from the ER membrane and is released into the cytosol but still maintains dynamic membrane contacts with the ER, which enable bidirectional cargo transfer [77,78]. Many components of the lipid droplet biogenesis machinery are transferred to the nascent lipid droplet and enable the growth, fusion, and fission of these dynamic organelles. Lipid droplets undergo a dynamic cycle of biogenesis and breakdown in response to

nutrient cues from the environment. They differ in size, number and distribution within cells in a population and in different cell types and these properties change depending on the physiological state of the cell [79]. Different lipid droplet pools are thought to have different protein compositions and functions [71,80–82].

Lipid droplets are broken down by two major mechanisms: lipolysis and lipophagy [51,57,83,84]. Lipolysis enables a highly regulated release of FAs from TAGs by the sequential action of adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL), and monoacylglycerol lipase (MAGL) [85–87]. Lipid droplet breakdown in adipocytes is hormonally regulated and provides FAs for mitochondrial energy production in non-adipose tissues during fasting [66,85,86]. In non-adipose tissues, lipolysis is involved in the regulation of mitochondrial oxidative metabolism, FA oxidation, membrane synthesis, cell growth, ER homeostasis, and lipid mediator synthesis [25,40,43,63,66,88–90]. Lipophagy is a recently discovered selective form of autophagy, whereby lipid droplets are enclosed within autophagosomal membranes and fused with lysosomes for degradation by hydrolytic enzymes [62]. Neutral and lysosomal lipolysis have overlapping roles in cellular lipid degradation and a cross-talk between them has been proposed [51,56,91]. This and other aspects of lipid droplet turnover in cells exposed to stress is discussed in more detail in the following sections.

3. Cancer Cells Depend on Lipids to Survive and Grow in a Challenging Environment

During tumorigenesis, cancer cells acquire various metabolic alterations to overcome the metabolic challenges of rapid proliferation and survival under inhospitable conditions. The changes in cancer metabolism are driven by oncogene activation or loss of tumour-suppressor genes, and by alterations in cellular signalling [2,92,93]. Recent evidence has revealed that the reprogramming of lipid metabolism, including changes in de novo lipogenesis, FA oxidation, as well as phospholipid and neutral lipid metabolism, is essential for various aspects of tumourigenesis [12,94–98]. While most normal cells preferentially use extracellular lipids for the synthesis of new structural lipids, cancer cells can elevate de novo FA synthesis irrespective of the levels of extracellular lipids to satisfy their requirements for lipids or to sustain proliferation in a lipid-poor microenvironment [94,98]. Upregulated SREBP signalling contributes to increased phospholipid, TAG, and cholesterol syntheses, and promotes cell survival and tumour growth [99,100]. On the other hand, FA oxidation is essential for the survival of cancer cells undergoing metabolic stress due to starvation, hypoxia, and matrix-detachment [95,101]. Furthermore, cancer cells display alterations in nutrient acquisition pathways and are able to scavenge extracellular lipids to sustain proliferation even in nutrient- and oxygen-deprived conditions [3–5,8,10]. In times of need, cancer cells use opportunistic modes of nutrient acquisition [2] and extract lipids from unusual sources, such as lysophospholipids [5] and even necrotic cellular corpses [10]. Cancer cells also engage in symbiotic relationships with neighbouring adipocytes to obtain FAs for energy production [102–104]. Numerous lipids, such as sphingolipids, FAs, and their oxygenated metabolites eicosanoids and pro-resolving lipid mediators, act as signalling molecules and second messengers that alter cancer cell proliferation, migration, angiogenesis, and immune responses [69,94,105]. Finally, membrane lipid composition, levels of saturation and cellular distribution of lipids are underexplored aspects that are crucial for organelle homeostasis, cell signalling, and the management of nutrient and oxidative stress [9,94,106,107]. It is becoming clear that the regulation of lipid metabolism, and in particular FA availability, is critical for the survival and proliferation of the stressed cancer cell. It is thus not surprising that cancer cells have multiple sources of FAs at hand that may be interchangeably used according to their particular metabolic needs and nutrient availability. Cancer cells exposed to limiting levels of nutrients and oxygen may acquire FAs from at least three main lipid pools (Figure 3), which may all drive lipid droplet biogenesis: (1) extracellular sources, including unusual, "alternative" sources of lipids and opportunistic modes of acquisition that provide FAs from extracellular lysophospholipids [5], from neighbouring adipocytes through ATGL-mediated lipolysis [102,103], through membrane phospholipid hydrolysis of healthy or damaged stromal cells and extracellular vesicles by enzymes such as secreted phospholipases A_2

(sPLA$_2$s) [7,108–111] and by engulfing damaged cells and cell remnants, such as apoptotic cells and cellular corpses [2,10]; (2) de novo FA synthesis, which is significantly upregulated in several cancers and is activated in the absence of extracellular lipids [94]; and (3) mobilisation of endogenous, structural lipids via breakdown of membranous organelles by autophagy and/or membrane remodelling by phospholipases (see Sections 6 and 8.3 and Figure 4) [7,9,15,23,24,108,112]. Lipid droplets are emerging as consolidating and regulatory hubs for these pathways of cellular lipid acquisition and are thus attractive targets for the management of cellular stress in cancer.

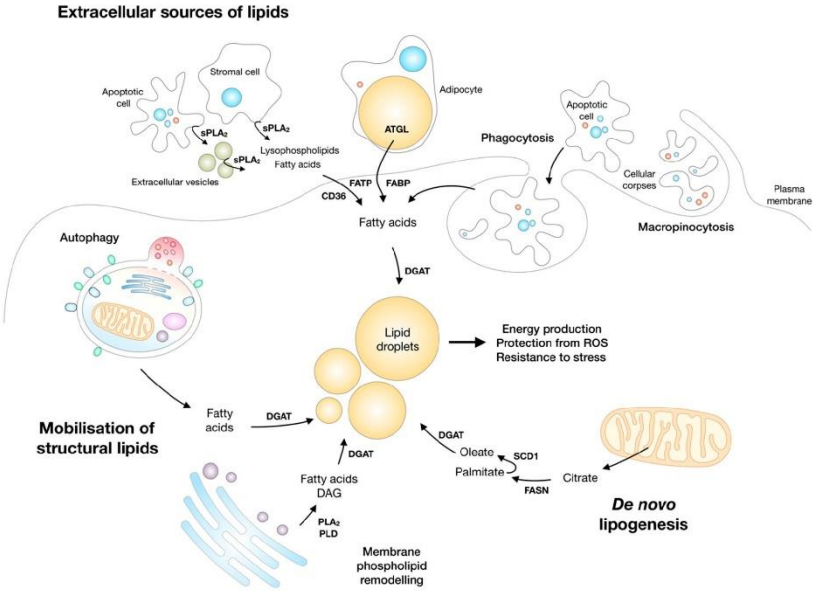

Figure 3. Different pathways of lipid acquisition may drive lipid droplet biogenesis in stressed cancer cells. Depending on the level of nutrient and oxygen deprivation and on the availability of extracellular lipids, cells may interchangeably use three main lipid pools: (1) extracellular sources, particularly when access to circulating lipids is limited, cancer cells display alternative and opportunistic modes of lipid uptake and may acquire fatty acids (FAs) from extracellular lysophospholipids, from stromal adipocytes through adipose triglyceride lipase (ATGL)-mediated lipolysis, via membrane phospholipid hydrolysis of neighbouring cells, extracellular vesicles and apoptotic cells by enzymes such as secreted phospholipase A$_2$ (sPLA$_2$), and by engulfing various material, such as apoptotic cells and even cellular corpses; (2) endogenous de novo FA synthesis, which becomes dominant when extracellular lipid sources are exhausted; and (3) mobilisation of endogenous, structural lipids via breakdown of membranous organelles by autophagy and/or membrane phospholipid hydrolysis by phospholipases A$_2$ (PLA$_2$) and D (PLD), which is activated during severe stress, such as complete nutrient deprivation. CD36, cluster of differentiation 36, platelet glycoprotein 4, fatty acid translocase; DAG, diacylglycerol; DGAT, diacylglycerol acyltransferase; FABP, fatty acid-binding protein; FASN, fatty acid synthase; FATP, fatty acid transport protein; SCD1, stearoyl-CoA desaturase 1.

Lipid Droplets Accumulate in Various Cancers

Some of the earliest evidence for the existence of cytoplasmic lipid droplets in cancer cells was provided in 1957 when numerous lipid droplets were found in the cytoplasm of a rat liver

tumour [113]. The first lipid droplets in human cancer cells were observed in 1963 when a high content of stainable "lipid vesicles" was observed in mammary carcinoma [114]. In a breakthrough study, Ramos and Taylor [115] performed a morphologic characterization of tumours from clinical samples and found that lipid-containing mammary carcinomas display a more aggressive behaviour. Since then, lipid droplets have been described in various animal and human cancers [116–118] and their accumulation has been associated with neoplastic processes and tumour invasiveness. Lipid droplet accumulation has been observed in many human cancers including breast [114,119], brain [118], liver [116], lung [120], cervical [121], prostate [122], colon [14], skin, bile duct [123], clear-cell renal cell carcinoma [124], ovarian [103], and pancreatic cancers [125]. A high aggressive potential has been ascribed to lipid-rich tumours and it has been associated with a poor clinical outcome [125,126]. Elevated lipid droplet accumulation has also been observed in hypoxic tumour areas and cancer cell lines deprived of oxygen [8,29,99,127]. It has been suggested that elevated lipid droplet levels in tumours are a consequence of acidic pH, glucose deprivation, or hypoxia [11]. These days, it is clear that cancer cells display significant alterations in lipid metabolism [94] and emerging evidence suggests that changes in lipid droplet metabolism are an important hallmark of cancer metabolic reprogramming [12,13,92,128].

4. Lipid Droplet Biogenesis and Lipotoxicity

Cells maintain FA homeostasis by coordinating the various cellular processes that generate or deliver FAs with processes that use these molecules. They have to balance their endogenous FA levels with FA availability from the circulation and according to their needs for energy production in mitochondria, for use as building blocks for membranes, and as precursors for more complex signalling lipids. The main sources of exogenous lipids in the circulation are serum albumin-FA complexes, released by adipocytes during fasting/lipolysis and lipoproteins, providing FAs released by lipoprotein lipase or other lipases/phospholipases [57,129]. The major sources of cellular endogenous FAs are de novo FA synthesis and TAG lipolysis from existing lipid droplets, but mobilisation of structural lipids by autophagy of membranous organelles and by membrane phospholipid hydrolysis also contributes to the endogenous pool of FAs, particularly during nutrient deprivation [7,15,23,25,112] (discussed in more detail in Sections 6 and 8.3; Figures 3 and 4). Any imbalance in these processes may lead to excess amounts of free FAs in the cell, which leads to lipotoxicity. Lipotoxicity is thus most often a consequence of a disparity between cellular lipid uptake, storage, and utilization [130]. Cells exposed to increased levels of FAs experience cellular stress, dysfunction and damage associated with elevated lipid turnover, ROS production, ER stress, mitochondrial damage, defective cell signalling pathways, impaired insulin signalling, elevated production of inflammatory mediators and cell death [130–132]. Lipid overload increases the rates of processes associated with FAs, such as β-oxidation, which may consequently lead to the over-production of ROS, lipid peroxidation, membrane, DNA and protein damage [133]. It is therefore not surprising that chronically elevated circulating levels of lipids and ectopic lipid accumulation in tissues have been linked with debilitating chronic diseases such as obesity, metabolic syndrome, heart failure, liver diseases, and diabetes [21,130,133]. In addition, besides diabetes and liver diseases, obesity is now a well-recognized risk factor for the development of many cancer types and it has been associated with reduced patient responses to chemotherapy [134,135].

As discussed in the following paragraphs in more detail, accumulation of excess FAs in the protective lipid droplet core diverts FAs from pathways causing lipotoxicity, thereby reducing the biogenesis of lipid species with a greater lipotoxic and pro-inflammatory potential, such as DAGs, ceramides, and acylcarnitines [31,39,136–138], and protecting sensitive, oxidation-prone polyunsaturated FAs from lipid peroxidation [9,24]. Lipid droplets also suppress nutrient stress associated with lipid overload by reducing excess mitochondrial FA oxidation and ROS overflow [132,139]. Indeed, mechanisms that control lipid droplet breakdown are critical for balancing FA-mediated energy production, FA trafficking, and oxidative metabolism. A poorly explored factor important for lipotoxicity is the "lipid quality" within lipid droplets, i.e., the FA composition of

TAGs and CEs stored in lipid droplets. The acyl-chain composition of lipid droplets, together with enzymes regulating lipid droplet synthesis and breakdown, governs the availability of particular FA species for various cellular processes, including the regulation of membrane unsaturation, which is tightly connected with ER and redox homeostasis during stress. Lipid droplets thus generally serve as protective organelles, which is seemingly at odds with the fact that lipid accumulation in non-adipose tissues has been associated with impaired cellular function and metabolic diseases. This paradox likely occurs due to the fact that in pathologic states, lipotoxicity may occur over time when the cellular capacity for neutral lipids is exceeded leading to dysregulation of lipid droplet biogenesis and/or breakdown mechanisms. In such conditions, lipolysis may be chronically elevated leading to cell damage associated with increased free FA levels in the cytosol [55,130,132].

4.1. Lipid Droplet Biogenesis Protects from Lipotoxicity

Excess FAs may be either removed by oxidation in mitochondria or stored in the form of neutral lipids to reduce their lipotoxicity [21]. Directing FAs to lipid droplets for storage in the form of TAGs has been shown to protect cells from lipotoxicity and prevent insulin resistance in various tissues, including the skeletal muscle, liver, and heart [27,47,140–143]. In fact, DGAT1-dependent lipid droplet formation mitigates lipotoxicity under various conditions of exogenous and endogenous lipid overload, including diet-induced obesity [144,145], cardiomyopathy [146,147], neuronal hypoxia [24], incubation with exogenous saturated and unsaturated FAs [9,27,144], and during periods of high autophagic flux [31]. Interestingly, even in adipose tissue, the lipolytic flux of FAs from lipid droplets into the cytosol causes cell damage and adipocytes undergo a "futile" ATP-driven cycle of FA release from TAGs by lipolysis and their re-esterification into TAGs, mediated by the DGAT1 enzyme, in order to reduce ER stress and adipose tissue inflammation [47].

Important evidence for the beneficial role of DGAT-mediated lipid droplet biogenesis at the organismal level has been obtained from studies focused on the connection between lipid accumulation and metabolic diseases. Overexpression of DGAT1 in white adipose tissue of mice exposed to high-fat diet leads to increased accumulation of TAGs in adipose tissue and to obesity, but it also improves insulin sensitivity in comparison with wild-type mice [145]. Similarly, mice overexpressing DGAT1 or DGAT2 in the liver develop steatosis but do not show any defects in insulin sensitivity and signalling, suggesting that lipid accumulation in the liver is not sufficient to cause insulin resistance [148]. Moreover, although intramyocellular fat deposits have been associated with insulin resistance, physical exercise elevates DGAT activity and TAG accumulation in skeletal muscle and reduces DAG and ceramide levels, a phenomenon referred to as "the athlete's paradox" [149]. Furthermore, transgenic overexpression of myocellular DGAT1 reduces high-fat diet-induced insulin resistance in mice. Humans with severe heart failure have a marked reduction in FA oxidation and DGAT1 mRNA levels in the heart and accumulate DAG and ceramides [150]. In accordance, cardiomyocyte-specific DGAT1 knockout mice also accumulate toxic lipids, mostly DAG and ceramides, in the heart and have increased mortality due to heart failure [147]. It has also been shown that elevated DGAT1 expression in macrophages increases their capacity for TAG storage and protects from diet-induced insulin resistance and inflammation [144]. Moreover, the protective effects of antidiabetic PPARγ agonists against FA-induced inflammatory macrophage activation were dependent on DGAT1. In the endothelium, DGAT1-mediated lipid droplet biogenesis is important for buffering postprandial surges of FAs and reducing their lipotoxicity [151]. Therefore, in most tissues exposed to elevated levels of circulating lipids, lipid droplet biogenesis protects cells from lipotoxicity and mitigates the effects of lipid overload on the development of insulin resistance, inflammation, and metabolic disorders.

4.2. Lipid Droplets Mediate the Protective Effects of Unsaturated FAs Against Lipotoxicity

In a seminal study, Listenberger et al. (2003) have shown that interfering with lipid droplet biogenesis by depletion of DGAT1 leads to cell death in fibroblasts exposed to the otherwise non-toxic oleate [27]. They found that palmitate is poorly incorporated into the TAG pool, whereas

exogenously-added or stearoyl-CoA desaturase 1 (SCD1)-derived unsaturated FAs were readily incorporated into lipid droplets. Moreover, oleate reduced palmitate-induced apoptosis by channelling the saturated FA into lipid droplets and reducing ceramide and ROS production associated with its toxicity. Exogenous oleate also prevented palmitate-induced inflammation, insulin resistance, and apoptosis in skeletal muscle cells by channelling palmitate into lipid droplets and stimulating β-oxidation via AMP-activated protein kinase (AMPK) activation [152,153]. In human cardiomyocytes, acyl-CoA synthetase long-chain family member 1 (ACSL1) and PPARγ overexpression augmented TAG accumulation and alleviated ER stress induced by exogenous palmitate, suggesting that channelling palmitate to neutral lipid stores reduces its lipotoxicity [40]. On the contrary, knockdown of DGAT1 in cultured rat hepatocytes exposed to exogenous FAs did not enhance palmitate lipotoxicity or prevent the ability of oleate to reduce palmitate-induced cell damage [154]. In accordance, Robblee et al. found that oleate mitigates palmitate-induced ER stress in myeloid cells independently of DGAT1 activity or palmitate incorporation into TAGs. Instead, the flux of saturated FAs into phospholipids was responsible for activation of the ER stress sensor inositol-requiring kinase 1α (IRE1α) and the NLR family pyrin domain-containing 3 (NLRP3) inflammasome, and this was counteracted by oleate incorporation into phospholipids [155]. Thus, the beneficial effects of oleate may also be based on its competition against palmitate for esterification into phospholipids [154,155]. Therefore, although a number of studies have shown that lipid droplet biogenesis mediates the protective effects of unsaturated FAs against saturated FA-induced lipotoxicity, more research is warranted to confirm this concept and define the underlying mechanisms.

Studies describing the relationship between FA lipotoxicity and lipid droplet metabolism in cancer cells are scarce. The roles of different FA species in supporting lipid droplet accumulation and cell growth in different cancer cell types in different environmental conditions are also not clear. It is generally accepted that excess amounts of saturated FAs are toxic to cancer cells, while unsaturated FAs, such as oleate, are most often cytoprotective, induce proliferation, and even provide protection from long-term starvation in some cancer cells [7,17,156,157]. However, as discussed below, the protective role of oleate may not be universal to all cancer cells. It has been shown that palmitate and other saturated FAs induce apoptosis in different breast cancer cell lines and are not incorporated into TAGs as readily as unsaturated FAs [156,157]. On the contrary, oleate reduces starvation-induced apoptosis and mitigates the lipotoxicity of saturated FAs in highly invasive MDA-MB-231 triple-negative breast cancer cells, likely by channelling saturated FAs toward TAG synthesis [157]. However, although oleate induces lipid droplet formation in MDA-MB-231, MCF-7, and T-47D breast cancer cells, it suppresses serum starvation-induced cell death only in MDA-MB-231 cells and it is toxic to the less aggressive hormone-responsive T-47D cells [7,9]. Similarly, exogenous oleate induces lipid droplet biogenesis, but it does not provide a survival advantage to serum-deprived HeLa cells [158]. More studies are necessary to explain the basis for the different effects of oleate and other unsaturated FAs on lipid droplet accumulation and survival of different types of cancer cells, but it is likely that rather than the level of lipid droplet accumulation at a given time point, the balance between lipid droplet biogenesis/breakdown and other FA metabolic pathways that determine FA flux and availability will be critical determinants. The underlying mutational landscape and the specific metabolic alterations of different cancer cells most probably dictate the ability of unsaturated FAs and lipid droplets to protect or harm cells in particular microenvironmental conditions.

A number of studies have demonstrated that polyunsaturated fatty acids (PUFAs) have distinct and contrasting effects in cancer, with the pro-inflammatory ω-6 PUFAs, such as arachidonic acid (AA; 20:4, ω-6), mostly displaying pro-tumorigenic effects and ω-3 PUFAs, such as eicosapentaenoic (EPA; 20:5, ω-3) and docosahexaenoic acid (DHA; 22:6, ω-3), showing anti-tumorigenic, anti-inflammatory, and pro-apoptotic effects in cancer cells [159,160]. However, most in vitro studies have been performed with high micromolar, non-physiological concentrations of PUFAs [160–163] and often disregard the need for proper complexation of free FAs to serum albumin prior to application [164]. The total free (non-esterified) FA concentration in human plasma is approximately 200 μM after overnight

fasting [165], with oleate being the most abundant FA (80 μM), followed by linoleate (15 μM), AA (3 μM), DHA (1 μM), and EPA (0.4 μM). Thus, cells exposed to high micromolar concentrations of PUFAs are under a massive overload of easily oxidizable FAs, suggesting that their apparent toxic effects may be largely exaggerated and may mask potential underlying effects. Indeed, we have recently found that both ω-3 and ω-6 PUFAs, used at low-micromolar, near physiological concentrations, display similar effects as oleate in inducing lipid droplet biogenesis and promoting breast cancer cell survival during stress [9]. As expected, higher micromolar concentrations of PUFAs, but not oleate or linoleate, induced oxidative stress-dependent cell death. Importantly, suppression of TAG synthesis by DGAT1 inhibition augmented PUFA-induced lipotoxicity, while stimulation of lipid droplet biogenesis reduced PUFA-induced cell death. The protective effect of lipid droplets was observed when their biogenesis was induced either by exogenous oleate, linoleate, or by the group X secreted phospholipase A_2 enzyme ($sPLA_2$). The enzyme binds very well to the phosphatidylcholine-rich plasma membrane of breast cancer cells and releases a mixture of mono- and polyunsaturated FAs, in which oleate and linoleate are by far the dominant FA species [9]. At first sight, these results suggest that the $sPLA_2$, and its products oleate and linoleate, channel excess PUFAs into neutral lipid storage and thus prevent their oxidation and cell damage. Instead, we found that the enzyme enriches TAGs with FAs with low unsaturation levels and reduces the fraction of highly unsaturated PUFA-TAGs stored in lipid droplets. This effectively leads to "dilution" of the most oxidation-prone PUFAs within lipid droplets, reducing their availability for lipolytic release [9]. In accordance, the levels of PUFAs in the phospholipid pool were reduced. Moreover, suppression of TAG lipolysis by ATGL depletion reduced PUFA-induced oxidative stress and cell death, confirming that the release of PUFAs from lipid droplets is responsible for cell damage. Importantly, as described below (Section 7.3), a similar anti-lipotoxic and antioxidant role for lipid droplets has been recently described in vivo in *Drosophila* larvae exposed to hypoxia, whereby the sequestration of membrane-derived PUFAs in lipid droplets reduces their lipotoxicity and has a critical role in enabling neuronal cell proliferation during development [24]. Therefore, lipid droplet biogenesis, TAG acyl chain remodelling, and lipid droplet breakdown are all determinants of PUFA lipotoxicity, suggesting that differences in basal or stress-induced levels of these processes in cancer and other cell types may strongly influence the lipotoxic potential of PUFAs. The capacity of cancer cells to balance (poly)unsaturated FA sequestration and release from lipid droplets is thus important for their ability to cope with FA-induced lipotoxicity and to use FAs for cell survival.

4.3. Lipid Droplets Also Store Acylceramides and Reduce Ceramide Accumulation-Induced Cell Damage

Interestingly, it was shown recently that acylceramides are also stored in lipid droplets, thus further expanding the roles of lipid droplets in their capacity to act as a sink for diverting not only lipotoxic FAs and DAGs, but also ceramides, from a bioactive to a storage pool [39]. It was found that acylceramides are synthesized by a complex involving ACSL5, ceramide synthase (CerS) and DGAT2 at the ER/lipid droplet interface in cultured cells and in the livers of mice on a high-fat diet. The conversion of ceramide into acylceramide and its sequestration into lipid droplets was associated with prevention of cell death. In colorectal carcinoma cells, stimulation of acylceramide biogenesis led to protection from ceramide-mediated 5-fluorouracil-induced cell death, whereas a blockade of acylceramide biogenesis led to elevated ceramide accumulation and apoptosis. Thus, the storage of acylceramide in lipid droplets in cancer cells may improve their resistance to chemotherapy by reducing pro-apoptotic ceramide levels. Interestingly, both DGAT1 and DGAT2 displayed ceramide acyltransferase activity, although DGAT2 is likely the predominant isoform responsible for acylceramide synthesis in vivo [39]. Thus, DGAT enzymes directly regulate the lipotoxicity of both DAG and ceramide by acylating and diverting these lipids into storage. Likewise, it may be anticipated that lipases that release ceramide from lipid droplets would also strongly impact the level of cell damage instigated by ceramide [39]. This previously unknown mechanism of reduction of ceramide toxicity calls for a re-evaluation of many previous studies on the lipotoxicity associated with saturated FA-induced ceramide and DAG accumulation. Thus, lipid droplets act as central

anti-lipotoxic organelles that control FA, DAG, cholesterol and ceramide lipotoxicity by coordinating TAG, CE and acylceramide storage.

4.4. Lipid Droplets Accumulate Cholesterol Esters to Regulate Cholesterol Availability and Promote Tumour Growth

Although the majority of studies addressing the role of lipid droplets in cancer have focused on FA metabolism and TAG accumulation, recent reports suggest that CE accumulation in cancer cells is also associated with tumour growth. CE accumulation has been associated with a poor clinical outcome in breast cancer patients [126] and with the aggressiveness of glioblastoma, prostate, and pancreatic cancer [166–168]. Elevated accumulation of CEs in prostate cancer has been associated with upregulated PI3K/Akt signalling and an increased uptake of exogenous lipids [166]. Importantly, inhibition of cholesterol esterification impaired cancer cell aggressiveness and suppressed tumour growth in mouse xenograft models. In glioblastoma, inhibition of ACAT1 increased cholesterol levels, leading to inhibition of SREBP-1 and suppression of lipogenesis and tumour growth [168]. In a mouse model of pancreatic cancer, depletion of ACAT1 suppressed tumour growth and metastasis by increasing intracellular free cholesterol levels, causing elevated ER stress and cell death. A lipid accumulating phenotype has been described in highly invasive and tumourigenic triple-negative (ER–, PR–, HER2–) breast cancer cells and it has been associated with increased cell proliferation, migration and cell survival [7,17,169,170]. In comparison with less tumorigenic hormone-responsive breast cancer cells, triple-negative breast cancer cells accumulate significantly greater amounts of TAGs upon exposure to exogenous unsaturated FAs and display a superior ability to use lipid droplets and FA oxidation for cell survival during prolonged starvation [7,9,17]. These cells also display a greater uptake of both exogenous oleate and lipoproteins and have a higher expression of ACAT1 in comparison with less aggressive ER+ breast cancer cells. Importantly, the suppression of CE accumulation by inhibition of ACAT1 reduced low-density lipoprotein (LDL)-stimulated proliferation and migration [169,170]. Thus, although the underlying mechanisms have not been clarified, the ability of cancer cells to increase their storage of CEs in lipid droplets and thus remove cholesterol from its bioactive pool is beneficial for the progression of several aggressive types of cancer.

4.5. Lipid Droplets Protect Cancer Cells From Chemotherapeutic Drugs

Cancer drug resistance is a major obstacle in cancer therapy and lipid droplets may be important for the ability of cancer cells to survive chemotherapeutic stress [12,18]. Cotte et al. [16] have recently investigated the role of lipid droplets in colorectal cancer under chemotherapeutic conditions. They found that the common chemotherapeutic agents 5-fluorouracil and oxaliplatin induce lysophosphatidylcholine acyltransferase 2 (LPCAT2)-dependent lipid droplet accumulation. They found that LPCAT2-driven lipid droplet biogenesis protects cancer cells from chemotherapy-induced ER stress and cell death both in vitro and in vivo. The authors also show that lipid droplets sequester calreticulin and prevent its exposure on the plasma membrane, thus impairing CD8+ T cell infiltration and immunogenic cell death under chemotherapeutic treatment [16]. The sequestration of calreticulin to lipid droplets suggests that limiting the exposure of danger-associated molecular patterns (DAMPs) may be another important mechanism through which lipid droplets protect stressed cancer cells from cell death. Besides identifying LPCAT2 as a potential target to improve the efficacy of chemotherapy in colorectal cancer, this study also suggests that the presence of elevated levels of lipid droplets may be diagnostically useful to predict tumour chemoresistance.

5. Lipolysis and Lipotoxic Stress

5.1. ATGL Regulates Fatty Acid-Mediated Energy Production, Signalling, and Lipotoxic Stress

Relatively little is known about the role of lipolytic lipid droplet breakdown in the regulation of lipotoxicity and cancer cell survival during stress [12,57]. Nevertheless, studies on the role of lipolysis and, in particular, ATGL, in cellular and organismal lipid homeostasis, provide important clues for cancer biology. ATGL catalyses the rate-limiting step in TAG lipolysis and is the major cytosolic TAG hydrolase in adipose tissue, the heart and liver, and other tissues [57,85,88,171]. The enzyme has a crucial role in supplying skeletal muscle with FAs from adipose tissue and is involved in the mobilisation of intramyocellular TAG reserves for energy conversion in the working muscle [86]. ATGL also enables FA transfer from lipid droplets to mitochondria in cardiomyocytes, hepatocytes, starved mouse embryonic fibroblasts, and, presumably, in most other cell types and tissues [25,66,88]. Lipolytic lipid droplet breakdown fuels the mitochondrial oxidative metabolism and enables cell survival during nutrient deprivation in various cells, including several cancer cell types [7,9,15,25]. Aside from being substrates for energy production, ATGL-derived FAs promote signalling pathways leading to an elevated mitochondrial and oxidative gene expression, thus, matching β-oxidation with FA mobilisation from lipid droplets [66,67,88,172]. ATGL deficiency in humans causes systemic TAG accumulation and neutral lipid storage disease with cardiomyopathy, but not obesity [173]. In accordance, mice with global ATGL deletion accumulate TAG in many tissues and exhibit lethal cardiac dysfunction due to a massive accumulation of TAG in the heart [174]. Paradoxically, ATGL-deficient mice are only moderately obese and display improved glucose tolerance and insulin sensitivity despite TAG accumulation in multiple tissues [174–176]. This unexpected adipose tissue phenotype was explained by a study using ATGL-deficient mice expressing ATGL only in the heart [177]. These mice were resistant to diet-induced obesity and had normal insulin sensitivity and adipogenic differentiation, however, they displayed impaired signalling through PPARγ and SREBP-1c. Therefore, reduced lipogenesis may, in fact, be responsible for the resistance of ATGL-deficient mice to high-fat diet-induced obesity and obesity-induced insulin resistance. In line with this, inhibition of ATGL in mice using Atglistatin leads to improvement of insulin signalling and a reduction of diet-induced obesity and hepatosteatosis, likely due to a reduced release of adipose tissue-derived FAs in the circulation [178]. Importantly, the pharmacological inhibition of ATGL did not cause ectopic lipid accumulation or cardiac dysfunction in mice even after long-term treatments. The inhibition of ATGL is thus a promising therapeutic strategy to treat obesity, metabolic disorders, and potentially also cancer [178].

While the prevailing role of ATGL in non-adipose tissues is most likely providing FAs for mitochondrial energy production and stimulating signalling associated with oxidative metabolism, TAG lipolysis may also lead to lipotoxicity. Excessive lipolysis is often associated with states of lipid overload and this may cause cell damage by increasing the pool of free FAs in the cytosol, altering signalling pathways that regulate oxidative metabolism, ER homeostasis, and stimulating β-oxidation and ROS production [53–55]. The inhibition of ATGL-mediated lipolysis by perilipin 5 provides a "lipolytic barrier" against uncontrolled TAG lipolysis in oxidative tissues, thus preventing cardiac, hepatic and muscle lipotoxic injury and insulin resistance [179–183]. Perilipin 5 couples TAG lipolysis with the metabolic demand for FAs, prevents ceramide accumulation and protects tissues against oxidative stress induced by excessive β-oxidation and FA peroxidation [89,139,180,184]. Additionally, perilipin 5 forms transcriptional complexes with PPARγ coactivator-1α (PGC-1α) and sirtuin 1 (SIRT1) and promotes the transcription of mitochondrial and oxidative genes in response to catecholamine-triggered signalling [185]. Similarly, the absence of ATGL protects mice treated with tunicamycin from hepatic ER stress and inflammation by inducing TAG accumulation and enrichment with oleate, thus shifting the balance in FA composition to reduce the flux of lipotoxic palmitate in the liver [90]. Moreover, stimulation of lipolysis by ATGL overexpression leads to ER stress in cardiomyocytes treated with oleate, which, in contrast to palmitate, does not cause ER stress

in cells with basal levels of ATGL [40]. In accordance with these studies, ATGL depletion in breast cancer cells challenged with lipotoxic concentrations of PUFAs leads to lipid droplet accumulation and reduction of oxidative stress and cell death, suggesting that the sequestration of PUFAs in lipid droplets is beneficial for protection from PUFA lipotoxicity [9]. On the contrary, ATGL deficiency in macrophages leads to TAG accumulation and cell death associated with ER stress, elevation of ROS, and mitochondrial damage [186], suggesting that ATGL may also play a protective role against lipotoxicity. Thus, both excessive TAG accumulation and lipolysis may induce cell damage and the precise regulation of ATGL activity may be critical in some cases. Collectively, depending on the cell type, state and environmental conditions regulating cellular stress, ATGL-mediated lipolysis may be responsible for providing FAs for mitochondrial energy production, regulation of oxidative metabolism, and cell survival, but it may also cause lipotoxic cell damage. Notably, lipid droplet lipolysis and mitochondrial metabolism are coupled and regulated at multiple levels and their coordination is critical for the removal of toxic FA species and the prevention of lipotoxicity.

5.2. ATGL and Cancer

The role of lipolysis in cancer has not been a subject of many studies to date [97]. Elevated expression of MAGL has been observed in several cancers and a pro-tumourigenic role has been suggested for the enzyme based on its ability to promote cancer aggressiveness by upregulating the endocannabinoid and FA signalling pathways [87,187,188]. On the other hand, there are conflicting reports about the role of ATGL in cancer [189]. ATGL depletion leads to growth inhibition in several cancer cell lines [190,191], suggesting a pro-tumourigenic role for the enzyme. Additionally, recent studies suggest that ATGL activity in both tumours and neighbouring adipose tissue contributes to the adipocyte-induced metabolic reprogramming and aggressiveness of breast, pancreatic, and ovarian cancer [102–104,192]. Activated lipolysis in omental fat adipocytes provides lipids for energy production in ovarian cancer cells and promotes their growth at the metastatic site [103]. ATGL is overexpressed in high-grade mammary tumours and breast cancer cell lines in vitro [104]. Breast cancer cells take up FAs released via lipolysis from neighbouring adipocytes, whereas ATGL activity in breast cancer cells enables FA translocation to mitochondria and stimulates the induction of uncoupled FA oxidation. The latter sustains AMPK activation and enables a persistent metabolic reprogramming that stimulates tumour invasion and metastasis [104]. However, recent in vivo studies suggest a tumour suppressor role for ATGL since its expression is reduced in several human cancers, including breast invasive adenocarcinoma, and it correlates with a reduced patient survival [193]. Importantly, mice lacking ATGL spontaneously develop lung tumours [193] and an adipose tissue-specific combined deficiency of ATGL and HSL leads to the development of liposarcoma [194]. Clearly, more studies are necessary to clarify the role of ATGL in cancer. However, in light of these conflicting reports, and given the pleiotropic roles of ATGL and lipolysis in different tissues in energy metabolism and beyond, a universal pro-tumourigenic or tumour suppressor role for ATGL should not be expected. Its role is most likely dependent on the particular type and stage of cancer, specific metabolic profile, nutrient status, and the level of environmental stress imposed by the presence or absence of nutrients and oxygen that critically affects lipid droplet metabolism. Importantly, the involvement of peritumoural adipocyte-derived ATGL in the provision of FAs for tumour growth suggests that ATGL may be a potential therapeutic target in tumours that depend on FAs derived from neighbouring adipose tissue.

6. Lipid Droplets are Synthesized and Broken Down during Nutrient Stress to Enable Cell Survival

6.1. Lipid Droplet Biogenesis is Enhanced during Nutrient Deprivation

Lipid droplet biogenesis is a general cellular response to exogenous lipid overload to reduce the accumulation of lipotoxic lipids, as described above. However, lipid droplet biogenesis also occurs in response to nutrient deprivation. This has been demonstrated in various types of cells

exposed to complete or partial nutrient deprivation, such as primary neurons and astrocytes, CHO-K1 cells, HeLa cervical carcinoma, Huh7 liver carcinoma, U2OS osteosarcoma, LN18 glioblastoma cancer cells, and starved mouse embryonic fibroblasts (MEFs) [15,23,25,31,195]. Importantly, nutrient deprivation-induced lipid droplet biogenesis occurs even in the absence of exogenous lipids and is independent of endogenous de novo lipid synthesis. Rather, it is driven by the autophagic and/or phospholipase-mediated mobilisation of the available endogenous, structural lipids (Figures 3 and 4) [23,25,31]. Paradoxically, cells mobilise endogenous lipids and engage in an ATP-consuming TAG synthesis process despite the absence of sufficient nutrients. Lipid droplet biogenesis must, therefore, be essential for cell survival, but it was not clear until recently why a futile cycle of FA esterification into TAG and lipolysis would be necessary for energy production and cell survival. Studies in starved MEFs have shown that structural lipids are mobilised through autophagy of membranous organelles and that lipid droplets are necessary for at least two main reasons: (1) to prevent the lipotoxicity of FAs released by autophagy into the cytosol [31] and (2) to enable efficient transfer of FAs from lipid droplets to the network of tubulated mitochondria for oxidation [25,31,34,41]. The lipid droplet biogenesis in starved MEFs depends on the activation of autophagy (see discussion in Section 8.3) and on DGAT1 activity. DGAT1 depletion or inhibition leads to mitochondrial damage due to FA overload and a concomitant accumulation of toxic acylcarnitine species. Thus, DGAT1-mediated lipid droplet biogenesis in starved cells is necessary to buffer autophagy-liberated FAs and prevent mitochondrial damage.

Previous studies have suggested that endogenous FAs required for TAG synthesis may also be derived from phospholipase A_2 (PLA_2)-mediated phospholipid hydrolysis (Figure 4). Namely, TAG synthesis in nutrient-deprived CHO-K1 cells is dependent on the activity of calcium-independent group VIA PLA_2 (also referred to as $iPLA_2\beta$ and PNPLA9; encoded by the *PLA2G6* gene), an enzyme traditionally ascribed with a role in membrane remodelling and repair [196,197]. Interestingly, the process of lipid droplet biogenesis, but not TAG synthesis, required the subsequent phosphorylation and activation of another PLA_2 enzyme—the calcium-dependent cytosolic group IVA PLA_2 (also $cPLA_2\alpha$; encoded by the *PLA2G4A* gene)—which is most likely responsible for facilitating lipid droplet nucleation by regulating ER membrane shape and curvature [23,76,112,198]. Interestingly, besides being implicated in the regulation of the formation of the organelle [76,112], several other PLA_2s [7,9,112,199] and phospholipases D (PLD) [24] have been implicated in channelling phospholipid-derived FAs into TAGs and CEs for lipid droplet biogenesis (Figure 4). Since a comprehensive study addressing the involvement of autophagy and PLA_2s in the provision of FAs for lipid droplet biogenesis in starved cells has not been performed, it is possible that PLA_2s act, in fact, as part of the broader autophagy-induced process of lipid droplet biogenesis. Phospholipase activity-derived FAs could be preferred over autophagy when a particular phospholipid and TAG acyl-chain remodelling is required (see Section 7.3), instead of the bulk FA provision by the lysosomal degradation of membranous organelles. In such cases, phospholipase-derived FAs could provide a subtler and safer channelling of FAs from membrane phospholipids to TAGs, simultaneously optimizing membrane saturation to adapt to cellular stress and providing FAs for TAG synthesis and energy production in mitochondria. It is thus possible that, depending on the conditions and cell type in question, autophagy and/or phospholipase activity may participate in lipid droplet biogenesis during stress.

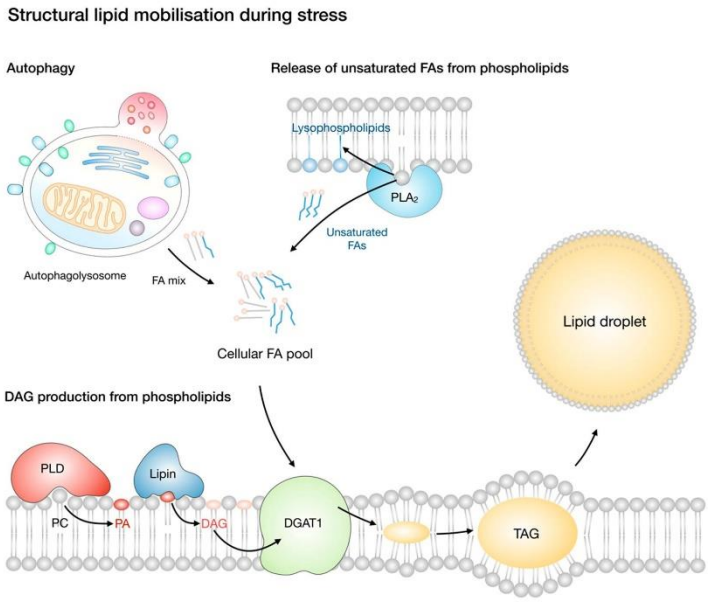

Figure 4. Autophagy and phospholipase-mediated phospholipid hydrolysis stimulate lipid droplet biogenesis during stress. Nutrient starvation activates the autophagy-mediated breakdown of membranous organelles leading to the release of various saturated and unsaturated fatty acids (FAs) that drive diacylglycerol acyltransferase 1 (DGAT1)-mediated lipid droplet biogenesis. Cellular stress may also lead to phospholipase A_2 (PLA_2) activation and membrane phospholipid hydrolysis that predominantly results in the release of unsaturated and polyunsaturated fatty acids (FAs), which are typically found at the *sn*-2 position of membrane phospholipids. Intracellular phospholipases A_2, such as the calcium-independent group VIA PLA_2 ($iPLA_2\beta$ or PNPLA9), or secreted isoforms, such as the group X secreted PLA_2, may drive lipid droplet biogenesis. Oxidative stress may lead to phospholipase D (PLD)-mediated redistribution of FAs from membrane phospholipids to triacylglycerols (TAGs) stored within lipid droplets in order to protect polyunsaturated FAs from peroxidation. Phospholipase D (PLD) converts phosphatidylcholine (PC) to phosphatidic acid (PA), whereas phosphatidate phosphatases (lipins) convert PA to diacylglycerol (DAG). The latter is then available for TAG synthesis by DGAT1 or DGAT2. Phospholipase activity-derived FAs could be preferred when a specific phospholipid and/or TAG acyl-chain remodelling is required instead of the bulk FA mixture provided by autophagy. Future studies will provide clues on the possible crosstalk between these pathways and the conditions that govern the predominant mechanisms of lipid droplet biogenesis during various conditions of stress in different cell types.

6.2. Lipid Droplet Breakdown is Necessary for Cell Survival during Starvation

Several lines of evidence suggest that FAs stored in starvation-induced lipid droplets are mobilised by cytoplasmic lipases, and not lipophagy (see also Section 8.3), to fuel the mitochondrial oxidative metabolism and to enable cell survival during nutrient deprivation [9,15,25,31]. The transfer of FAs from lipid droplets to mitochondria in acutely starved MEFs is dependent on ATGL and is inhibited by etomoxir, an inhibitor of carnitine palmitoyltransferase 1A (CPT-1A) and of FA translocation into mitochondria [25]. Furthermore, a close spatial proximity of lipid droplets and tubulated mitochondria is required for efficient transfer and redistribution of FAs within mitochondria [25,41]. This requirement

for precise channelling of FAs from lipid droplets to mitochondria may explain why lipolysis, rather than lipophagy, is the preferred process for FA transfer to mitochondria in Hanks' balanced salt solution (HBSS)-starved cells [25]. However, under milder and prolonged starvation conditions, lipophagy may be activated and may become the predominant mechanism of lipid droplet breakdown [25,51,62]. Interestingly, in serum-starved MEFs, autophagy-driven lipid droplet biogenesis does not occur [31], but lipophagy contributes to the lipid droplet breakdown [25]. We have found that while ATGL is involved in TAG lipolysis in breast cancer cells exposed to prolonged starvation, its depletion is not sufficient to fully prevent lipid droplet breakdown and significantly reduce the pro-survival effect of lipid droplets [9], suggesting the involvement of other lipases and/or lipophagy in serum starvation-induced lipid droplet breakdown in breast cancer cells. Therefore, the importance of autophagy in lipid droplet biogenesis and the predominant role of lipophagy vs. lipolysis in lipid droplet breakdown most likely depends on the cell type, its particular metabolic requirements, and the type and length of nutrient stress imposed.

7. Lipid Droplets and Oxidative Stress

Cancer cells display elevated rates of reactive oxygen species (ROS) production due to oncogenic mutations, loss of tumour suppressors, accelerated metabolism necessary to sustain rapid growth, and adaptation to the various stresses imposed by the tumour microenvironment (i.e., nutrient and oxygen deprivation, infiltrating immune cells) [200,201]. Cancer cells may thus experience oxidative stress during both oncogene-driven nutrient excess and rapid metabolism and during nutrient and/or oxygen deprivation in poorly vascularized tumours [1]. Importantly, it is now clear that ROS- induced pro-tumourigenic signalling pathways are necessary for cancer cell survival, growth, proliferation, and metastasis. High levels of ROS, in turn, oxidize other molecules, such as proteins, lipids, and DNA, thus, further supporting oncogenic transformations. However, prolonged exposure to elevated ROS may lead to oxidative stress-dependent cell death and cancer cells simultaneously upregulate antioxidant pathways in order to maintain redox homeostasis. Oxidative stress also affects cellular lipid metabolism on several levels. The availability of glucose carbons for lipogenesis is highly restricted during hypoxia and cancer cells shift to a reductive glutamine metabolism or cytosolic acetate carboxylation for lipid synthesis [94,202,203]. Hypoxic cancer cells may upregulate FA uptake to compensate for impaired de novo lipogenesis [5,8]. Moreover, hypoxia inhibits FA oxidation and can induce the formation of lipid droplets as a temporary storage of energy for use during reoxygenation [8,204]. However, during metabolic stress, FA oxidation maintains the production of NADPH and is critical for protection against oxidative stress and cancer cell survival [95,101]. On the contrary, upregulated lipolysis and FA oxidation may also result in an elevated production of ROS and cell damage [53,180,205]. Imbalances in redox homeostasis are thus associated with significant alterations in lipid metabolism and it is not surprising that they also affect the lipid droplet metabolism. In this section, we discuss how lipid droplets support the cellular response to oxidative stress, and conversely, how hypoxia/oxidative stress regulates lipid droplet biogenesis and degradation.

7.1. Lipid Droplets Accumulate in Hypoxia and Protect Cancer Cells from Oxidative Stress

Tumour cells often face restricted access to nutrients and oxygen due to an aberrant vascularization and poor blood supply [206]. Hypoxia, a non-physiological level of low oxygen tension, is a common feature of most solid and large tumours [207]. The hypoxic tumour microenvironment affects cancer metabolism by upregulating hypoxia-inducible factor (HIF) signalling, thereby promoting angiogenesis, metastasis, aggressiveness, chemoresistance, resistance to radiotherapy, and a poor overall outcome [208,209]. Short- and long-term exposure to hypoxia has been shown to promote cell survival by activating cellular stress response mechanisms—such as autophagy—and enhance tumour aggressiveness [210,211]. Hypoxia-associated induction of lipid droplet biogenesis has been observed in different cancers, including brain, breast, renal, and prostate cancers [8,12,26,28,29,204,212]. Studies

in C6 rat models of glioma have shown that lipid droplets accumulate in the perinecrotic and necrotic hypoxic areas of the tumour core [212,213]. Primary glioma cultures obtained from patients also accumulate lipid droplets and have an increased expression of PPARα [204]. Hepatocellular carcinoma and cervical adenocarcinoma cells exposed to hypoxia accumulate lipid droplets through a direct HIF-1α-dependent stimulation of lipin 1 expression, a phosphatidate phosphatase involved in the production of DAG [28]. Constitutive HIF signalling and abundant lipid droplet accumulation are hallmarks of clear-cell renal carcinoma and it was shown recently that HIF2α-dependent perilipin 2 (PLIN2) expression stimulates lipid droplet accumulation, protects from ER stress, and promotes cell proliferation and xenograft tumour growth [26]. Several studies have shown that hypoxia leads to a blockade in the endogenous production of unsaturated FAs due to the inhibition of oxygen-dependent SCD1-mediated FA desaturation and that it renders cancer cells dependent on the uptake of unsaturated FAs to reduce hypoxia-induced ER stress and prevent cell death [4,5,107]. In accordance, glioblastoma and breast cancer cells exposed to hypoxia shift from de novo lipogenesis to FA uptake and accumulate lipid droplets, which are necessary to prevent ROS accumulation and to enable cancer cell survival [8]. Lipid droplet biogenesis was dependent on the HIF-1α-mediated upregulation of fatty acid-binding proteins (FABPs) 3 and 7, members of a family of proteins involved in FA uptake, metabolism and transport. Knockdown of FABP3, FABP7 or of the structural lipid droplet protein PLIN2 significantly impaired lipid droplet biogenesis during hypoxia, reduced NADPH levels, and increased ROS accumulation, suggesting that FA uptake and lipid droplet biogenesis are crucial for redox homeostasis. Accordingly, exogenous addition of the unsaturated FA oleate also protected cells from ROS toxicity. Importantly, the inhibition of lipid droplet formation by depletion of FABP3/7 led to suppression of xenograft tumour growth in vivo. Thus, lipid droplet biogenesis in hypoxia is most likely a protective mechanism that promotes cancer cell survival under oxygen-deprived conditions by reducing ROS accumulation. The accumulated lipid droplets provide a temporary storage of FAs during hypoxia and release them after reoxygenation to enable ATP production and cell growth [8]. Similarly, hypoxia-induced changes in lipid mobilisation have also been associated with increased TAG accumulation in prostate cancer cells and in extracellular vesicles released by these cells. Following reoxygenation, lipid droplets provided FAs for β-oxidation and enabled cancer cell growth [29]. Collectively, the studies described above show that cancer cells exposed to hypoxia upregulate FA uptake and accumulate lipid droplets, which enable ER and redox homeostasis during oxygen deprivation and provide FAs for mitochondrial energy production to boost cell growth following reoxygenation.

7.2. Excessive Lipolysis is Damaging to Hypoxic Cancer Cells

Recent studies have established that hypoxia affects the expression of several lipid droplet-associated proteins involved in lipid droplet breakdown [205,214]. Gimm et al. have shown that hypoxia-activated HIF-1-mediated signalling upregulates the expression of hypoxia-inducible lipid droplet-associated protein (HILPDA)/hypoxia-inducible gene 2 (HIG2)—a protein localized on the lipid droplet surface—in a number of cell lines and mouse tissues in vivo [214]. HIG2 binds to the surface of lipid droplets and its overexpression promotes neutral lipid accumulation in HeLa cells. Furthermore, HIG2 co-localization with lipid droplets was observed in HIF-1α-positive areas in renal cell carcinoma samples, suggesting that HIG2 may be used as a marker of hypoxia-induced lipid droplets [214]. Moreover, HIG2 expression is elevated during anti-angiogenic treatment and it may promote tumour resistance to therapy [215]. Recently, the basis for the HILPDA/HIG2-induced lipid droplet accumulation was explained by studies from two laboratories showing that the protein is a direct inhibitor of ATGL [205,216]. Zhang et al. found that the observed downregulation of ATGL-mediated lipolysis during hypoxia is due to HIF-1-induced upregulation of HIG2 [205]. Importantly, HIG2 depletion enhanced lipid droplet breakdown and β-oxidation, leading to elevated ROS production and impaired xenograft tumour growth. Depletion of ATGL reversed the effects of HIG2 knockout, suggesting that HIG2-mediated ATGL inhibition enables the survival of hypoxic

cancer cells by reducing lipid droplet degradation and sequestering FAs away from mitochondrial oxidative and ROS-producing pathways [205]. Thus, ATGL-mediated lipid droplet breakdown is damaging to cancer cells during hypoxia and the ability of cancer cells to modulate lipolysis may determine their potential to survive oxygen deprivation. Other inhibitors and activators of ATGL, such as perilipin 5, G0/S2 switch regulatory protein 2 (G0S2), and abhydrolase domain-containing protein 5 (ABHD5/CGI-58), whose roles in cancer are currently under investigation [189–191,217,218], could have an important impact on lipid droplet metabolism in hypoxic cancer cells. As discussed above, based on its involvement in the protection of several tissues from lipolysis-induced oxidative stress, including ischemia/reperfusion injury in the heart [180], it will be particularly interesting to find out whether perilipin 5 is involved in the survival of some cancer cell types exposed to fluctuating levels of oxygen in the tumour microenvironment.

7.3. Sequestration of PUFAs in Lipid Droplets Reduces Membrane Lipid Peroxidation and Protects Cells from Oxidative Stress

Besides hypoxia and the accumulation of endogenous lipid metabolites that disrupt mitochondrial and ER homeostasis, such as saturated FAs, ceramides, and DAG, oxidative stress can also arise due to chemotherapeutic drugs, exogenous pro-oxidants, and the accumulation of oxidized PUFAs. PUFAs are significantly more sensitive to oxidation in comparison to saturated and monounsaturated FAs. Cell membrane phospholipids containing PUFAs are particularly vulnerable to lipid peroxidation chain reactions during oxidative stress, thereby further amplifying ROS production [219]. Accordingly, increasing the relative degree of membrane (poly)unsaturation by inhibition of de novo lipogenesis sensitizes cancer cells to lipid peroxidation and oxidative stress-induced cell death [106]. Recently, an antioxidant role for lipid droplets against PUFA-induced lipotoxicity and oxidative stress was reported in neuronal and cancer cells [9,24]. In *Drosophila* larvae exposed to oxidative stress, it was shown that lipid droplet biogenesis occurs in niche glial cells to prevent peroxidation of membrane PUFAs by redirecting them from membrane phospholipids to TAGs stored within lipid droplets [24]. This mechanism spares both glia and neuroblasts from hypoxia-induced oxidative stress and allows neuroblast proliferation. Interestingly, lipid droplet biogenesis was not dependent on HIF-1 and could be induced by exposing larvae to various pro-oxidants, suggesting that ROS are the main inducers of lipid droplets during both pro-oxidant treatment and hypoxia. In agreement, studies from another laboratory have shown that oxidative stress in neurons leads to ROS-dependent accumulation of lipid droplets in neighbouring glial cells [220,221]. Notably, both hypoxia and pro-oxidants induced PUFA redistribution from plasma membranes to TAGs where the PUFAs were less vulnerable to peroxidation than in membranes [24]. The redistribution was dependent on PLD, lipin 1, and DGAT1 enzymes, suggesting that FAs are transferred from membrane phospholipids via PLD-dependent production of phosphatidic acid, its dephosphorylation to DAG by lipin 1 and a final DAG acylation mediated by DGAT1 (Figure 4) [24]. Lipid droplets also protected cellular proteins from harmful products of peroxidation, such as 4-hydroxynonenal (4-HNE), suggesting an even wider antioxidant role for the organelle. Thus, hypoxia and oxidative stress induce lipid droplet accumulation in glial cells to sequester oxidation prone PUFAs away from oxidative pathways, thereby reducing stress and enabling the proliferation of neighbouring neuronal stem cells. As discussed in more detail above (Section 4.2), a similar antioxidant role of lipid droplets in aggressive breast cancer cells exposed to lipotoxic levels of PUFAs has been recently described [9]. These cells benefited from sequestration of PUFAs in lipid droplets and both $sPLA_2$-mediated stimulation of lipid droplet biogenesis, as well as suppression of ATGL-mediated lipolysis, protected cells from PUFA-induced oxidative stress. $sPLA_2$-induced protection from PUFA lipotoxicity was associated with extensive TAG remodelling and reduced levels of PUFAs present in cellular membranes, revealing an important connection between TAG and phospholipid remodelling for cancer cell resistance to stress [9]. Interestingly, an enrichment of PUFA-TAGs in lipid droplets has been observed during the activation of hepatic stellate cells [222,223], a process critical for the development of liver fibrosis and

associated with elevated levels of oxidative stress [224]. Moreover, fasted mice have elevated levels of very long-chain PUFA-TAGs in their hepatocyte lipid droplets in comparison with mice fed a high-fat diet [225]. Cisplatin treatment has been associated with an elevated accumulation of oleate and linoleate in lipid droplets of cancer cells [226]. An enrichment of TAGs with PUFAs has also been found in visceral adipose tissue samples from colon cancer patients, along with elevated expression of markers of inflammatory lipid metabolism, including phospholipases and prostaglandin synthesis–related enzymes, such as the group X $sPLA_2$ and cyclooxygenase-2 (COX-2) [227]. Lipid droplet biogenesis is also induced during apoptosis [36,228] and PUFA-TAG enrichment has been suggested as a general feature of apoptosis [37]. Thus, the sequestration of PUFAs in lipid droplets and away from membrane phospholipids may be a general cytoprotective mechanism activated during various conditions associated with oxidative stress, to prevent further damage due to membrane lipid peroxidation. It will be interesting to see in future studies whether similar PUFA sequestration mechanisms protect cancer cells from various stress conditions characterized by an elevated ROS production.

8. Lipid Droplets and Autophagy/Lipophagy

Autophagy is an evolutionarily conserved catabolic process of recycling dispensable cytoplasmic material by lysosomal degradation, thus providing building blocks and metabolic substrates for processes essential for cell survival [229–231]. Under physiological conditions, constitutive autophagy maintains cellular homeostasis by degrading potentially toxic products of normal cellular metabolism, such as damaged mitochondria and protein aggregates. Importantly, autophagy is crucial for the ability of both normal and malignant cells to cope with stress and adapt to adverse environmental conditions, such as nutrient and oxygen deprivation [229,232]. Autophagy is activated under various stressful conditions, including nutrient deprivation, hypoxia, elevated ROS levels, and, interestingly, by lipid overload and exogenous unsaturated FAs [129,233,234]. It acts as a tumour suppressor by preventing the accumulation of damaged cellular components, but cancer cells also use autophagy to survive stressful conditions, such as nutrient deprivation, hypoxia, and the high metabolic demand during rapid proliferation. Autophagy has thus been under investigation as an attractive therapeutic target in efforts to reduce tumour growth and chemoresistance. However, autophagy also promotes tumour-specific immune responses and recent studies suggest that the activation of autophagy using safe nutritional interventions may, in fact, improve the efficacy of anticancer agents [233].

There are three main types of autophagy and they are all involved in lipid droplet metabolism: macroautophagy, microautophagy, and chaperone-mediated autophagy (CMA). Macroautophagy (hereafter named "autophagy") refers to the packaging of cytosolic cargo into an autophagosome and its fusion with the lysosome for enzymatic breakdown of the engulfed material. Although initially considered a non-selective process, several types of selective autophagy have been discovered [230] and named according to their substrate specificity: protein aggregates (aggrephagy), peroxisomes (pexophagy), mitochondria (mitophagy), ER (reticulophagy), pathogens (xenophagy), lysosomes (lysophagy), and lipid droplets (lipophagy) [235]. CMA is a specialized form of selective autophagy, so far characterized only in mammalian cells, and includes a motif-based recognition of damaged proteins by the heat shock cognate protein of 70 kDa (Hsc70) and lysosomal intake mediated by the lysosome-associated membrane protein type 2 (LAMP-2A) [236]. In contrast, microautophagy enables the uptake of cytoplasmic material by a direct invagination of the lysosomal membrane and it has been characterized in yeast [46,237], while its importance in mammalian cells is still questionable.

Recent studies have established strong links between autophagy and the maintenance of cellular and whole-body lipid homeostasis under physiological conditions and during stress, including that induced by lipid imbalance. The relationship is bidirectional since lipids also regulate autophagy. Lipid overload affects autophagic activity in multiple tissues, which has important implications for diseases associated with excessive lipid accumulation, such as metabolic disorders and cancer [51,129]. Interestingly, while short-term high-fat feeding induces autophagy in pancreatic β-cells to protect from lipotoxicity [129,238], long-term, chronic lipid overload impairs autophagic flux in β-cells,

hepatocytes, and cardiomyocytes [129]. The crosstalk between autophagy and lipid metabolism is also reflected in their common hormonal, nutrient, and transcriptional regulators, such as mTORc1, AMPK, PPARα, PGC-1α, TFEB, TFE3, FoxO1, CREB, SREBP, and FXR [52,83,239,240]. Since lipid droplets are central to lipid homeostasis, there are also multiple ways of crosstalk between lipid droplets and autophagy (Figure 5). Autophagy may participate in lipid droplet biogenesis [25,31,34,241,242] and breakdown [62], but lipid droplets may also promote autophagy through several mechanisms [58–61]. Indeed, recent studies have shown that lipid droplets may be sites for autophagy initiation or provide lipids for the assembly of the autophagic machinery. Thus, autophagy and lipid droplet metabolism are connected through a number of different mechanisms, and the prevalence of each may depend on several factors, including the cell type in question, its nutritional status, and environmental context. Future studies will help us decipher the complex associations between these two important processes, thereby providing new clues for the understanding of metabolic diseases and cancer. In the following text, we have attempted to give a broad overview of the mechanisms of crosstalk between autophagy and lipid droplet metabolism. The detailed molecular mechanisms and pathophysiological aspects of autophagy/lipophagy have been reviewed recently in excellent reviews and will not be covered here [229,232,243,244].

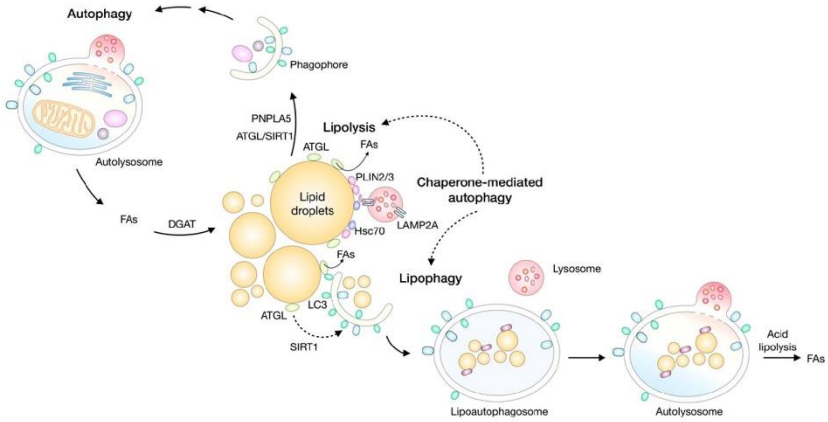

Figure 5. Crosstalk between autophagy and lipid droplets. Autophagy provides fatty acids (FAs) for lipid droplet biogenesis in starved cells, but it may also participate in lipid droplet breakdown though lipophagy. Lipid droplets promote autophagy through several possible mechanisms: acting as sites for autophagy initiation, providing lipids for the assembly of the autophagic machinery (e.g., via patatin-like phospholipase domain-containing 5 (PNPLA5)-mediated TAG lipolysis), maintaining endoplasmic reticulum (ER) homeostasis that enables autophagy initiation or stimulating sirtuin 1 (SIRT1) signalling by adipose triglyceride lipase (ATGL) that promotes both autophagy and lipophagy. Chaperone-mediated autophagy (CMA) promotes both lipophagy and ATGL-mediated lipolysis by degrading the lipid droplet-coating proteins perilipin 2 and 3 (PLIN2 and PLIN3), thereby freeing the access to the lipid droplet surface for lipases such as ATGL and for the lipophagic machinery. CMA is executed following recognition of PLINs by the chaperone Hsc70, their unfolding, and translocation into the lysosome through the lysosomal membrane glycoprotein receptor LAMP-2A. A coordinated regulation of lipophagy and lipolysis has also been suggested through a direct interaction between microtubule-associated protein light chain 3 (LC3) and ATGL. DGAT, diacylglycerol acyltransferase.

8.1. Lipophagy is an Important Pathway for Lipid Droplet Breakdown during Stress

The role of autophagy in lipid droplet catabolism has been widely studied in the past decade after the surprising discovery of lipophagy. Indeed, besides lipolysis, lipophagy is emerging as a major pathway of lipid droplet breakdown in multiple tissues and pathophysiological settings [51]. Lipolysis is catalysed by neutral lipases acting on the surface of lipid droplets, whereas lipophagy uses the autophagic machinery to deliver lipid droplets or parts of lipid droplets to the lysosome for degradation by acidic lipases. Lipolysis predominantly affects TAGs, DAGs, and CEs, leading to shrinkage of lipid droplets, which is accompanied by remodelling of surface phospholipids and proteins. On the contrary, lysosomal lipid droplet breakdown degrades all storage and structural lipid droplet lipids and associated lipid droplet proteins, thereby releasing FAs, cholesterol, amino acids, and other products into the cytosol. Recent evidence suggests that the prevalent mechanism of lipid droplet breakdown depends on physiological requirements, nutrient status, and the type of stress but also that a significant cross-talk exists between lipolysis and lipophagy [51,56,91]. A number of studies have reported pivotal roles of lipophagy in the maintenance of lipid stores, the regulation of nutrient and energy balance, protection against oxidative stress, ER stress and lipotoxicity, protein quality control and even viral replication [48–52,245]. These and other studies have implicated lipophagy in important pathophysiological processes such as embryogenesis, lipid digestion in the gut, neural control of food intake and organismal energy balance, obesity, metabolic disorders, liver disease, atherosclerosis and cancer.

The discovery of lipophagy in 2009 was based on findings that autophagy regulates lipid metabolism in mouse hepatocytes in vitro and in vivo [62]. Singh et al. showed for the first time that lipid droplets interact with the autophagic machinery during nutrient deprivation and that the inhibition of autophagy leads to TAG and lipid droplet accumulation. Depletion of the essential autophagic protein autophagy related 5 (Atg5) reduced TAG breakdown and β-oxidation rates in starved and oleate-fed cells indicating that autophagy regulates mitochondrial oxidation by providing free FAs from TAG hydrolysis. In line with this, autophagic deficiency leads to impairment of mitochondrial energy production and to oxidative stress-dependent cell death in hepatocytes [246]. Importantly, the study by Singh et al. [62] suggests that rather than de novo lipid synthesis, the dysfunction of lipophagy may contribute to elevated lipid accumulation in diseases associated with lipid storage in the liver. Namely, they found that a high-fat diet-induced lipid accumulation in the liver interferes with the execution of lipophagy, revealing a detrimental cycle in which elevated lipid storage reduces lipid clearance by autophagy, leading to a further increase in lipid accumulation. In accordance, it has been suggested recently that lipophagy facilitates the clearance of toxic ceramides arising from elevated de novo sphingolipid synthesis in the livers of obese mice [247]. Moreover, hormonal stimulation of autophagic activity reduces lipid droplet accumulation and prevents hepatotoxicity in mice on a high-fat diet [248], whereas both lipophagy and mitophagy protect against ethanol-induced liver injury [249]. These findings suggest that constitutive lipophagy may protect the liver against lipotoxicity by reducing the accumulation of toxic lipids, sustaining mitochondrial energy production and maintaining redox homeostasis.

In contrast to its importance in liver metabolism, the role of lipophagy in other tissues is less defined. Current findings have not yet explained the role of lipophagy in adipocytes, mostly because of difficulties in differentiating between the effects of autophagy and lipophagy, but a protective role of lipophagy against obesity-induced inflammation and lipotoxicity has been proposed [129]. In accordance, stimulation of lipophagy by a caloric restriction mimetic in differentiated adipocytes leads to lipid droplet breakdown and a reduction of TAG levels associated with the activation of AMPK [250]. In response to atherogenic lipoprotein accumulation, lipophagy reduces the accumulation of CE-rich lipid droplets in macrophage foam cells [251] and it has been recognized as a potential target in preventing atherosclerosis and cardiovascular diseases [252]. Enhanced lipophagy has been shown to regulate lipid droplet turnover in enterocytes exposed to alimentary lipid micelles, likely to protect cells against lipotoxicity and ER stress [45,253]. Lipophagy also maintains energy homeostasis in the

kidney during prolonged starvation by providing FAs for energy production in mitochondria [254]. Intriguingly, the packaging of circulating FAs into lipid droplets and lipophagy are necessary for channelling exogenous FAs into mitochondria and energy production in starved kidney proximal tubular cells. In hypothalamic neurons, lipophagy has been implicated in the regulation of food intake and energy balance by regulating the release of endogenous FAs that modulate neuropeptide levels and affect signalling pathways controlling food intake [255]. Moreover, autophagy in the central nervous system activates lipophagy and lipolysis in peripheral tissues as a response to cold exposure [256]. The induction of forced lipophagy in mouse embryos reduces lipid droplet levels during development and results in retardation, suggesting a novel role of lipid droplets and lipophagy during embryogenesis [257]. A sudden depletion of glucose in yeast cells activates AMPK-dependent lipid droplet breakdown by microautophagy, i.e., microlipophagy, and provides energy for long-term survival [258]. Microlipophagy has also been implicated in the management of ER lipid and protein quality control during phospholipid imbalance-induced ER stress [46,237]. It was found that lipid droplets are formed at ER aggregates and act as carriers for the transfer of damaged proteins and toxic lipids from the ER to the vacuole through microlipophagy [46,237]. Whether microautophagy occurs in mammalian cells is still unknown, but an alternative pathway of endosomal autophagy shares similar characteristics [259] and has a physiological role in erythropoiesis in vivo [260]. In summary, lipophagy is involved in important pathophysiological processes in various tissues by regulating lipid mobilisation, energy production, and lipotoxic cell damage, but its exact roles and the underlying mechanisms are yet to be defined.

The role of lipophagy in cancer has not been addressed by many studies so far. While several reports have indicated tumour promoting effects of lipophagy [261,262], most studies so far suggest that lipophagy restricts tumourigenesis [263–266]. Microtubule-associated protein 1S (MAP1S)-mediated lipophagy promotes lipid droplet clearance and higher levels of MAP1S have been associated with reduced tumour growth and metastasis and an increased patient survival in clear-cell renal cell carcinoma [263]. Another study has shown that overexpression of ATG14 facilitates lipid droplet breakdown in Hela cells and induces free FA accumulation leading to ER stress- and ROS-mediated apoptosis, whereas suppression of lipophagy by 3-methyladenine (3-MA) or inhibition of lysosomal acid lipase (LAL) reverses these effects [264]. LAL catalyses both TAG and CE hydrolysis in the lysosome and its deficiency or inhibition leads to several pathologies associated with elevated lysosomal lipid accumulation in mice and humans [56]. Recent evidence suggests that LAL plays a tumour suppressor role and its deficiency in mice has been linked with spontaneous tumourigenesis. Importantly, LAL re-expression in hepatocytes prevents liver metastases [265] and its reintroduction in the lung reduces inflammation and metastasis in lung cancer [266]. Interestingly, it was found recently that adipocyte-derived FAs upregulate FA oxidation and stimulate AMPK-dependent autophagy in neighbouring cancer cells [267]. Inhibition of autophagy suppressed lipid droplet breakdown and attenuated the growth-promoting effects of adipocytes, suggesting that adipocytes stimulate lipophagy in cancer cells to foster energy production and enable cancer cell survival. Finally, recent studies suggest that the tumour suppressor protein p53 stimulates lipid catabolism by regulating the expression of several autophagy and lipid metabolism genes, thereby enhancing lipophagy and FA oxidation [268–270]. Thus, current findings on the involvement of lipophagy in cancer provide mixed conclusions and more studies are necessary to delineate its roles. In analogy with the contrasting roles of lipolysis and ATGL in cancer and cell stress, as discussed above, it is likely that the role of lipophagy will be determined by the metabolic and oncogenic dysregulation of particular cancer cells and their exposure to stress.

8.2. Crosstalk between Lipolysis and Lipophagy/Autophagy

CMA has been recently described as a regulator of lipid droplet breakdown by acting as an activator of both lipolysis and lipophagy (Figure 5) [271]. In nutrient-deprived conditions, CMA facilitates the removal and lysosomal degradation of the lipid droplet-associated PLIN2 and

PLIN3 proteins, thus, enabling lipid droplet surface access to ATGL and the autophagic machinery to initiate lipolysis and/or lipophagy. In principle, CMA may thus regulate both macro- and microautophagy-mediated lipid droplet breakdown as well as lipolysis mediated by neutral lipases [83]. Targeted deletion of the LAMP-2A CMA receptor in murine liver revealed that defective CMA causes hepatic glycogen depletion and steatosis and compromises the adaptation to energetic requirements under nutrient stress [272]. Additionally, AMPK-mediated PLIN2 phosphorylation was required for CMA-mediated PLIN2 degradation, revealing a new role of AMPK in stimulating lipid droplet breakdown during starvation [273]. The role of CMA in the adaptation of cancer cells to stress is currently unknown, but elevated CMA activity has been recently associated with increased growth and metastasis of breast cancer cells in vitro and in vivo [274]. Future studies will provide insights into the potential connections between CMA and lipid droplet metabolism in regulating the cancer cell stress response.

A coordinated regulation of lipophagy and lipolysis has also been suggested through a direct interaction between microtubule-associated protein light chain 3 (LC3) and ATGL (Figure 5). Cold exposure induces autophagy in the central nervous system, which, in turn, activates both lipolysis and lipophagy in peripheral tissues, such as the brown adipose tissue and liver [256]. Interestingly, an interaction between LC3 and ATGL was observed after cold exposure in peripheral tissues and it was necessary for efficient lipolysis, suggesting that LC3 regulates lipolysis. Thus, autophagosomal membranes and proteins may serve as scaffolds for regulating the recruitment and activity of neutral lipases, ensuring a coordinated regulation of lipolysis and lipophagy [256].

The crosstalk between lipid droplets and autophagy/lipophagy also extends to recent discoveries, suggesting that lipid droplets are necessary for the proper initiation and execution of autophagy (Figure 5). The consumption of lipid droplets during starvation has been associated with lipid droplets not as substrates for lipophagy, but rather as sources of lipids for the biogenesis of nascent autophagosomal membranes [58]. The lipid droplet-localized TAG lipase patatin-like phospholipase domain-containing 5 (PNPLA5) was necessary for autophagy, suggesting that TAG lipolysis contributes to autophagosomal formation [58]. Studies in yeast have shown that lipid droplet lipids are required for starvation-induced formation of autophagosomes and that several TAG and sterol ester lipases are essential for autophagy [59,275]. However, another study has shown that lipid droplets may be dispensable as a source of lipids for autophagosome formation but are instead necessary for buffering FA fluxes and the maintenance of ER homeostasis that is, in turn, critical for autophagosome biogenesis [60]. The mechanisms that determine the source of lipids for autophagic initiation are still unknown, but these studies suggest that lipid droplets may be required for autophagy in multiple ways, acting as sources of essential lipids for autophagosomal membrane biogenesis and/or as regulators of lipid trafficking that is critical for ER homeostasis and thus indirectly affecting autophagy.

Furthermore, it has been shown recently that lipolysis mediated by ATGL (PNPLA2) may stimulate autophagy/lipophagy in the liver [61]. It was found that ATGL promotes autophagic flux in a SIRT1-dependent manner and promotes interactions between LC3 and lipid droplets (Figure 5). Furthermore, inhibition of autophagy suppressed ATGL-mediated lipid droplet breakdown and FA oxidation, suggesting that ATGL activates lipophagy. On the contrary, in colon epithelial cells, ATGL deficiency induces autophagic flux, whereas its co-factor ABHD5 promotes autophagy but in an ATGL-independent manner [276]. It was found that ABHD5 binds to and prevents the cleavage of the essential autophagy regulator Beclin 1 (BECN1), thus stimulating autophagy, which reduces colon cancer tumorigenesis [276]. Another enzyme from the PNPLA family has been recently shown to stimulate lipophagy [277]. PNPLA8, also named calcium-independent phospholipase A_2 γ (iPLA$_2\gamma$), displays PLA_2 activity and preferentially hydrolyses arachidonic acid-containing membrane phospholipids [278]. PNPLA8 plays an important role in mitochondrial lipid and energy metabolism and its transgenic expression in the heart leads to TAG accumulation and cardiac dysfunction [196,197,279]. A recent study has shown that PNPLA8 interacts with autophagosomes and stimulates hepatic lipid droplet breakdown via autophagy in a mouse model of non-alcoholic fatty

liver disease [277]. These studies add to the notion that an intricate relationship exists between lipid droplet lipolysis and autophagy/lipophagy and that lipases and their co-factors may act as important upstream regulators of autophagy and lipophagy [83].

8.3. Autophagy Drives Lipid Droplet Biogenesis to Protect Cells from Stress

As discussed above, the importance of autophagy for lipid droplet breakdown has been well established in the last decade, but autophagy also affects lipid droplet biogenesis. Studies in 3T3-L1 preadipocytes have shown that the depletion of Atg5 and Atg7 leads to a reduced lipid droplet accumulation and impaired differentiation [33]. Similar results were obtained in Atg5-depleted primary mouse fibroblasts, which failed to differentiate into mature adipocytes [280]. Accordingly, in vivo experiments have revealed that adipose-specific deletion of Atg7 significantly reduces adiposity, causes a shift towards a brown adipose tissue phenotype, including an increase in mitochondrial biogenesis and rates of FA oxidation, leading to an enhanced insulin sensitivity and a lean phenotype [33]. Furthermore, targeted deletion of Atg5 in late-stage embryos and neonatal pups reduced subcutaneous fat [280]. These findings illustrate that autophagy is required for normal adipocyte differentiation and the maintenance of lipid storage in adipose tissue in vivo but do not directly implicate autophagy in the mechanism of lipid droplet biogenesis. Two studies by Shibata et al. [241,281] have reported that autophagy is involved in lipid droplet synthesis in the liver, heart, and in various cultured cell lines. Atg7 deficiency led to a reduction in lipid droplet biogenesis in mouse hepatocytes and a co-localization of conjugated LC3-II with lipid droplets was observed in hepatic and cardiac tissues of starved mice [241]. In addition, depletion of LC3 resulted in a reduced lipid droplet accumulation in several cell types, including the cancer cell lines HeLa, HepG2, and PC12 [281], suggesting that the LC3 conjugation system is involved in lipid droplet formation. These studies suggested that the autophagic machinery regulates both autophagosome and lipid droplet biogenesis as a coordinated mechanism for the maintenance of cellular homeostasis. Recently, it was demonstrated that a liver-specific knockout of Atg5 leads to impaired fasting-induced lipid droplet biogenesis, suggesting that autophagy is involved in lipid droplet biogenesis from exogenous lipids provided from the circulation during fasting [242]. Interestingly, lipid droplet biogenesis was restored in Atg5/nuclear factor-like 2 (Nrf2) double-knockout mice, suggesting that persistent Nrf2 activation is responsible for the impaired lipid droplet biogenesis in autophagy-deficient livers. Nrf2 is a stress-activated transcription factor that regulates the expression of a range of antioxidant and stress-responsive genes [282]. This suggests that autophagy-dependent lipid droplet biogenesis in the liver is involved in the maintenance of redox homeostasis and protection from oxidative stress during fasting.

The first clues for understanding the reasons for lipid droplet biogenesis in starved cells came from recent studies showing that autophagy contributes to lipid droplet biogenesis in acutely starved MEFs [25,31]. Genetic depletion of Atg5, inhibition of autophagosome formation by 3-MA and of autophagosome-lysosome fusion by bafilomycin A1, all prevented lipid droplet biogenesis during starvation in HBBS. Using a fluorescently-tagged phosphatidylcholine, Rambold et al. also show that autophagosomal degradation of membrane phospholipids provides FAs for lipid droplet synthesis. Their results further suggest that the role of autophagy in acutely starved cells is a constant replenishment of lipid droplets with FAs, which are then released by ATGL-mediated lipolysis and transferred to mitochondria for energy production. In the same model system, Nguyen et al. [31], have recently discovered that the function of autophagy-dependent lipid droplet biogenesis in starved MEFs is also to reduce the lipotoxicity of FAs released by autophagy. They show that lipid droplets serve as a protective buffer system that prevents mitochondrial damage and allows for a controlled lipolytic release of FAs for mitochondrial energy production. Importantly, lipid droplet biogenesis in starved MEFs occurs specifically in the absence of amino acids, but not during glucose or serum starvation, and is controlled by the major nutrient sensor and cell growth-associated kinase mTORC1 [31]. mTORC1 is active in the presence of sufficient amino acids to promote anabolic growth and is a potent negative regulator of autophagy [283]. Inhibition of mTORc1 in the presence

of nutrients led to activation of autophagy and was sufficient to induce lipid droplet biogenesis, suggesting that autophagy-derived lipid droplet synthesis is not limited to starvation, but it may be important for various conditions characterized by high autophagic flux, including cancer [31]. Furthermore, the authors suggest that since mTORc1 responds to various nutrient and stress signals, mTORc1-dependent autophagy could explain the increase in lipid droplet accumulation under other stress conditions, such as ER stress [23,284], proteasome inhibition [285,286], and hypoxia/oxidative stress [8,24,28,204]. Indeed, several studies suggest that mTOR-dependent autophagy is activated during hypoxia [287,288], which opens the possibility that autophagy contributes to lipid droplet biogenesis during hypoxia and other conditions of oxidative stress.

Autophagy-induced lipid droplet biogenesis has been recently associated with the protection of mesenchymal stem cells against nutrient and oxidative stress [34]. Carnitine palmitoyltransferase 1C (CPT1C) is an isoform of CPT1A with minimal catalytic activity and is expressed in the brain and many cancer cells [289,290]. In human mesenchymal stem cells exposed to glucose and oxygen deficiencies, CPT1C promoted autophagy-dependent lipid droplet biogenesis rather than directly affecting FA oxidation [34]. Inhibition of ATGL blocked the pro-survival effect of CPT1C and reduced ATP production, suggesting that ATGL-mediated lipolysis and energy production are required for its pro-survival effect [34]. In cancer cells, CPT1C is induced by hypoxia and glucose deprivation, its expression inversely correlates with mTORc1 activation and it promotes cell survival by stimulating FA oxidation and ATP production [290]. It is thus possible that CPT1C activates autophagy and lipid droplet biogenesis to promote cancer cell survival through lipid droplet lipolysis, which, in turn, provides FAs for oxidation in mitochondria and stimulation of PPARα-mediated mitochondrial biogenesis and oxidative metabolism [67]. Additionally, the link between autophagy and lipid droplet biogenesis during stress has been observed in other organisms. Rapamycin-induced autophagy contributes to TAG synthesis in yeast [291] and autophagy is required for TAG accumulation under nitrogen-deprived conditions in *Chlamydomonas* [292]. Clearly, autophagy-driven lipid droplet biogenesis is beneficial for a variety of cells in different stress conditions, but it remains to be confirmed whether lipid droplet biogenesis is indeed a general protective response to high levels of autophagy. The tight bonds between autophagy, lipid droplet metabolism, and the cellular stress response are only beginning to emerge and future studies will surely provide important clues on the molecular mechanisms involved and their pathophysiological relevance. This will help us improve our understanding of the specific alterations of lipid metabolism in cancer cells and their metabolic advantage during stress.

9. Conclusions and Perspectives

Lipid droplets have long been regarded as inert fat storage depots and we know very little about their lipid and protein composition, dynamics, and functions in the cell. Along with the increasing interest in lipid metabolism in cancer in the last decade, there is an emerging appeal for studying lipid droplets and their role in the cellular stress response. The extra- and intracellular sources of lipids used by cells for lipid droplet biogenesis and the reasons behind lipid droplet accumulation in different pathophysiological settings are only beginning to emerge. It is also not clear how different stress conditions affect the choice of lipids for lipid droplet biogenesis and how lipid droplets regulate the release of particular species of lipids from their core. Likewise, one of the outstanding questions in cancer research is which lipid sources are used by cancer cells for survival in particular stress conditions and how cells switch between them. Furthermore, it will be important to get a broader and quantifiable picture of the lipid species stored in lipid droplets in various cells and tissues and improve our ability to follow the dynamic changes in the lipid droplet lipidome and proteome. For example, it will be fascinating to discover how different stress conditions and diseases are reflected by changes in the lipidome/proteome and what the roles of the numerous fatty acid, cholesterol and ceramide species stored in lipid droplets are. The potential of lipid droplets as lipid mediator signalling hubs is also emerging and provides exciting prospects for their role in inflammation and immunity.

Identification of lipid droplet-associated proteins involved in sensing and responding to nutrient and oxidative stress will improve our understanding of lipid droplet functions in the stressed cancer cell. Studies will also provide important clues on the identity of lipid droplet proteins that are specifically recognized by the lipophagic machinery. It will also be important to see how cells switch between lipolysis and lipophagy and how these complementary processes of lipid droplet breakdown are regulated. Moreover, lipid droplet biogenesis may be a general cellular response to conditions of high autophagic flux. Clearly, we are only at the beginning of our understanding of the complex network of interactions between lipid droplets and the various forms of autophagy. Finally, interfering with lipid droplet biogenesis and/or breakdown may be beneficial for reducing the ability of cancer cells to cope with stress in therapeutic settings. The inhibition of lipid droplet biogenesis could compromise lipid acquisition and usage pathways in cancer cells by concomitantly increasing lipotoxicity and shutting down critical survival mechanisms. Current studies imply that several molecular targets, such as DGAT1, ATGL and LPCAT2, hold promise for reducing the availability of lipids critical for the survival, growth, and metastatic potential of cancer cells. However, given the pleiotropic roles of lipid droplets, it will be critical to first define the dependence of particular cancer types on lipid droplet metabolism. For example, targeting lipid droplet lipolysis (e.g., via inhibition of adipose tissue ATGL) may be particularly useful for the treatment of cancers that depend on and strive in the vicinity of adipose tissue, such as breast and ovarian cancer. ATGL inhibitors are currently under investigation as promising therapeutic agents for the treatment of metabolic disorders, but they may also prove to be beneficial in the prevention of cancers associated with obesity and diabetes. Future studies of the roles of lipid droplets in challenged (cancer) cells will surely provide important answers to many of these questions and will improve our ability to target the specific vulnerabilities of different cancer cells in their respective environments.

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Appendix B.

Review Paper "Lipid Droplets and the Management of Cellular Stress"

The paper “Lipid Droplets and the Management of Cellular Stress” by Eva Jarc and Toni Petan was published in the *Yale Journal of Biology and Medicine*, a journal that has been continuously published since 1928 and is edited by Yale medical and graduate students. This invited review paper was prepared for the September 2019 special issue of the journal entitled “Organelles” and was selected as an editor’s pick upon publication. Here, we discussed the emerging roles of lipid droplets as fat storage organelles and major regulators of cellular metabolism. Lipid droplets provide protection against excess dietary fat, but also enable optimal energy production in the muscle and heart, complement autophagy during starvation and regulate inflammatory and immune responses. In each cell, they support the homeostasis of membrane lipid composition and dynamics, take care of damaged proteins and lipids and patrol the cell to form dynamic contacts with other organelles. I contributed to this work by performing extensive literature search, writing several sections, proof-checking the drafts and preparing some of the figures.

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REVIEW

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Lipid Droplets and the Management of Cellular Stress

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Lipid droplets are cytosolic fat storage organelles present in most eukaryotic cells. Long regarded merely as inert fat reservoirs, they are now emerging as major regulators of cellular metabolism. They act as hubs that coordinate the pathways of lipid uptake, distribution, storage, and use in the cell. Recent studies have revealed that they are also essential components of the cellular stress response. One of the hallmark characteristics of lipid droplets is their capacity to buffer excess lipids and to finely tune their subsequent release based on specific cellular requirements. This simple feature of lipid droplet biology, buffering and delayed release of lipids, forms the basis for their pleiotropic roles in the cellular stress response. In stressed cells, lipid droplets maintain energy and redox homeostasis and protect against lipotoxicity by sequestering toxic lipids into their neutral lipid core. Their mobility and dynamic interactions with mitochondria enable an efficient delivery of fatty acids for optimal energy production. Lipid droplets are also involved in the maintenance of membrane and organelle homeostasis by regulating membrane composition, preventing lipid peroxidation and removing damaged proteins and lipids. Finally, they also engage in a symbiotic relationship with autophagy and act as reservoirs of bioactive lipids that regulate inflammation and immunity. Thus, lipid droplets are central managers of lipid metabolism that function as safeguards against various types of cellular stress.

INTRODUCTION

Cells rely on lipids to provide an efficient means of fuel storage and energy production. Lipids are also the major building blocks of cellular membranes and are precursors for hundreds of signaling molecules. Most eukaryotic cells have the capacity to store lipids in the form of lipid droplets. Being only recently recognized as dynamic, independent organelles rather than inert fat depots, lipid droplets are now emerging as central regulators

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†Abbreviations: AMPK, AMP-activated protein kinase; ATGL, adipose triglyceride lipase; CE, cholesterol ester; COX, cyclooxygenase; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FA, fatty acid; HSC, hepatic stellate cell; HSL, hormone sensitive lipase; LOX, lipoxygenase; LAL, lysosomal acid lipase; MAGL, monoacylglycerol lipase; mTORC1, mammalian target of rapamycin complex 1; MEF, mouse embryonic fibroblast; PLIN, perilipin; PC, phosphatidylcholine; PPAR, peroxisome-proliferator activated receptor; PUFA, polyunsaturated fatty acid; RA, retinoic acid; RE, retinyl-ester; TAG, triacylglycerol; UPR, unfolded protein response.

Keywords: lipid droplets, fatty acids, nutrient stress, oxidative stress, autophagy, lipophagy, lipotoxicity, mitochondria, β-oxidation, lipid mediators, eicosanoids

Author Contributions: T.P. conceptualized the study. E.J. and T.P. wrote the manuscript and designed the figures.

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of lipid uptake, metabolism, trafficking, and signaling in the cell. However, their roles extend well beyond lipid metabolism and an increasing number of studies suggest that they play vital roles in the cellular stress response [1-4].

Lipid droplets are dynamically synthesized and broken down in response to cellular needs and environmental signals [5]. Their biogenesis is induced in cells exposed to excess amounts of lipids, to nutrient and oxidative stress, and to various other conditions characterized by energetic and redox imbalances [2]. Intriguingly, lipid droplet biogenesis also occurs in response to complete nutrient deprivation, suggesting that the expense of their synthesis must be worthwhile for the cell and is in fact essential for its response to stress. Indeed, emerging studies reveal that lipid droplets perform numerous tasks crucial for the protection of cellular integrity and function during stress: they sequester potentially toxic lipids and proteins, maintain energy and redox balance, preserve membrane and organelle homeostasis, modulate autophagy, and provide lipids acting as signaling mediators [1,2,4]. Many of these functions are engaged simultaneously and in cooperation with other organelles and we are only beginning to understand their interplay. One of the major properties of lipid droplets is their ability to buffer excess lipids, thereby providing immediate protection from lipotoxicity, and to release them later, gradually and according to cellular needs (Figure 1). This property is particularly important for cells exposed to rapidly changing conditions of stress and it is, for example, exploited by cancer cells during alternating periods of feeding/starvation or hypoxia/reoxygenation [6-10]. This simple aspect of lipid droplet biology – buffering and delayed release of lipids – is indispensable for most of their pleiotropic roles in the cellular stress response.

In this article, we discuss emerging functions of lipid droplets in the management of cellular stress. We focus on how lipid droplet turnover and associated mechanisms are engaged during nutrient stress to maintain cellular homeostasis.

LIPID DROPLET BIOGENESIS AND BREAKDOWN IN STRESSED CELLS

Lipid droplets are unique among organelles with their central hydrophobic core of neutral lipids surrounded by a single layer of phospholipids and proteins (Figure 2A). They are derived from the endoplasmic reticulum (ER†), whereby triacylglycerols (TAGs) are synthesized between the two leaflets of the ER membrane by sequential addition of fatty acids (FAs) to a glycerol backbone (Figure 2B) [11]. Diacylglycerol acyltransferases (DGATs) 1 and 2 catalyze the last and committed step in the TAG biosynthesis pathway. When sufficient neutral lipids accumulate, nascent lipid droplets bud from the ER membrane and are released into the cytosol [4,12,13]. Lipid droplets are broken down by two major mechanisms: lipolysis and lipophagy (Figure 2C) [14-16]. Lipolysis enables a highly regulated release of FAs from TAGs by the sequential action of adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) [17-19]. Lipophagy is a recently discovered selective form of autophagy, whereby parts or whole lipid droplets are engulfed within autophagosomal membranes and fused with lysosomes for degradation by hydrolytic enzymes. At the organismal level, lipid droplet breakdown in adipocytes is hormonally regulated and provides FAs for mitochondrial energy production in non-adipose tissues during fasting and exercise [17,18,20]. However, lipid droplets in non-adipose tissues also undergo cycles of biogenesis and breakdown in response to nutrient and other cues from the environment. Their composition, number, size and distribution within cells is dynamically changing depending on the physiological state of the cell [21]. We are only beginning to understand how lipid droplet structure and dynamics define the multitude of their functions.

Enhanced lipid droplet accumulation has been observed in various types of cells exposed to complete or partial nutrient restriction [22-26]. Lipid droplet biogenesis in starved cells is essential for buffering autophagy-derived lipids, for protection of mitochondria from lipotoxic damage and for storing lipids for future consumption [25-28]. Lipid droplets also finely regulate the distribution and consumption of lipids during stress in order to maintain energy and redox homeostasis. As discussed in more detail below, starvation-induced dispersion of lipid droplets, their extensive contacts with the network of fused mitochondria and ATGL-mediated lipolysis likely provide the optimal means of FA transfer from lipid droplets to mitochondria [25,27]. In various cells and tissues, including adipocytes, hepatocytes and muscle cells, the delivery of FAs from lipid droplets to mitochondria and its coordination with mitochondrial and oxidative gene expression depends on ATGL activity [18,20,25,29]. In cells exposed to oxidative stress, lipid droplets accumulate in order to protect membranes from peroxidation reactions, maintain membrane saturation and organelle homeostasis, and enable a long-term supply of lipids for energy production and cell survival [6,7,30,31]. By stimulating FA oxidation and increasing NADPH levels in stressed cells, which is required for the maintenance of the glutathione-dependent antioxidant system, lipid droplet-derived FAs simultaneously provide energy and protect from oxidative stress [6,32,33]. As discussed in more detail below, it is therefore not surprising that dysregulated lipolysis may result in lipotoxicity and cell dysfunction. Thus, lipid droplets are dynamic

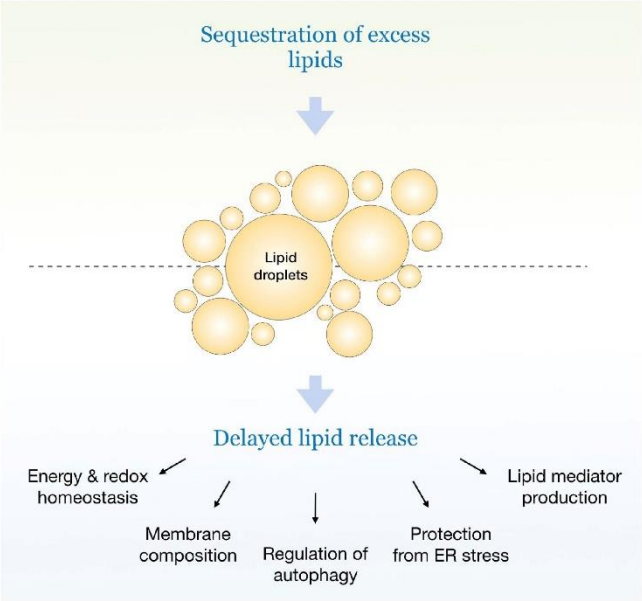

Figure 1. Lipid droplet buffering and delayed release of lipids. Various conditions of cellular stress are characterized by lipid overload, arising from an excess of exogenous (*e.g.* increased uptake of lipids from the circulation) or endogenous lipids (*e.g.* starvation-induced autophagic breakdown of membranous organelles). Lipid droplets have the capacity to sequester excess lipids, thereby preventing their lipotoxicity, and to release them gradually based on cellular needs to preserve energy, redox, membrane, and organelle homeostasis through various mechanisms. This essential property of lipid droplets – buffering and delayed release of lipids – is particularly important for cells exposed to rapidly changing conditions of nutrient and oxidative stress.

organelles that are essential for the cellular response to metabolic stress.

LIPID DROPLETS RESPOND TO NUTRIENT AND ENERGY IMBALANCES IN STRESSED CELLS

During evolution, cells have developed mechanisms of metabolite sensing in order to cope with the unreliable supply of sugars, amino acids and lipids from the environment [34]. The metabolite-sensing machinery facilitates the coordination of mechanisms that govern nutrient preference, uptake, distribution, recycling, and storage in order to maintain cellular homeostasis. AMP-activated protein kinase (AMPK) and mammalian target of rapamycin complex 1 (mTORC1) are highly conserved and fundamental reciprocal regulators of cellular metabolism [34-37]. AMPK is activated by nutrient and oxidative stress, genotoxins and xenobiotics [37]. It senses minute changes in the intracellular AMP:ATP ratio and responds to low energy states by suppressing anabolic processes, including *de novo* FA, cholesterol, and TAG synthesis [37-39], while stimulating ATP production through the activation of glycolysis, mitochondrial biogenesis, and FA oxidation [32]. Besides responding to levels of adenine nucleotides, recent reports have shown that AMPK activation may also occur at the lysosomal membrane, where an AMPK-activation complex senses glucose levels independently of the cellular energy status [35,40]. The mTORC1 kinase also localizes to lysosomes, where it is activated by nutrient abundance to promote anabolic cell growth pathways. By regulating the sterol regulatory element-binding protein (SREBP) transcription factors it stimulates FA, cholesterol, and glycerolipid synthesis, whereas it inhibits lipid catabolism by repressing the activity of the transcription factor EB (TFEB) [41-43]. mTORC1 is negatively regulated by AMPK, amino acid, or glucose starvation and is a strong suppressor of

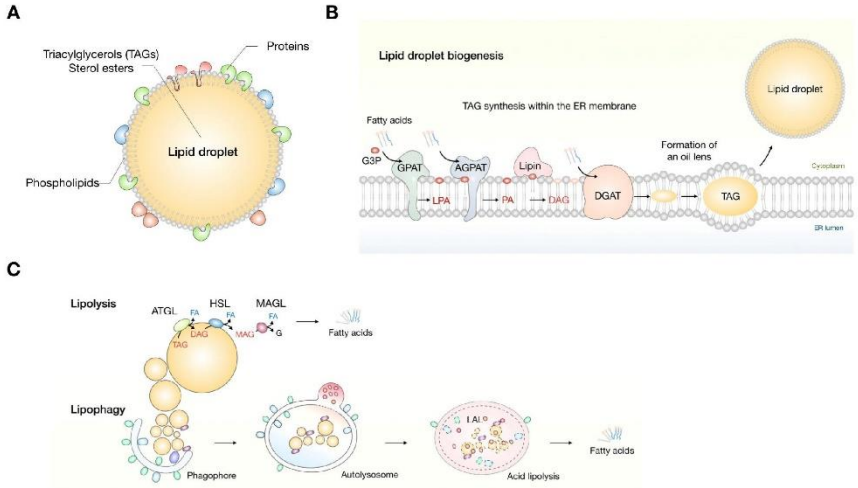

Figure 2. Lipid droplet structure, biogenesis, and breakdown. (**A**) Lipid droplets are composed of a central hydrophobic core of neutral lipids, mostly composed of triacylglycerols (TAGs) and sterol esters, surrounded by a monolayer of phospholipid molecules, wherein numerous proteins are embedded. (**B**) TAG synthesis occurs in the endoplasmic reticulum membrane by sequential addition of fatty acids (FAs) (in their activated acyl-CoA form) to a glycerol-3-phosphate (G3P) backbone, yielding lysophosphatidic acid (LPA), phosphatidic acid (PA), and diacylglycerol (DAG). These reactions are catalyzed by several acyltransferase enzymes, including glycerol-3-phosphate acyltransferases (GPATs), acylglycerol-3-phosphate acyltransferases (AGPATs), and phosphatidic acid phosphatases (lipins). The last and committed step in the TAG synthesis cascade is catalyzed by the diacyglycerol acyltransferase enzymes (DGATs). (**C**) Lipid droplet breakdown occurs through lipolysis or lipophagy. TAG lipolysis is carried out by the sequential action of adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL), and monoacylglycerol lipase (MAGL). Lipophagy is a selective form of autophagy wherein lipid droplets are engulfed within autophagosomal membranes and delivered to lysosomes for degradation by acid lipolysis. Lysosomal TAG lipolysis is catalyzed by lysosomal acid lipase (LAL).

autophagy [35,40,44]. Autophagy/lipophagy may be directly regulated by AMPK via a "dual safe" mechanism, involving not only mTORC1 inhibition, but also direct phosphorylation of the Unc-51 like autophagy activating kinase 1 (ULK1) [45].

Different types and severities of nutrient restriction may distinctly affect cellular sensing mechanisms thereby resulting in specific cellular responses. Emerging studies have shown that lipid droplet metabolism is affected by the sensing and regulatory networks of AMPK and mTOR. In growing yeast cells, lipid precursors are mainly used for membrane synthesis. Following rapid exponential growth, nutrients such as glucose or amino acids are eventually exhausted, which leads to a metabolic shift towards mitochondrial oxidative metabolism. This is facilitated by the activation of Snf1 (the yeast homologue of AMPK), which senses glucose depletion and derepresses oxidative gene expression [40]. Intriguingly, the switch from fermentation to respiration also includes a rapid burst of lipid droplet biogenesis in order to redirect lipid precursors away from membrane synthesis and preserve them to enable cell survival during the ensuing starvation [3,46,47]. The shift in lipid metabolism is also dependent on the activation of phosphatidate phosphatase 1 (Pah1), which is necessary for TAG synthesis in the stationary phase and is indirectly regulated by TORC1 [48,49]. And how are lipid droplets consumed during starvation? Yeast cells undergoing sudden glucose restriction consume lipid droplets through microautophagy, *i.e.* microlipophagy, which is triggered by Snf1/AMPK activation, in order to survive during prolonged nutrient stress [47]. Intriguingly, yeast cells exposed to gradual glucose reduction, amino acid starvation, or TORC1 inhibition cannot activate microlipophagy and do not survive in the long-term, likely because they fail to maintain Snf1/AMPK activation [47].

The mTORC1 kinase may regulate lipid droplet turnover during stress through autophagy-dependent and autophagy-independent mechanisms. In *Drosophila*, hypoxia-induced TORC1 inhibition leads to autophagy-independent lipid droplet growth and lipid storage in the fat body, which is required for tolerance to hypoxia [50]. In mammalian cells, acute and complete nutrient restriction promotes lipid droplet biogenesis [24]. Experiments in starved mouse embryonic fibroblasts (MEFs) have revealed that amino acid depletion, but not glucose or serum limitation, drives lipid droplet biogenesis [26], which depends on mTORC1 inactivation-induced autophagy [26]. Interestingly, AMPK activation succeeded mTORC1 inhibition and was not required for autophagy initiation, indicating that it might instead be important for sustaining autophagic flux and oxidative metabolism during prolonged starvation [26]. Intriguingly, even in conditions of nutrient sufficiency AMPK may contribute to basal autophagy by activating the ULK1 kinase [51]. Given that lipophagy is activated in milder and prolonged conditions of serum restriction and in the presence of glucose and amino acids [9,25,52], it is not surprising that AMPK stimulates lipophagy through mTORC1-independent activation of ULK1 [51,53]. Thus, it seems that the intricate interplay between mTORC1 and AMPK signaling that regulates autophagy/lipophagy and lipid droplet metabolism depends on the type of nutrients being restricted, the severity and length of stress and the physiological context.

Interestingly, lipolysis and lipophagy are also transcriptionally regulated by common pathways downstream of AMPK and mTORC1 in response to changes in nutrient and energy levels [16,34]. AMPK and mTORC1 signal transduction pathways converge at the TFEB, peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α)-PPARα-retinoid receptor X (RXR) and forkhead box protein O (FOXO) transcription factors that activate the transcription of ATGL and lysosomal acid lipase (LAL), thus promoting both neutral and acid lipolysis [16]. By promoting catabolism and elevating NAD+ levels, AMPK also indirectly activates the deacetylase sirtuin 1 (SIRT1) and its targets PGC1α and FOXO. On the contrary, starvation-induced mTORC1 inhibition leads to translocation of TFEB into the nucleus, thereby increasing the expression of genes that are involved in lysosomal biogenesis and function, autophagy and lysosomal exocytosis [54]. AMPK also regulates the expression and activation of PLIN3, which promotes lipid droplet dispersion during starvation, and the autophagic degradation of PLIN2, which regulates the binding of cytosolic lipases to the lipid droplet surface [55-58]. At the tissue/organismal level, the role of AMPK in regulating mammalian lipid droplet breakdown is still not clear, with numerous contradictory reports suggesting that AMPK has a pro- or anti-lipolytic function, or has no effect on lipolysis [16]. This suggests a marked tissue- and cell type-specific function of AMPK-mediated regulation of lipid droplet breakdown.

LIPID DROPLETS ARE CELLULAR MANAGERS OF LIPOTOXICITY

Lipotoxicity is a condition that occurs when cellular mechanisms fail to overcome cell damage caused by lipids [59]. It is often a consequence of imbalances between lipid uptake, storage and utilization. Cellular mechanisms of protection against lipotoxicity act on the principle of eliminating damaged or surplus lipids by transfer into inert storage pools, breakdown through oxidation or expulsion from the cell. Due to their central role in coordinating lipid uptake, storage, trafficking, and oxidation, lipid droplets are particularly well-suited among organelles to prevent, respond to and alleviate lipotoxic insults. A hallmark feature of lipid droplet biology is their ability to take up large amounts of lipids and release them gradually when needed in response to cellular requirements (Figure 1). Importantly, they do not act solely as simple buffers that minimize cellular lipid fluctuations, but also regulate the multitude of pathways that control the fate of stored lipids, thereby affecting all aspects of cellular homeostasis. Lipid droplets protect cells and tissues against lipotoxicity by mitigating the overload of both exogenous and endogenous lipids [2,4]. They reduce the lipotoxicity of "free," non-esterified lipids by storing them in their less hazardous, esterified forms within the neutral lipid core [9,26,60-62]. FAs, diacylglycerols (DAGs), cholesterol and ceramides are stored within lipid droplets in the form of TAGs, cholesterol esters (CEs) and acylceramides, respectively. Cells require lipid droplets for protection against lipotoxicity in a variety of stressful conditions, including during lipid overload, e.g. exposure to exogenous FAs [9,61,63-65], hypoxia and oxidative stress [9,30,31], high autophagic flux [26,66] and dysfunctional lipolysis [67,68].

Intriguingly, the protective role of lipid droplets extends also to neighboring cells and tissues, and this cell non-autonomous function of lipid droplets is critical for proper maintenance of tissue homeostasis and stress responses [4,65,69,70]. This phenomenon is not limited to adipose tissue, which is specialized for the protection of cells and tissues from lipotoxicity both locally and at the organismal level, but is also important, for example, during neuronal development, whereby glial lipid droplets protect neighboring neuroblasts from lipotoxicity [30,69,71]. A cell-to-cell heterogeneity in the capacity for storing lipids has also been observed within subpopulations of cells, whereby a subset of cells accumulates more lipid droplets to reduce the risk for lipotoxic damage for

the whole population, but also to supply stored lipids to neighboring cells when needed [72]. At the organismal level, lipid droplet biogenesis in different tissues improves insulin sensitivity [73], reduces accumulation of DAG and ceramide [74], and buffers postprandial surges of FAs in order to reduce their lipotoxicity [70]. Dysfunctional lipid droplet turnover may interrupt the ability of cells and tissues to fight against lipotoxic damage and can lead to persistent cellular stress, which is a hallmark of many diseases, including obesity, cardiovascular diseases, metabolic syndrome, diabetes, inflammation, and cancer [2,73,75,76]. The manipulation of lipid droplet turnover thus represents an attractive target for reducing the detrimental consequences of lipotoxicity.

Lipolytic lipid droplet breakdown fuels and regulates mitochondrial oxidative metabolism and is essential for cell survival during nutrient deprivation in various cells [8,9,20,24,25,77]. However, upregulated lipolysis is a hallmark of cellular states and diseases associated with lipid overload. Elevated lipolysis may cause cell damage by increasing the pool of free FAs in the cytosol, altering cell signaling pathways affecting oxidative metabolism, ER homeostasis, and stimulating β-oxidation and reactive oxygen species (ROS) production [78,79]. Under these conditions, inhibition of ATGL-mediated lipolysis protects cells and tissues from lipotoxicity, oxidative stress, insulin resistance, and ER stress [9,67,68,80-83]. The lipid droplet coating protein perilipin 5 (PLIN5) plays a critical role in the regulation of lipolysis and lipotoxicity. It inhibits ATGL and provides a "lipolytic barrier" that suppresses uncontrolled TAG lipolysis, thereby reducing lipotoxic injury and insulin resistance in oxidative tissues (Figure 3) [67,80-82]. It controls the tight coupling between lipolysis and the metabolic demand for FAs, thus preventing ceramide accumulation and reducing oxidative stress, which may be induced by excessive FA oxidation and peroxidation [80,84,85]. The coordination of lipid droplet lipolysis and mitochondrial metabolism is thus critical for the removal of toxic FA species and the prevention of lipotoxicity. On the contrary, ATGL deficiency in macrophages leads to TAG accumulation and apoptotic cell death, suggesting that lipolysis may also play a protective role against lipotoxicity in some cases [86]. Therefore, the precise regulation of ATGL activity is critical for the balance between the protective effects of lipolysis on energy and redox homeostasis, and lipotoxic cell damage.

LIPID DROPLETS INTERACT WITH OTHER ORGANELLES TO MAINTAIN CELLULAR HOMEOSTASIS

Lipid Droplets Interact with Mitochondria to Optimize Lipid Transfer and Energy Production

Energy production during glucose restriction depends on FA oxidation that primarily occurs in mitochondria in mammals, and in peroxisomes in yeast and plants [87]. Recent studies suggest that for an efficient delivery of FAs from lipid droplets to mitochondria a close proximity of both organelles is required, which could minimize the exposure of FAs to the cytosol and prevent lipotoxicity [25,26]. Indeed, lipid droplets form contacts with mitochondria and other organelles including the ER, Golgi, lysosomes, and peroxisomes [4,88,89]. The architecture of lipid droplet–organelle contact sites is mostly unknown, but recent studies suggest that they may include both protein complexes that function as tethers between the organelles and membrane bridges that connect the outer phospholipid monolayers of the opposing membranes [90]. The ability to form membrane bridges at organelle contact sites is unique to lipid droplets, due to the monolayer structure of their phospholipid coat, which may form continuous structures with the outer leaflet of bilayer membranes of other organelles. It may be envisaged that, in contrast to protein tethers, membrane bridges enable direct and efficient transfer of lipids and proteins between the two organelles. Notably, the transport between lipid droplets and mitochondria is probably bidirectional and not limited to FA transfer for the purpose of energy production [90]. For example, FA transfer in the reverse direction may protect mitochondria from lipotoxicity, and mitochondria may also contribute to phospholipid and TAG synthesis for lipid droplet growth [89,90]. Indeed, it has been recently shown in brown adipocytes that a subset of "peridroplet" mitochondria, which display elevated Krebs cycle activity but low FA oxidation capacity, in fact support lipid droplet biogenesis by providing ATP for the synthesis of TAG [91]. Benador *et al.* [92] have suggested that cells contain two major populations of mitochondria that enable the simultaneous occurrence of lipid synthesis and breakdown: peridroplet mitochondria, that promote lipid droplet biogenesis and protect from lipotoxicity, and non-lipid droplet-bound cytoplasmic mitochondria that specialize in FA oxidation and energy production.

Changes in mitochondrial structure and lipid droplet distribution are necessary for efficient FA trafficking during starvation [25,27]. In nutrient replete conditions, lipid droplets form clusters and are clumped around the nucleus [27]. On the contrary, in starved cells they adopt a dispersed distribution within the cell, which promotes their recruitment to mitochondria. Lipid droplets relocate from the cell center to the periphery by moving on detyrosinated microtubules. Activation of the energy sensor AMPK is essential for lipid droplet mobility along the microtubule network and stimulates their consumption during starvation [27]. Moreover, mitochondrial fusion is necessary for efficient β-oxidation in starved cells, be-

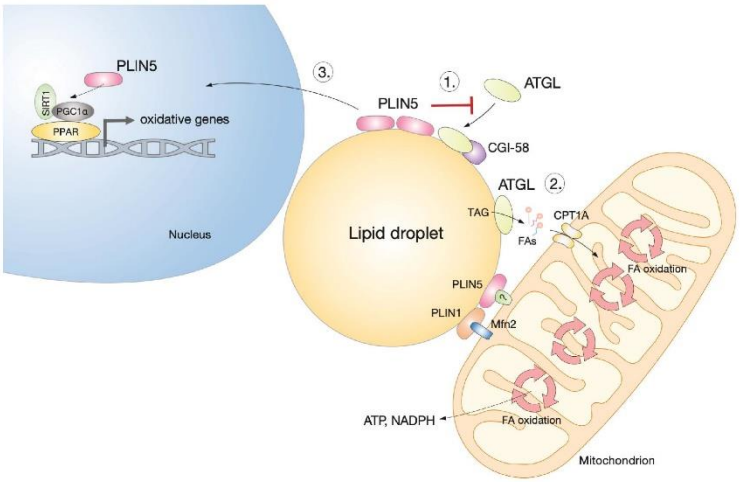

Figure 3. Perilipin 5 regulates lipid droplet and mitochondrial metabolism through metabolic and signaling actions. The lipid droplet coating protein perilipin 5 (PLIN5) plays a critical role in the regulation of lipolysis and mitochondrial metabolism. At least three mechanisms of its action have been implicated in the cellular stress response. 1) In conditions of lipid overload, PLIN5 limits the activity of adipose triglyceride lipase (ATGL) and its co-activator lipid droplet-binding protein CGI-58 and provides a "lipolytic barrier" that suppresses uncontrolled triacylglycerol (TAG) lipolysis, thereby reducing lipotoxic injury and insulin resistance. 2) During nutrient starvation, PLIN5 facilitates fatty acid (FA) flux from lipid droplets to mitochondria and provides a physical link between the organelles. Their close proximity enables a more efficient FA transfer and supports energy and redox homeostasis. Its homologue PLIN1, which is specifically expressed in adipocytes, has been shown to directly interact with the mitochondrial protein mitofusin 2 (Mfn2), a mediator of mitochondrial fusion and mitochondria–lipid droplet interactions. 3) Catecholamine- or fasting-stimulated lipolysis leads to protein kinase A-dependent translocation and enrichment of PLIN5 in the nucleus, whereby it forms transcriptional complexes with sirtuin 1 (SIRT1) and peroxisome proliferator-activated receptor γ co-activator 1α (PGC1α), leading to the transcription of target genes involved in mitochondrial biogenesis and oxidative metabolism.

cause it ensures efficient FA intake and uniform distribution of FAs within the network of tubulated mitochondria [25]. Mitochondrial fusion in mammals is controlled by mitofusins (Mfn) 1 and 2, GTPase enzymes that control the fusion of the outer mitochondrial membrane [93]. Mfn2 deficiency in murine brown adipose tissue leads to lipid droplet accumulation and mitochondrial dysfunction [94]. In accordance, defects in mitochondrial fusion dynamics reduce β-oxidation and mitochondrial respiration, while promoting lipid droplet accumulation and FA efflux from cells [25]. ATGL activity, but not lipophagy, is essential for supplying mitochondria with lipid droplet-derived FAs in acutely starved MEFs [25], but it is not clear how the transfer of FAs is executed at the molecular level.

Several proteins have been shown to regulate the interaction between lipid droplets and mitochondria, including members of the PLIN family [94,95], however, it is still not clear how they contribute to FA transport from lipid droplets to mitochondria. The lipid droplet-coating protein PLIN5 regulates FA flux from lipid droplets to mitochondria [67] and provides a physical link between the organelles (Figure 3) [91,95]. In accordance, overexpression of PLIN5 has been shown to promote clustering of mitochondria around lipid droplets [95,96]. Interestingly, in adipocytes, Mfn2 binds to PLIN1 to facilitate the interaction between mitochondria and lipid droplets (Figure 3) [94]. Clearly, the protein composition, structure and dynamics of lipid droplet–mitochondria contact sites remain to be elucidated in future studies.

Lipid Droplets Associate with Peroxisomes to Support Lipid Metabolism

Lipid droplets also associate with peroxisomes [88,97,98]. In yeast, lipid droplets form stable interactions with peroxisomes and are enriched with peroxisomal β-oxidation enzymes, suggesting a coupling of lipid

droplet lipolysis and peroxisomal FA oxidation [98]. The latter is essential for the breakdown of very-long chain and branched chain FAs [4,87] and for the shortening of polyunsaturated fatty acids (PUFAs), eicosanoids, and epoxy FAs before their final catabolism by mitochondrial oxidation [99]. Furthermore, lipid droplets and peroxisomes interact even in the presence of nutrients, when peroxisomes are not required for growth, indicating that lipid droplets may provide membrane lipids for peroxisome synthesis [98]. In addition, the organelles share a similar ER-mediated pathway for their biogenesis [97]. In mammalian cells, lipid droplets associate with peroxisomes through a tethering complex formed by spastin and ATP binding cassette subfamily D member 1 (ABCD1), which allows direct channeling of FAs from lipid droplets to peroxisomes [100]. Spastin enables lipid droplet–peroxisome interactions by attaching to the lipid droplet phospholipid monolayer and binding to ABCD1 in peroxisomes via its peroxisome-interacting region. Importantly, spastin also recruits two membrane-shaping endosomal sorting complexes required for transport (ESCRT)-III proteins, which facilitate FA trafficking, most likely by inducing morphological changes in the lipid droplet membrane. Moreover, the tethering complex is necessary for the removal of peroxidized lipids from lipid droplets, which suggests a novel role for the lipid droplet–peroxisome relationship in protecting cells against lipotoxicity and oxidative stress. Thus, contact sites between lipid droplets and mitochondria/peroxisomes enable bidirectional communication and effective lipid transfer, which contributes to energy production, maintenance of organelle integrity and prevention of lipotoxicity.

Lipid Droplets Regulate Nuclear Function

Interactions between lipid droplets and the nucleus are an emerging and exciting field of research, currently being addressed mostly from two major perspectives: the involvement of cytosolic lipid droplets in the regulation of nuclear function and the existence of nuclear lipid droplets and their poorly explained origins and function [101]. Cytosolic lipid droplets are often found in close proximity to the nucleus and affect its function via different mechanisms [101]. They are involved in protein exchange with the nucleus, chromatin assembly, and regulation of gene transcription [102-107]. These modulatory functions of lipid droplets are typically associated with the cellular response to stress. For example, the lipid droplet-associated fat-specific protein 27 (Fsp27) sequesters the transcriptional factor nuclear factor of activated T cells 5 (NFAT5) on cytosolic lipid droplets and away from the nucleus, thus directly affecting the execution of an osmoprotective gene expression program that depends on NFAT5 [103]. Furthermore, catecholamine signaling, which is responsible for activation of lipolysis during stress, may stimulate the translocation of the lipid droplet-associated protein PLIN5 to the nucleus, where it physically interacts with PGC-1α/SIRT1 complexes and regulates the transcription of genes that mediate mitochondrial oxidative metabolism (Figure 3) [104]. Thus, besides regulating lipase activity and mitochondria-lipid droplet interactions [67,81,85,91,95], PLIN5 also directly affects the transcription of genes essential for efficient coupling of lipolysis with oxidative metabolism. Intriguingly, bacterial lipid droplets bind directly to DNA using the microorganism lipid droplet small (MLDS) protein, thereby regulating MLDS gene transcription and cell survival under low nitrogen conditions [102]. Finally, lipid droplets are also modulators of chromatin assembly and organization. During early embryonic development of *Drosophila*, lipid droplets store excess maternal histones to ensure a sufficient supply for rapid nuclear division in the embryo [105]. The sequestration of excess histones also protects embryos from DNA damage [106,107] and, surprisingly, histone-bound LDs also protect the fly against bacterial infections [108]. These findings have begun to reveal many as yet unknown roles of cytosolic lipid droplets in the regulation of nuclear function that are involved in various aspects of the cellular stress response.

Recently, it has been reported that lipid droplets are also present in the nucleus [109-111]. Nuclear lipid droplets have been found in yeast and mammalian cells, and they are particularly abundant in hepatocytes [112,113]. Although it was initially not clear whether these are in fact cytoplasmic lipid droplets trapped in the nucleus [110], it has been confirmed recently that nuclear lipid droplets are formed through distinct mechanisms in the nucleus. These include budding from the inner nuclear membrane (INM) or from the lumen of the type I nucleoplasmic reticulum [112,114]. Studies in yeast have recently shown that nuclear lipid droplets bud from the INM and remain attached to it through seipin-mediated membrane bridges [112]. The latter suggest a dynamic relationship between the two structures, likely enabling bidirectional lipid and protein exchange depending on requirements for membrane synthesis vs storage, protection from lipotoxicity, or regulation of gene expression [4,112]. In hepatocytes, lipid droplets grow within the nucleus and their biogenesis depends on interactions with promyelocytic leukemia nuclear bodies, which are involved in regulation of transcription [113]. A recent study reported that hepatic nuclear lipid droplets derive from a precursor of very low density lipoprotein (VLDL), that arise from the lumen of the type I nucleoplasmic reticulum, and turn into nucleoplasmic lipid droplets by disintegration of the surrounding INM [114].

During nutrient stress caused by lipid overload, cells are faced with a great demand for additional phosphatidylcholine (PC), the main phospholipid constituent

of the lipid droplet monolayer membrane. PC acts as a surfactant that prevents lipid droplet coalescence and its availability is essential for lipid droplet growth and expansion [115]. In *Drosophila*, local PC synthesis on the lipid droplet surface is mediated by the rate-limiting enzyme in the PC synthesis pathway CTP:phosphocholine cytidylyltransferase alfa (CCTα) [115]. In this case, the coordination of membrane PC synthesis with lipid droplet expansion is mediated by the reversible translocation of CCTα from the nucleus to growing cytosolic lipid droplets [115]. However, in other cell types CCTα is mostly localized to the nucleus, suggesting the presence of other coupling mechanisms [4,116]. Interestingly, new evidence suggests that hepatocyte nuclear lipid droplets are involved in the regulation of phospholipid synthesis [114]. They recruit and activate CCTα to promote PC synthesis, which is additionally modulated by competitive binding of PLIN3 to the nuclear lipid droplet surface. The nuclear regulation of PC synthesis is important for the maintenance of membrane homeostasis in response to ER stress [114]. The important discovery of nuclear lipid droplets elicits new questions about their functions in cell biology. It is worth speculating that they are predominantly involved in the regulation of gene expression, since they are located in the nucleus, but they may also exhibit similar roles to those established for cytosolic lipid droplets.

LIPID DROPLETS MAINTAIN MEMBRANE AND ER HOMEOSTASIS

Cell integrity and function depend on the preservation of membrane and ER homeostasis. The ER is composed of a network of membrane cisternae and tubules that extends throughout the cell. It comprises the largest pool of lipids in the cell and forms membrane contact sites with all other organelles [117]. It has a pivotal role in the synthesis, storage, and secretion of proteins and lipids, and is important for calcium dynamics. Various pathophysiological events that interrupt protein folding processes, calcium uptake, or alter ER lipid composition may cause ER stress. Cells cope with ER stress by triggering the unfolded protein response (UPR) in order to restore ER homeostasis. The UPR is an adaptive mechanism whose activation leads to reduced protein translation, increased transcription of genes involved in the ER stress response, and elevated ER-associated protein degradation (ERAD). If any of these mechanisms fail or ER stress is prolonged, the UPR shifts from being an adaptive to a pro-apoptotic mechanism [1,4,118,119]. It is not surprising that lipid droplets, as organelles arising from and intricately associated with the ER, respond to disturbances in ER homeostasis. ER stress promotes lipid droplet formation [120,121] and lipid droplet accumulation has been observed as a general downstream effect of ER stress, including that induced by pharmacological agents, lipid overload, starvation, and impaired lipid biosynthetic pathways (Figure 4) [68,83,120,122,123]. Conversely, dysfunctional lipid droplet turnover also contributes to the induction of ER stress [68,122,124], suggesting a complex and bidirectional relationship between lipid droplets and ER stress. Emerging evidence has revealed that lipid droplets protect against ER stress by removing misfolded proteins, rebalancing ER lipid homeostasis and by regulating other stress response mechanisms, such as autophagy [68,122-127]. It has been shown that lipid droplets act as buffers that not only sequester excess free FAs but also unfolded or misfolded proteins in order to alleviate ER stress (Figure 4) [68,127,128]. The storage of calcium in the ER is affected by ER stress, which leads to the release of calcium in the cytosol and cell death [118,126]. However, it has been suggested that lipid droplets protect from cell death by sequestering calcium and preventing cytosolic calcium overload [129]. Interestingly, ER stress also promotes the formation of nucleoplasmic lipid droplets, which act in a feed-back mechanism that activates the synthesis of PC to alleviate ER stress [114]. Although the mechanisms linking lipid droplets and ER stress are not yet clear, changes in lipid droplet turnover seem to be tightly associated with ER stress. Through their ability to buffer toxic lipids and proteins, and by regulating lipid flux to membranes and organelles, lipid droplets may be essential for protein quality control as well as membrane synthesis, composition, and dynamics. Depending on the particular conditions and cell type, they may act both to prevent the onset of ER stress and to restore ER homeostasis upon UPR activation. Finally, the role of lipid droplets in the regulation of ER stress has been implicated in several pathologies, including insulin resistance, dyslipidemia, hepatic steatosis, heart failure, and inflammation [68,83,130].

Pharmacological inducers of ER stress, including tunicamycin, brefeldin A, and thapsigargin, promote lipid droplet biogenesis in yeast [120] and mammalian cells [121]. Interestingly, although lipid droplet accumulation correlates with the level of ER stress in yeast cells, it is not essential for their survival [120]. In cardiomyocytes, treatments with exogenous palmitate, which is poorly incorporated into TAGs [61,131,132], upregulates the expression of genes associated with ER stress and causes lipotoxicity, but does not induce lipid droplet biogenesis [68]. When lipid droplet biogenesis was augmented by increasing the expression of peroxisome proliferator-activated receptor γ (PPARγ), palmitate-induced ER stress was mitigated, suggesting that sequestration of palmitate into neutral lipid stores reduces ER stress. However, inhibition of TAG synthesis by DGAT1 knockdown in cultured rat hepatocytes exposed to exogenous FAs did

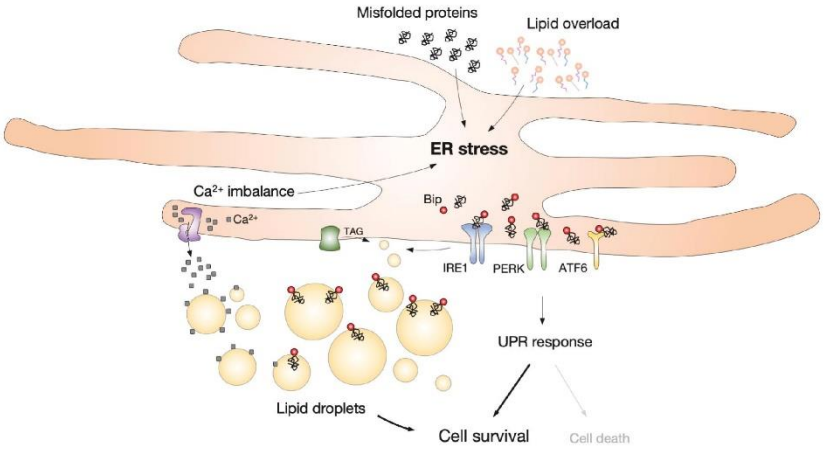

Figure 4. Lipid droplet formation is induced by ER stress to maintain lipid, protein and calcium homeostasis. ER stress may be triggered by various conditions, including the accumulation of misfolded proteins, excess or damaged lipids and calcium imbalance. Cells respond to ER stress by activating the unfolded protein response (UPR) in order to restore homeostasis and enable cell survival, but its prolonged activation may lead to cell death. In mammals, the UPR activation is regulated by three distinct transmembrane proteins: inositol-requiring enzyme 1 (IRE1), PKR (double-stranded-RNA-dependent protein kinase)-like endoplasmic reticulum kinase (PERK), and activating transcription factor 6α (ATF6α). These proteins are activated by dissociating from the ER chaperone imunoglobulin heavy-chain-binding protein BiP/GRP78, which binds to misfolded proteins. ER stress promotes the formation of lipid droplets that bud from the ER membrane. Lipid droplets act as buffers, which sequester misfolded proteins and excess lipids in order to alleviate ER stress. Lipid droplets may also sequester free Ca^{2+} and protect from cytosolic Ca^{2+} overload, which has been associated with ER stress. Lipid droplets are thus crucial for the maintenance of lipid, protein and calcium ER homeostasis.

not enhance palmitate lipotoxicity or abolish the ability of oleate to reduce palmitate-induced ER stress [133]. Similarly, rather than promoting the flux of palmitate into TAG, oleate protects myeloid cells from palmitate-induced ER stress by counteracting its incorporation into phospholipids and reducing membrane saturation [134]. In accordance, the protective effect of monounsaturated FAs against ferroptosis, an iron- and lipid peroxidation-dependent form of cell death, was not dependent on lipid droplet formation, but on the displacement of easily oxidizable PUFAs from membrane phospholipids and the concomitant suppression of membrane lipid peroxidation [135]. Thus, the protective effects of monounsaturated FAs against ER stress and lipotoxicity are not always associated with lipid droplet metabolism. Future studies will help elucidate the currently obscure links between lipid droplets, ER stress, FA lipotoxicity, and ferroptosis [136].

In adipocytes, ER stress stimulates lipolysis by activating signaling pathways involved in the regulation of neutral lipases [137] and may contribute to lipotoxicity because of the persistently elevated FA efflux. Conversely, physiological conditions like fasting or intense exercise increase adipose tissue lipolysis to supply other tissues with FAs, but the large amounts of lipolytically released FAs could lead to ER stress and lipotoxicity even in the adipocytes themselves [122]. Therefore, a seemingly futile cycle of lipolysis followed by ATP-consuming FA re-esterification into TAGs occurs simultaneously in adipocytes in order to reduce ER stress. This is indicative of a cell-autonomous protective role of lipid droplet biogenesis against ER stress induced by high levels of lipolysis. In line with this, stimulation of ATGL-mediated lipolysis leads to ER stress even in cells treated with the otherwise beneficial, ER stress-reducing FA, oleate [68]. Similarly, facilitating autophagy-related 14 (ATG14)-mediated lipophagy results in accumulation of free FAs, ER stress, and apoptotic cell death [124]. On the contrary, the absence of ATGL reduces FA saturation levels in the liver and protects mice against tunicamycin-induced hepatic

ER stress [83]. These results suggest that the excessive amounts of free FAs released through elevated lipid droplet breakdown are a major cause of ER stress and tissue damage in diseases associated with lipid overload.

ERAD is a mechanism for the elimination of unfolded or misfolded proteins from the ER through ubiquitination and subsequent degradation by the proteasome [138]. Genetic impairment of ERAD-mediated protein degradation or protein glycosylation pathways in yeast leads to lipid droplet accumulation, opening the possibility that lipid droplets are employed by cells to alleviate ER stress associated with protein misfolding [120]. Lipid droplets have been proposed to act as "escape hatches" for removal of damaged ER proteins [128]. Indeed, their role in maintaining protein quality and ER homeostasis has been recently demonstrated [127]. Impairment of yeast PC synthesis led to ER stress, elevated lipid droplet accumulation, and altered the morphology and localization of the ER. The ER was aggregated in the perinuclear area and co-localized with lipid droplets. In addition, chaperone proteins, including heat shock protein 104p, were recruited to the aggregated ER. Importantly, elevated levels of polyubiquitinated proteins were found in lipid droplet isolates, suggesting that unfolded proteins have been transferred from ER aggregates to lipid droplets. Finally, this study suggests that stress-induced lipid droplets are engulfed by and degraded in the vacuole via a process similar to microlipophagy [127]. Thus, lipid droplets likely serve as vehicles for the removal of damaged ER proteins and their delivery to the vacuole independently of the ERAD mechanism. In accordance, other studies have suggested the existence of ERAD-independent vesicle-mediated mechanisms of protein delivery and lysosomal elimination [139,140]. Thus, lipid droplets may regulate the elimination of damaged proteins from the ER by acting as transporters of unfolded or misfolded proteins intended for further elimination.

LIPID DROPLETS AND AUTOPHAGY WORK IN SYMBIOSIS

Autophagy is an evolutionarily conserved process of recycling dispensable cytoplasmic material by lysosomal degradation. It provides building blocks and metabolic substrates for processes essential for cell survival [141-143]. Strong links have been suggested between autophagy and the maintenance of cellular lipid homeostasis under physiological conditions and during stress [14,144]. Both lipid droplet turnover and autophagy are activated during stress and emerging evidence points to a complex, intertwined relationship between these processes [2].

Autophagy may participate in lipid droplet biogenesis in starving cells [25,26,28]. As discussed above, in acutely starved MEFs, autophagy of membranous organelles drives lipid droplet formation by providing a constant supply of FAs, which are then released by ATGL-mediated lipolysis and transferred to mitochondria for energy production [25]. The protective role of autophagy-induced lipid droplet biogenesis has also been observed in other organisms including yeast [145], algae [146], and plants [147]. In white adipose tissue, liver and heart, autophagy-driven lipid droplet biogenesis has been implicated in the regulation of adipocyte differentiation, maintenance of lipid storage, redox homeostasis, and energy metabolism [148-151]. Thus, autophagy contributes to lipid droplet biogenesis under various conditions, and it is possible that lipid droplet biogenesis is a general protective response to high levels of autophagy [26].

Autophagy is also involved in lipid droplet breakdown and control of lipid distribution through a lipid droplet-selective form of autophagy called lipophagy [52]. In lipophagy, whole or parts of lipid droplets are engulfed within autophagosomal membranes and delivered to lysosomes for degradation by acid lipolysis (Figure 2C). In yeast, lipid droplet contacts with vacuoles lead to direct invaginations of the vacuolar membrane and lipid droplet breakdown [127]. In mammalian cells, interactions between lipid droplets and lysosomes occur in both prolonged and brief kiss-and-run manners [4] and play an important role in the regulation of lipid droplet breakdown by lipolysis and lipophagy [55]. Lipophagy provides FAs for mitochondrial energy production in hepatocytes [52], participates in the clearance of toxic ceramides in the liver [152], prevents high-fat diet-induced hepatotoxicity and, together with mitophagy, protects against ethanol-induced liver injury [153]. Liver homeostasis may thus depend on constitutive lipophagy. Lipophagy in other tissues is less characterized, but it has been observed in adipocytes [144], macrophages [154], enterocytes [155], hypothalamic neurons [156], and embryos [157]. These studies suggest that lipophagy is involved in food-intake control, lipid distribution and protection against ectopic lipid accumulation. In yeast, microlipophagy, a special form of autophagosome-independent vacuole-mediated lipid droplet breakdown, is involved in the management of ER stress, protein quality control and energy metabolism [47,127,158]. Taken together, lipid droplet mobilization through autophagy occurs in different cell types and has important roles in the protection against cellular stress by reducing lipotoxicity, preserving organelle homeostasis, and maintaining energy metabolism.

Finally, lipid droplets may also promote the autophagic process in several ways, including the provision of lipids for the formation of autophagosomal membranes [159,160], regulation of FA flux and ER homeostasis [123], and through signaling-mediated regulation of autophagy [161]. Interestingly, in hepatocytes, ATGL acts as a signaling node that promotes autophagy/lipophagy

through SIRT1-mediated signaling [161]. By orchestrating the extracellular and intracellular lipid fluxes necessary for energy production and membrane synthesis, lipid droplets may be an essential element of the autophagic stress response. In summary, autophagy-mediated lipid droplet metabolism is involved in the maintenance of lipid homeostasis and cell survival under various conditions of stress. On the one hand, autophagy participates in lipid droplet biogenesis or breakdown, while on the other, lipid droplets may provide lipids and signals for the proper execution of autophagy.

LIPID DROPLETS ARE RESERVOIRS OF BIOACTIVE LIPIDS

One of the most underappreciated aspects of lipid droplet biology is their role as cellular storage pools of lipid signaling mediators, *i.e.* bioactive lipids, and their precursors. Broadly speaking, the lipid composition of lipid droplets depends on the cell type, metabolic state, and environmental conditions. Lipid droplets in adipocytes are predominantly composed of TAGs, those found in Leydig cells of the adrenal cortex and macrophage foam cells mainly contain CEs, whereas retinyl-ester (RE)-rich lipid droplets may be found in hepatic stellate cells (HSCs) [154,162-164]. The fatty acyl group composition of neutral lipids also differs between cells and changes according to cell state. For example, PUFA enrichment in TAGs has been observed in hepatocytes of fasted mice [165], in HSCs during activation [166,167], in glial cells exposed to oxidative stress [30], in visceral adipose tissue of cancer patients [168], cancer cells exposed to exogenous PUFAs [9], and in apoptotic cells [169]. Lipids released from lipid droplets may act directly as signaling molecules, such as FAs, or serve as precursors for the synthesis of other bioactive lipid mediators, including eicosanoids, retinoic acid (RA), endocannabinoids, and ceramides [19,170-173]. Lipid droplet-mediated signaling has been shown to affect mitochondrial function and lipid metabolism [20,164], cell activation [172,173], inflammation [170,171,174-176], and may contribute to tumorigenesis [176-178].

Lipolysis has been associated with the production of inflammatory mediators in several types of immune cells. ATGL-mediated lipolysis has been shown to regulate the generation of pro-inflammatory eicosanoids in mastocytes [171] and mouse neutrophils [170]. In human activated mastocytes, blocking ATGL-mediated lipolysis resulted in lipid droplet accumulation and reduced levels of prostaglandin D_2 (PGD_2) and leukotriene C_4 (LTC_4), suggesting that TAG lipolysis is a source of arachidonic acid, a precursor for eicosanoid synthesis [171]. Similarly, pharmacological inhibition of ATGL in murine neutrophils led to a significant decrease in the release of several eicosanoids, including PGD_2, LTE_4, LTB_2, and thromboxane B_2. Importantly, exudates from ATGL deficient mice had reduced amounts of eicosanoids, suggesting that the enzyme participates in inflammatory signaling processes *in vivo* [170]. Additionally, it has recently been suggested that HSL is involved in the generation of lipid mediators in adipocytes [174]. Stimulation of adipocyte lipolysis with a non-selective β-adrenergic agonist resulted in elevated release of cyclooxygenase (COX)-, lipoxygenase (LOX)- and epoxygenase-derived eicosanoids, which was reduced by inhibition of HSL. In addition, elevated lipolysis led to COX-2 upregulation and infiltration of immune cells, suggesting that lipid mediators produced by lipolysis provoke an inflammatory response in adipose tissue [174]. In summary, lipolytic enzymes contribute to the synthesis of pro-inflammatory lipid mediators and are thus potential targets for therapeutic interventions in inflammatory diseases.

Lipid droplet breakdown may also occur through lipophagy and lysosomal degradation. In this case, LAL is involved in neutral lipid catabolism and it may contribute to bioactive lipid synthesis. Macrophages depleted in LAL accumulate neutral lipids within lysosomes, predominantly CEs [175]. These are particularly enriched with arachidonic and linoleic acids, both precursors of pro-inflammatory lipid mediators. Blocking LAL activity in macrophages resulted in a reduced release of several bioactive species derived from COX-, LOX- and cytochrome P450-mediated biosynthetic pathways, suggesting that lipid hydrolysis by LAL is an important source of precursors for bioactive lipid synthesis in macrophages. Further studies are required to establish the connection between LAL-mediated lipid mediator synthesis and lipid droplet breakdown in other immune and non-immune cells.

Lipid droplet-derived lipid mediators are also involved in HSC activation. In HSCs, different pools of lipid droplets have been observed. Quiescent HSCs mainly accumulate RE-rich lipid droplets that are gradually lost during cell activation. On the other hand, activated HSCs contain two structurally distinct pools of lipid droplets: "original," composed of TAGs and REs, and "new," transient lipid droplets that are enriched in TAGs containing PUFA acyl chains [163,179]. It has been suggested that the loss of REs from lipid droplets during cell activation promotes the synthesis of bioactive RA, which mitigates HSC activation [173]. Another study has suggested that during HSC activation lipid droplet breakdown and remodeling contribute to the generation of highly potent non-retinoid bioactive lipids, including PUFAs, endocannabinoids, N-acylethanolamides, and ceramides, which affect HSC activation and liver metabolism [172]. Thus, lipid droplets act as dynamic cellular reserves of bioactive lipids with distinct roles in cell metabolism.

CONCLUSION

Lipid droplets are emerging as major organelles implicated in organellar and membrane lipid homeostasis. Their dynamic turnover is under control of important cellular sensors and effectors of metabolic and oxidative stress, but much remains to be learned in order to understand the mechanisms of regulation of lipid droplet metabolism in stressed cells. It is now clear that lipid droplets interact with virtually all organelles through specific membrane contact sites, thereby engaging in bidirectional communication, which is essential for efficient exchange of lipids and proteins and is critical for various aspects of the cellular stress response. The detailed structures of the contact sites remain to be determined, the mechanisms of nutrient exchange revealed and their physiological significance confirmed in various settings. Likewise, we know very little about the biogenesis and function of nuclear lipid droplets and it will be exciting to witness future discoveries regarding their role in cell biology and diseases. We are only beginning to understand the heterogenous nature of lipid droplets, both in composition and function, within cells and within populations of cells. It will be important to find out how lipid droplets manage the trafficking of different lipid species and how their lipid composition and protein ensemble affects their function. Current evidence suggests that lipid droplet turnover has critical roles in the regulation of membrane lipid composition, lipid trafficking, mitochondrial function, and the protection of other organelles from lipotoxicity and oxidative stress. Their pleiotropic roles in managing lipids and stress will surely expand in the future as new functions and more detailed mechanisms are elucidated. Remarkably, lipid droplets also act as signaling platforms that affect nuclear function and gene expression in the cell, but also signal to neighboring cells through bioactive lipid mediators. Their involvement in the regulation of immune cell function, immunometabolism and inflammatory signaling will be an exciting field of discovery. The clarification of these and many other yet unasked questions in lipid droplet biology in stressed cells may help us uncover their roles in different pathologies, in particular metabolic disorders, cardiovascular diseases, cancer, and neurodegeneration, and may help us design strategies to prevent and fight against these debilitating diseases.

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Appendix C.

Review Paper "A Twist of FATe: Lipid Droplets and Inflammatory Lipid Mediators"

The paper “A twist of FATe: Lipid droplets and inflammatory lipid mediators” by Eva Jarc and Toni Petan was published upon invitation in the special issue of *Biochimie* entitled "Biogenesis and fate of lipid droplets". *Biochimie* is a journal published by Elsevier on behalf of the Société Française de Biochimie et Biologie Moléculaire and we were delighted to have the opportunity to contribute to this issue. In this article, we discuss the principal ways in which lipid droplets regulate the availability of fatty acids for the activation of inflammatory signaling pathways and for the production of lipid mediators. On the one hand, lipid droplets sequester lipids and proteins thereby limiting their availability for participation in signaling pathways. On the other hand, lipids derived from the neutral lipid core or the phospholipid monolayer of lipid droplets directly act as signaling mediators or are converted into ones. Lipid droplets thus act as signaling hubs that integrate metabolic and inflammatory processes. In this work I performed literature research and fact-checking, drafted parts of the manuscript and contributed to the preparation of figures.

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Review

A twist of FATe: Lipid droplets and inflammatory lipid mediators

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ABSTRACT

Lipid droplets are fat storage organelles present in most eukaryotic cells. They consist of a neutral lipid core containing mostly triglycerides and sterol esters and covered by a monolayer of phospholipids, wherein numerous proteins are embedded. In the cell, lipid droplets have a dynamic life cycle, rapidly altering their size, location, lipid and protein composition in response to environmental stimuli and cell state. Lipid droplets are primarily involved in the coordination of lipid metabolism with cellular requirements for energy production, membrane homeostasis and cell growth. However, they are also directly or indirectly engaged in signalling pathways. On the one hand, lipid droplets sequester lipids and proteins thereby limiting their availability for participation in signalling pathways. On the other hand, the lipolytic machinery provides a highly regulated, on-demand source of signalling lipids: lipids derived from their neutral lipid core, or the phospholipid monolayer, directly act as signalling mediators or are converted into ones. In fact, emerging studies suggest that these organelles are essential for various cellular stress response mechanisms, including inflammation and immunity, acting as hubs that integrate metabolic and inflammatory processes. Here, we discuss the ways in which lipid droplets regulate the availability of fatty acids for the activation of signalling pathways and for the production of polyunsaturated fatty acid-derived lipid mediators. We focus in particular on recent discoveries in immune cells and adipose tissue that have revealed an intricate relationship between lipid droplets and inflammatory signalling and may also be relevant for other tissues and various human diseases.

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1. Introduction

Lipid droplets are cytosolic fat storage organelles that are dynamically synthesised and broken down in response to environmental signals and according to cellular requirements [1,2]. They have a unique structure among organelles consisting of a neutral lipid core that contains mostly triacylglycerols (TAGs) and cholesteryl esters, covered by a phospholipid monolayer, in which numerous proteins are embedded [3,4]. Being only recently recognized as fully functional, independent organelles rather than inert fat depots, they are now emerging as major regulators of lipid metabolism, trafficking and signalling [5–8]. They actively control metabolic fluxes and signalling pathways that coordinate lipid uptake, synthesis and breakdown with cellular needs for energy production, biosynthesis, membrane and organelle homeostasis. It has become evident in recent years that the functions of lipid droplets extend beyond lipid metabolism and that they are also an integral part of the cellular stress response [2,9–11].

Lipid droplet biogenesis is initiated between the two leaflets of the endoplasmic reticulum (ER) membrane, whereby newly formed TAGs accumulate in the hydrophobic environment provided by the glycerophospholipid acyl chains [2,12]. Nascent lipid droplets bud from the ER membrane and are released into the cytosol, where their dynamic lifestyle begins: travelling along microtubules, they may be clustered and rather stationary in fed cells, but are rapidly dispersed during starvation; they can grow, fuse and shrink through several mechanisms, while all along communicating with other organelles through dynamic contacts that enable lipid and protein exchange [2,13–18]. They are most often broken down by lipolysis, which includes a sequential and highly regulated removal of fatty acids (FAs) from TAGs by adipose triglyceride lipase (ATGL), hormone sensitive lipase (HSL) and monoacylglycerol lipase (MAGL) [19–21]. In specific conditions and preferentially in certain cell types, lipid droplet breakdown also occurs by lipophagy, a selective process of autophagy that delivers parts of or whole lipid droplets to lysosomes for bulk degradation by hydrolytic enzymes, including the TAG and cholesteryl ester hydrolase lysosomal acid lipase (LAL) [9,22–25]. Lipid droplet turnover is induced in cells exposed to nutrient and oxidative stress, whereby they provide protection against energy and redox imbalances [2,9,10,26]. Lipid droplets are also formed in various cells exposed to inflammatory stimuli

Abbreviations: ATGL, adipose triglyceride lipase; COX, cyclooxygenase; DGAT, diacylglycerol acyltransferase; ER, endoplasmic reticulum; FA, fatty acid; HSL, hormone sensitive lipase; LOX, lipoxygenase; LAL, lysosomal acid lipase; MAGL, monoacylglycerol lipase; PLIN, perilipin; PLA_2, phospholipase A_2; PPAR, peroxisome proliferator-activated receptor; PUFA, polyunsaturated fatty acid; PGE_2, prostaglandin E_2; SPMs, specialized pro-resolving mediators; TAG, triacylglycerol.

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and infection, in particular in immune cells during activation and differentiation [27–30]. Emerging studies suggest that these fat-laden organelles regulate various aspects of inflammation and immunity by acting as hubs that coordinate metabolic and inflammatory processes in various conditions of acute and chronic cellular stress [9,29].

Lipid droplets store different lipids, which upon their release may directly act as signalling molecules or are converted into ones. The breakdown of TAGs and other esterified neutral lipids gives rise to free FAs that may activate numerous signalling pathways in immune and other cell types by directly binding to cell surface receptors, including G protein-coupled receptors (GPCRs) and Toll-like receptors (TLRs), intracellular binding/transport proteins and nuclear receptors. The latter include the peroxisome proliferator-activated receptors (PPARs), sterol regulatory element-binding proteins (SREBPs) and nuclear factor κB (NF-κB) transcription factors [6,31,32]. Notably, different FA species have distinct abilities to activate cellular signalling pathways and display context-dependent effects that result in diverse (patho)physiological outcomes [31,32].

Lipid droplets also store polyunsaturated FAs (PUFAs), which are precursors for the synthesis of numerous bioactive lipid mediators, such as eicosanoids and specialized pro-resolving mediators (SPMs). These mediators are released from various immune and other cell types to modulate inflammatory and immune responses in their local microenvironment by binding to cognate GPCRs ooon target cells [33,34]. Dozens, even hundreds of different eicosanoids, SPMs and related oxygenated FA species may be rapidly synthesised and simultaneously released from activated cells. This mixture of potent lipid mediators induces the initiation of cellular response programs that depend on the combined effects of the released species, the presence of specific receptors on target cells, the cell types involved and the conditions they are exposed to. In these rather complex and dynamic ways, FAs and their oxygenated metabolites control a plethora of signalling pathways and transcriptional programs that integrate metabolism, immunity and inflammation [33,35–39]. However, the contribution of different cellular FA pools, in particular those derived from lipid droplets, to the activation of these pathways is poorly understood.

Several recent studies have shown that lipid droplet lipolysis mediated by ATGL and HSL is involved in the production of eicosanoids in several types of immune cells, adipocytes and endothelial cells [40–44], thereby challenging the conventional view that membrane glycerophospholipids, and primarily their hydrolysis by phospholipase A_2 (PLA_2) enzymes, are the main sources of PUFAs for eicosanoid production. The concept that lipid droplets release FAs that in turn regulate important signalling pathways, most notably the PPAR transcriptional network [6], and also store FAs that may be potentially used for conversion into eicosanoids [45–47] is not new. However, these new studies using ATGL-deficient cells and specific inhibitors [40–44] provide the first evidence that TAGs stored in cytosolic lipid droplets are in fact sources of FA precursors for lipid mediator production and that their availability is controlled by neutral lipases. Additionally, the breakdown of cholesteryl esters by LAL in the lysosome has recently been shown to contribute to the production of eicosanoids, further expanding our view that the turnover of neutral lipids is important for lipid mediator production [48].

In this review, we provide an overview of the current evidence implicating lipid droplets in the regulation of signalling pathways mediated by FAs and their oxygenated metabolites. We focus on how lipid droplets contribute to the regulation of the availability of FAs for the activation of signalling pathways and for the production of lipid mediators, but also discuss recent studies that reveal the intricate, bidirectional relationship between inflammatory signalling pathways and lipid droplet metabolism.

2. Lipid droplets sequester lipids and proteins and modulate signalling pathways

Lipid droplets can affect signalling pathways by actively removing signalling lipids from their bioactive to a storage pool or by regulating their release and production from stored precursors [6,49]. Besides FAs, all other major intermediates of TAG synthesis and breakdown, namely acyl-coenzyme A, lysophosphatidic acid, phosphatidic acid, diacylglycerol (DAG) and monoacylglycerol (MAG), are potential signalling molecules [49]. In addition, by regulating the storage and release of sterols, retinoids and ceramides, lipid droplets also affect various signalling pathways that are directly or indirectly modified by these lipids and their metabolites [50–53]. The removal of lipids from membrane or cytosolic pools into the lipid droplet core is one of the major aspects of lipid droplet biology that is crucial for the regulation of lipid trafficking and prevention of lipotoxicity [10]. Their conversion into inert neutral lipids reduces direct cell damage incurred by their excess, which may be caused by altered membrane composition affecting protein and organelle function or by activation of specific signalling pathways, but it also precludes their conversion into other, potentially even more damaging metabolites and/or signalling lipids. Sequestration of these various lipids into the lipid droplet core thus reduces their availability for the activation of signalling pathways involved in cellular stress and inflammation (Fig. 1), including the activation of PPAR signalling, TLR and NF-κB inflammatory pathways by saturated FAs, the conversion of PUFAs into eicosanoids, the regulation of SREBP signalling by cholesterol and FAs, the activation of protein phosphatase 2A (PP2A) by ceramides, or of protein kinase C by membrane-resident DAG [6,31,32,54–57]. For instance, channelling of saturated FAs into TAGs reduces their availability for conversion into ceramides thus limiting both saturated FA- and ceramide-induced cell dysfunction and inflammatory signalling [58,59]. In cancer cells exposed to hypoxic stress, blocking lipid droplet biogenesis elevates lipid saturation and ceramide levels and activates the NF-κB signalling pathway [60]. Lipid droplet biogenesis also reduces macrophage inflammatory activation by palmitate and protects mice against diet-induced inflammation and insulin resistance in adipose tissue [61]. Similarly, the esterification of ceramides into acylceramides and their release from lipid droplets may also modulate the activation of ceramide-induced signalling pathways in the cell [50,62].

Lipid droplets have also been suggested to act as sinks that limit the availability of arachidonic acid for its conversion into pro-inflammatory eicosanoids and thus reduce potential tissue damage associated with extensive inflammation [63,64]. Similarly, cells exposed to oxidative stress redirect PUFAs from membrane phospholipids into the protective core of lipid droplets in order to prevent their oxidation and ensuing lipid peroxidation chain reactions, thereby preserving membrane homeostasis, but also limiting signal transduction pathways that regulate cell proliferation, growth and survival [65,66]. Namely, oxidized membrane lipids, such as oxygenated PUFA-containing phospholipids and cardiolipins are emerging as important signals that trigger various stress and programmed cell death pathways, including autophagy, apoptosis and ferroptosis [67,68]. Lipid droplets could thus limit cell damage, prevent cell death and reduce the inflammatory response by sequestering PUFAs and reducing their availability for oxygenation and action as danger signals.

Lipid droplets may also sequester oxidized, damaged lipids, most likely in order to protect the cell from oxidative damage, but their excessive accumulation may also disrupt lipid droplet metabolism or the function of lipid droplet-associated proteins. For example, elevated lipid accumulation has been associated with the impairment of the antigen cross-presentation capacity of tumour-associated dendritic cells, which is necessary for the induction of anti-tumour immune responses [69]. Intriguingly, oxidatively-damaged, truncated TAGs accu-

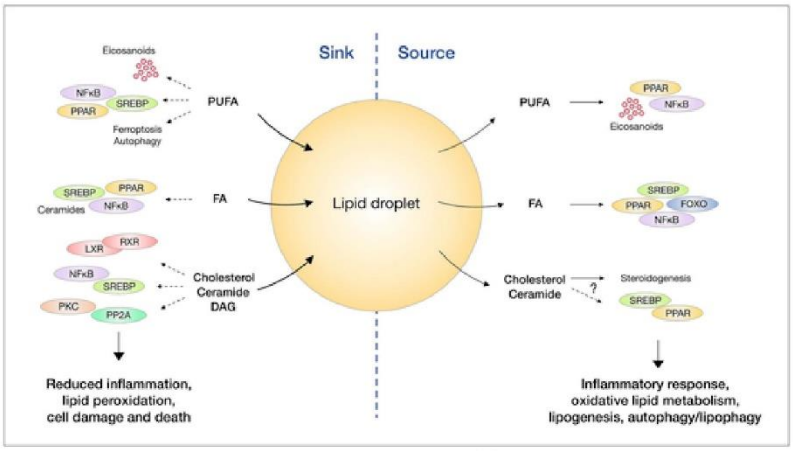

Fig. 1. Lipid droplets and lipid signalling pathways. In principle, lipid droplets may participate in signalling pathways by sequestering lipids or by actively controlling the provision of lipids for the activation of specific signalling pathways. On the one hand, lipid droplets act as sinks that limit the availability of various lipids for their participation in signalling pathways that may lead to lipotoxic cell damage and inflammation. Sequestration of fatty acids (FAs), diacylglycerols (DAG), cholesterol and ceramides into triglycerides, cholesteryl esters and acylceramides, removes these lipids from their bioactive pool and prevents their conversion into other signalling molecules, e.g., saturated FAs into ceramides or polyunsaturated FAs (PUFAs) into eicosanoid lipid mediators. On the other hand, lipid droplets may act via their lipolytic enzymes as on-demand sources of lipids that regulate numerous signalling pathways associated with oxidative metabolism, lipogenesis, adipogenesis, autophagy/lipophagy and inflammation. Strong evidence and mechanistic details for the involvement of lipid droplets in many of the depicted pathways are still lacking. See text for more details. SREBP, sterol regulatory element-binding protein; PPAR, peroxisome proliferator-activated receptor; NF-κB, nuclear factor κB; FOXO, forkhead homeobox type protein O; PP2A, protein phosphatase 2A; PKC, protein kinase C; LXR, liver X receptor; RXR, retinoid X receptor.

mulate in lipid droplets of tumour-associated dendritic cells and negatively affect dendritic cell function by covalently binding and thus "trapping" heat shock protein 70 (HSP70, encoded by *HSPA1A*) on the lipid droplet surface (Fig. 2A) [70,71]. The covalent lipidation of HSP70 is possible because of the highly reactive electrophilic groups of truncated and oxidized acyl chains that are most likely predominantly localized at the surface of lipid droplets [71]. This impairs antigen presentation by reducing the trafficking of peptide–MHC class I (pMHC) complexes to the cell surface. Interestingly, representatives of the HSP70 family of chaperones are consistently found in lipid droplet proteomes [72] and some have important roles in lipid droplet metabolism. For instance, heat shock cognate protein of 70 kDa (Hsc70; encoded by *HSPA8*) is one such molecular chaperone involved in many cellular processes [73], including the regulation of chaperone-mediated autophagy (CMA) [74]. CMA regulates both lipolysis and lipophagy by removing the lipid droplet-coating protein perilipin 2 (PLIN2) from

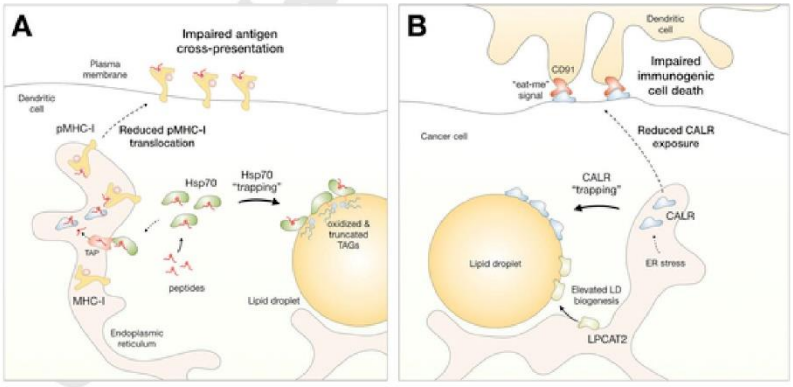

Fig. 2. Lipid droplets alter immune function by regulating chaperone trafficking and exposure. (A) In tumour-associated dendritic cells, lipid droplets accumulate oxidatively-damaged, truncated triacylglycerols (TAGs), which lipidate and sequester the cytosolic heat shock protein 70 (HSP70) to the lipid droplet surface. This "trapping" of HSP70 prevents its participation in the trafficking of peptide-MHC class I (pMHC-I) complexes to the cell surface, leading to inhibition of antigen cross-presentation. (B) Colon cancer cells expressing high levels of lysophosphatidylcholine acyltransferase 2 (LPCAT2) accumulate large amounts of lipid droplets upon exposure to chemotherapeutics. Lipid droplet accumulation supresses endoplasmic reticulum (ER) stress and drives the sequestration of the ER luminal chaperone calreticulin (CALR) to lipid droplets, thereby inhibiting its ER stress-induced translocation to the cell surface, where it serves as an "eat-me" signal for recognition by immature dendritic cells. This leads to supressed removal of dying cancer cells due to impaired dendritic cell maturation and T-cell activation. TAP, transporter for antigen presentation; CD91, cluster of differentiation 91 (also known as low density lipoprotein receptor-related protein 1, LRP1).

the lipid droplet surface [75]. The lipidation of HSP70 by the oxidatively-truncated electrophilic TAG acyl chains may only be one representative example of a more general mechanism of protein lipidation and retention on the lipid droplet surface and disruption of lipid droplet and/or protein function. It will be interesting to see whether other proteins may be regulated by similar mechanisms in other pathophysiological settings. Interestingly, the sequestration of another chaperone, the Ca^{2+}-binding protein calreticulin, on lipid droplets also impairs immune function [76] (Fig. 2B). Calreticulin serves as a phagocytic signal for immunogenic cell death and is also involved in antigen presentation [77]. In cancer cells treated with chemotherapeutics, calreticulin is sequestered to lipid droplets, thereby reducing the exposure of this "eat-me" signal on the plasma membrane, which leads to supressed removal of dying cancer cells, most likely due to impaired dendritic cell maturation and T-cell activation [76]. Cytosolic lipid droplets are also involved in the exchange of proteins with the nucleus, including histones [11,78,79], perilipin 5 (PLIN5) (see text below) [80] and the transcription factor nuclear factor of activated T cells 5 (NFAT5) [81], thereby affecting chromatin assembly, regulation of gene expression and even anti-bacterial defence. Intriguingly, emerging studies have shown that lipid droplets are also present in the nucleus [11,26,82,83], where they regulate various aspects of nuclear function, including phosphatidylcholine synthesis by affecting the recruitment of CTP:phosphocholine cytidylyltransferase α (CCTα) [84]. For a more detailed discussion on the emerging roles of lipid droplets in protein trafficking and quality control the reader is advised to turn to several recent excellent reviews [2,3,11,85–87].

3. Lipolysis regulates fatty acid-mediated signalling pathways

3.1. Lipolytic signalling couples fatty acid supply with oxidative metabolism and lipogenesis

In principle, lipid droplets could only act as storage facilities that are not involved in signalling *per se*, merely providing a temporary repository for lipids that act in downstream signalling pathways occurring at other cellular locations, where key rate-limiting and regulating steps occur (Fig. 1). The most convincing evidence that lipid droplet turnover is essential for the regulation of intracellular FA-mediated signalling pathways have emerged from studies on the pathophysiological roles of ATGL [24,88,89]. Namely, it is now clear that lipolytic products are required for the regulation of both lipogenesis and oxidative lipid metabolism mediated by the PPAR family of transcription factors in a number of tissues (Fig. 1) [24,89]. The three members of the PPAR family of ligand-activated nuclear receptors control the transcription of genes involved in metabolism, cell proliferation, differentiation and inflammation [90]. PPARs are activated by FAs and their derivatives, including endocannabinoids and eicosanoids, and act as major lipid sensors and master regulators of lipid metabolism. The products of ATGL-mediated lipolysis are necessary for the activation of PPAR-α and the stimulation of mitochondrial oxidative metabolism in several tissues, including the heart, liver and brown adipose tissue (BAT) [54,91,92]. For instance, this activity is essential for proper cardiac function and thermogenesis in BAT. Importantly, although exogenous FAs could enter the cell and bind to PPAR transcription factors directly or after incorporation and release from membrane phospholipids, lipid droplet turnover seems to be necessary for the activation of PPAR signalling by exogenously-derived FAs. In cardiomyocytes, the incorporation of exogenous FAs and their subsequent release by ATGL-mediated lipolysis produces ligands that are required for PPAR-mediated stimulation of mitochondrial gene expression and oxidative metabolism [54]. The importance of this mechanism is evident from the fact that ATGL-deficient mice display lethal cardiomyopathy, which can be prevented by pharmacological activation of PPAR-α [54]. Therefore, rerouting of exogenous FAs into lipid droplets is essential for their signalling effects, likely in order to precisely match FA supply from the circulation with mitochondrial capacity for oxidative metabolism and avoid lipotoxic cell damage [10,17,18]. It can be envisaged that a similar mechanism operates in other cell types as well, particularly in oxidative tissues and in those exposed to large amounts of circulating lipids.

Lipolysis also signals through PPAR transcription factors in order to regulate lipogenesis. Namely, ATGL activity exerts a feedback regulation on lipogenesis by activating PPAR-γ and sterol regulatory element-binding protein 1c (SREBP-1c) signalling in white adipose tissue, revealing an important interdependence between lipolysis and TAG synthesis [93,94]. The coordination of lipolysis and lipogenesis likely serves to optimize FA composition and enable glycerolipid acyl chain remodelling among different cellular lipid pools, thereby maintaining membrane and organelle homeostasis and reducing lipotoxicity [10,60,66]. In accordance, diacylglycerol acyltransferase 1 (DGAT1)-mediated FA re-esterification into TAG is required in adipose tissue during lipolysis to reduce lipotoxic cell damage and ER stress [95]. In fact, the coordination between lipolysis and lipogenesis is emerging as an important factor for the regulation of insulin signalling, obesity and adipose tissue inflammation [89]. However, the lipolytic ligands and signal transduction pathways involved in the coordination of the two processes are still poorly understood [24].

3.2. Lipolytic signals integrate metabolic and inflammatory transcriptional responses

Emerging studies suggest that lipolysis also activates other signalling pathways that regulate energy metabolism, cellular stress responses and inflammation, which may participate in or complement PPAR signalling. In the liver, Sirtuin 1 (SIRT1) is activated by ATGL to promote PPAR-α activation in response to β-adrenergic signalling [96]. SIRT1 is a NAD^+-dependent deacetylase that acts as a metabolic sensor and integrates metabolic stress and inflammatory responses [97]. It deacetylates and activates the transcriptional coregulator PPAR-γ coactivator-1α (PGC-1α) that binds to PPAR-α to promote mitochondrial biogenesis and oxidative metabolism. On the other hand, SIRT1 inactivates the transcription factor NF-κB and suppresses inflammatory responses [98]. The SIRT1-mediated activation of the PGC-1α/PPAR-α pathway by ATGL indicates that lipolysis may also activate PPAR-α through a ligand-independent mechanism [96]. In accordance, PPAR-α agonists cannot restore PPAR target gene expression in ATGL deficient hepatocytes and ATGL-mediated PPAR-α activation does not require fatty acid-binding protein 1 (FABP1), the major liver FA transporter [91,99]. In addition, it has recently been suggested that rather than being responsible for bulk TAG lipolysis in the liver, ATGL acts as a signalling node that activates lipophagy, which in turn performs the majority of lipid droplet breakdown [23,100]. This activity of ATGL was also dependent on SIRT1, but the mechanism of ATGL-mediated activation of SIRT1 remains unknown.

Moreover, recent evidence suggests that ATGL is in fact engaged in a feedback regulatory loop with SIRT1, FOXO1 and PGC-1α/PPAR signal transduction pathways that may coordinate lipid metabolism, inflammation and autophagy/lipophagy [24]. Namely, both acid (LAL) and neutral (ATGL) lipolysis are transcriptionally regulated by SIRT1-mediated activation of forkhead homeobox type protein O1 (FOXO1) and by PGC-1α/PPAR signalling. The FOXO1 transcription factor regulates adipogenesis, lipid droplet metabolism, autophagy and inflammation [24,101,102]. Accordingly, a recent study has shown that the innate immune transcription factor NF-κB/Relish represses FOXO-dependent ATGL/Brummer lipase gene expression to limit fasting-induced lipolysis and conserve triglyceride levels in the *Drosophila* fat body [103]. In addition, the elevated levels of lipid droplets in immune and colonic epithelial cells observed in inflamed intestinal tissue have been associated with repression of FOXO3 and increased NF-κB

and prostaglandin E_2 (PGE_2) signalling [104]. Finally, the lipid droplet-associated protein PLIN5, which regulates lipase activity at the lipid droplet surface and mediates lipid droplet contacts with mitochondria [105–107], is translocated to the nucleus upon catecholamine-induced activation of the protein kinase A pathway. In the nucleus, PLIN5 binds to SIRT1/PGC-1α complexes and stimulates mitochondrial oxidative gene expression [80]. PLIN5 thereby provides another mechanism that couples lipolytic events at the lipid droplet surface with mitochondrial oxidative metabolism, inflammatory signalling and corresponding transcriptional activation in the nucleus. Collectively, these studies reveal the multiple layers of complexity in the regulation of lipid droplet breakdown and its coupling with signalling pathways that regulate metabolism, cellular stress responses and inflammation.

4. Lipid mediator production is controlled by the availability of polyunsaturated fatty acid precursors

4.1. Eicosanoids and specialized pro-resolving mediators (SPMs) are potent lipid mediators involved in inflammation and immunity

Lipid mediators are bioactive lipids that are rapidly synthesised and secreted in response to diverse stimuli and act locally on target cells in their microenvironment. These are short-lived autocrine and paracrine signalling agents that bind to their cognate GPCRs and activate intracellular signalling cascades to elicit cellular responses. Several lipid classes have been classified as lipid mediators and are grouped according to their structure, primary source and biological function [33,38,108]. Eicosanoids are derivatives of arachidonic acid, an ω-6 PUFA, and are most often recognized for their role in the initiation and perpetuation of the inflammatory response, but they may also have anti-inflammatory activities [33]. There are hundreds of different eicosanoid lipid species that are formed by oxygenation of arachidonic acid and related PUFAs by a series of cyclooxygenase (COX), lipoxygenase (LOX) and cytochrome P450 epoxygenase enzymes, giving rise to prostaglandins, leukotrienes, thromboxanes, lipoxins and other related oxygenated lipid species [33]. Remarkably, the same biosynthetic machinery is employed for the conversion of ω-3 PUFAs, such as eicosapentaenoic (EPA) and docosahexaenoic (DHA) acids, into protectins, resolvins and maresins, collectively termed specialized pro-resolving mediators (SPMs) [34]. These relatively recently discovered lipid mediators predominantly display anti-inflammatory effects and are essential for the proper execution of the resolution phase of inflammation. Notably, lipoxins are arachidonic acid-derived LOX-produced eicosanoids that have been classified as SPMs due to their potent anti-inflammatory and pro-resolving activities [33,34]. Eicosanoids and SPMs have important roles in homeostatic tissue function, including vascular biology and mucosal integrity, and have been implicated in numerous chronic inflammatory and immune disorders, including asthma, rheumatoid arthritis, atherosclerosis, neurodegeneration, obesity, diabetes, cancer, metabolic and autoimmune diseases [33,34,39,108–111].

Cell activation at the onset of an inflammatory or immune response typically results in the synthesis of numerous lipid mediators that collectively act on target cells to elicit the desired response. The nature of this lipid mediator "storm" largely depends on the availability of particular PUFA precursors, the set of biosynthetic enzymes and specific receptors that are present in a given cell type [33]. The balance between ω-6 and ω-3 PUFAs that are available for the synthesis of lipid mediators strongly affects the composition of the lipid mediator pool that is produced and determines the short- and long-term outcome of the response. The coordinated action of pro- and anti-inflammatory lipid mediators defines the proper initiation, progression and resolution of acute inflammatory responses and any dysregulation in these processes may underlie the development of chronic inflammatory states associated with numerous diseases [33,34,108,109]. The initiation of inflammation is controlled by pro-inflammatory eicosanoids, which promote neutrophil migration and infiltration, however, they also regulate the resolution phase of inflammation by activating a temporal lipid class switch that activates the production of SPMs and limits neutrophil infiltration. SPMs act as endogenous immunoresolvents that enhance the clearance of microbes, inflammatory debris and apoptotic neutrophils through macrophage phagocytosis and efferocytosis, thereby actively shortening the resolution phase of inflammation, promoting wound-healing and preventing collateral tissue damage [112,113].

4.2. Glycerophospholipid hydrolysis by phospholipases A_2 is a major source of fatty acids for lipid mediator production

The production of lipid mediators is intrinsically linked to the availability of PUFA precursors required for their synthesis, which depends on the activities of numerous enzymes and proteins that regulate FA uptake, transport, hydrolysis, storage, remodelling and trafficking among the different cellular and extracellular lipid pools [114,115]. Traditionally, eicosanoid production has been associated with the activity of phospholipase A_2 (PLA_2) enzymes, which have access to one of the major reservoirs of readily mobilizable PUFAs – the *sn*-2 position of glycerophospholipids. In particular, the cytosolic $PLA_2\alpha$ ($cPLA_2\alpha$) enzyme has been widely recognized as the primary source of arachidonic acid for eicosanoid production, but other members of the PLA_2 superfamily have also been implicated in the provision of arachidonic acid and other PUFAs for lipid mediator production. Moreover, as described in more detail in the following sections, recent studies have revealed additional physiologically relevant sources of arachidonic acid for eicosanoid production (Fig. 3), including the hydrolysis of endocannabinoids by MAGL [21,116–118] and of neutral lipids by lipases, such as ATGL and LAL [40,41,48].

The PLA_2 superfamily consists of more than 30 enzymes that exhibit different selectivity and specificity for membrane phospholipids [119–121]. They all release FAs and lysophospholipids from the *sn*-2 position of the glycerophospholipid backbone and have been classically divided into at least three major families, including secreted phospholipases A_2 ($sPLA_2$s), intracellular, Ca^{2+}-independent ($iPLA_2$s) and Ca^{2+}-dependent cytosolic phospholipases A_2 ($cPLA_2$s). Moreover, the $iPLA_2$ enzymes also belong to the PNPLA family of lipid hydrolases, which comprises both phospholipases (PNPLA6–9) and lipases (PNPLA1–5) and also includes the major lipid droplet TAG lipase, ATGL (PNPLA2). For more details on the classification, biochemistry, cellular and pathophysiological roles of the PLA_2 and PNPLA protein families the reader is referred to several excellent comprehensive reviews [119–123].

Although many members of the PLA_2 superfamily may provide arachidonic acid for eicosanoid production, the group IVA PLA_2 enzyme, or $cPLA_2\alpha$, is the only one that displays a significant specificity for the hydrolysis of arachidonic acid-containing membrane phospholipids [120,122,124]. Upon cell activation by diverse inflammatory stimuli, the enzyme is translocated in a Ca^{2+}-dependent manner from the cytosol to perinuclear membranes of the Golgi, ER and the nuclear envelope membrane, an event that is essential for the initiation of eicosanoid production [120,124,125]. This allows its positioning in the immediate proximity of the COX/LOX enzymatic machinery, thereby optimizing the provision of arachidonic acid for rapid eicosanoid production (Fig. 3). The COX enzymes are primarily localized at the ER, but also the Golgi, while leukotriene synthesis most likely occurs at the nuclear envelope [120,124]. However, while being preferential, the perinuclear localization of $cPLA_2\alpha$ is not obligatory for its functional coupling with COX/LOX enzymes [126]. Importantly, studies in $cPLA_2\alpha$-deficient mice have confirmed that the enzyme is essential for stimulus-induced eicosanoid production in various cells and tissues [120,124,127–129]. Accordingly, numerous studies have suggested its involvement in inflammatory diseases, including asthma, ulceration, cystic fibrosis, Alzheimer's disease, rheumatoid arthritis, neu-

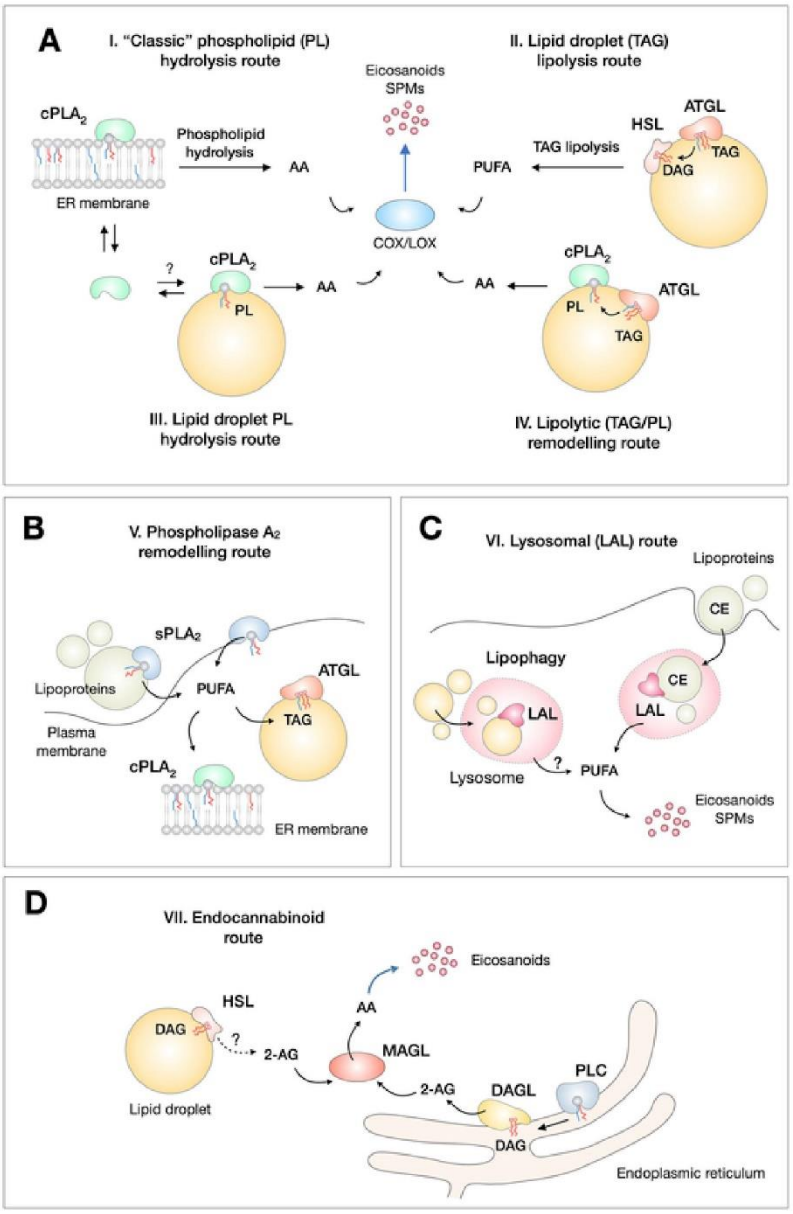

Fig. 3. Cellular sources of precursors for lipid mediator production. The availability of polyunsaturated fatty acid (PUFA) precursors for the production of bioactive lipid mediators, eicosanoids and specialized pro-resolving mediators (SPMs), is controlled by the activity of different phospholipases and lipases on glycerophospholipids and neutral lipids at different subcellular locations. (A) Membrane phospholipid hydrolysis by cytosolic phospholipase A_2 ($cPLA_2$) is considered to be the major source of arachidonic acid (AA) for stimuli-in-

duced eicosanoid production in most cell types (route I). Upon cell activation $cPLA_2$ translocates from the cytosol to perinuclear membrane locations, where it is positioned in close proximity to cyclooxygenases (COXs) and lipoxygenases (LOXs), thereby spatially and temporally coupling AA release with eicosanoid production. Triacylglycerol (TAG) and diacylglycerol (DAG) hydrolysis by adipose triglyceride lipase (ATGL) and hormone sensitive lipase (HSL), respectively, are also potential sources of PUFA precursors for lipid mediator synthesis in several types of cells (route II). $cPLA_2$ has also been found at the lipid droplet surface (route III), along with COX and LOX enzymes, suggesting that it may release AA from the lipid droplet phospholipid monolayer independently of ATGL activity. In principle, PUFAs released by ATGL from lipid droplets may be either directly converted into bioactive lipids by the COX/LOX machinery (route II), or reacylated into phospholipids (route IV) and then released by the activity of $cPLA_2$ (or other PLA_2s). (B) Secreted phospholipases A_2 ($sPLA_2$s) release PUFAs from lipoproteins, the plasma membrane and other extracellular phospholipids, and participate in lipid mediator production in different cells and tissues (route V). These PUFAs may be directly taken up by COX/LOX enzymes, they may be re-esterified into phospholipids and released by $cPLA_2$ (via route I) and/or incorporated into neutral lipids and released by neutral lipases from lipid droplets (via routes II and IV). The importance of these remodelling pathways for lipid mediator production remains to be confirmed in different cell types and conditions; similar remodelling mechanisms may occur at other subcellular locations, whereby the depicted roles of $sPLA_2$/$cPLA_2$ enzymes in route V are played by other members of the PLA_2 superfamily. (C) Endocytosis of cholesteryl ester (CE)-rich lipoproteins and their lysosomal degradation by lysosomal acid lipase (LAL) is also a potential source of PUFAs for eicosanoid and SPM synthesis (route VI). LAL may also contribute to lipid droplet breakdown via lipophagy, which is another possible route for the provision of lipid droplet-derived PUFA precursors that remains to be tested. (D) Monoacylglycerol lipase (MAGL) is an important source of AA for lipid mediator production in several tissues, whereby it controls the availability of AA for eicosanoid synthesis by hydrolysing the endocannabinoid 2-arachidonoylglycerol (2-AG) (route VII). The sequential actions of phospholipase C (PLC) and diacylglycerol lipase (DAGL) from intracellular membranes is considered the main source of 2-AG and other endocannabinoids. Endocannabinoids may also be derived from extracellular lipoprotein lipolysis, whereas the possible contribution of lipid droplets and neutral lipases as sources of 2-AG has not been demonstrated yet.

ronal injury, metabolic diseases and cancer [120,124,130]. Furthermore, many of the phenotypes observed in $cPLA_2\alpha$-deficient mice are recapitulated in mice lacking one or more eicosanoid biosynthetic enzymes or receptors, including PGE_2 synthase (mPGES-1), PGE_2 receptors (EP1–4), LTC_4 synthase (LTC4S) and several leukotriene receptors, reinforcing the concept that $cPLA_2\alpha$ is a major and pathophysiologically relevant regulator of eicosanoid production [122].

Although $cPLA_2\alpha$ plays a central role in arachidonic acid release for eicosanoid production, its ablation does not fully suppress the latter [131]. Indeed, other PLA_2 enzymes, which likely act at different subcellular sites to mobilise their respective FA pools and stimulate eicosanoid production under distinct conditions, have been implicated in the process, including $cPLA_2\zeta$ (encoded by *PLA2G4F*) [132] and $iPLA_2\gamma$ (encoded by *PNPLA8*) [133]. Interestingly, $iPLA_2\gamma$ has recently been shown to provide 2-arachidonoyl lysophospholipids for the production of novel, previously unrecognized eicosanoid-lysolipids derived from direct lysophospholipid oxygenation by COX-2 [134]. The prototypical $iPLA_2$ enzyme, $iPLA_2\beta$ (PNPLA9; group VIA PLA_2; encoded by the *PLA2G6* gene), displays poor *sn*-2 acyl chain selectivity, but it may also contribute to eicosanoid production in certain situations and its involvement in the production of ω-3 PUFA-derived SPMs has been suggested [120,135,136].

Besides the intracellular enzymes, there is another important group of PLA_2s that participates in lipid mediator production, the $sPLA_2$ family [123,137]. $sPLA_2$s are secreted from immune and other cells and primarily act on extracellular substrates, such as lipoproteins, microparticles, bacteria and viruses, and the external leaflet of the plasma membrane of mammalian cells (Fig. 3B). In particular, the groups IID, IIF, III and X $sPLA_2$ enzymes (encoded by *PLA2G2D*, *PLA2G2F*, *PLA2G3* and *PLA2G10*, respectively) have shown a preference for the release of PUFAs, including arachidonic acid and ω-3 PUFAs, when acting on their respective target membranes in physiologically relevant contexts [122,138]. The group IID $sPLA_2$ is expressed in lymphoid dendritic cells and displays an anti-inflammatory role by releasing ω-3 PUFAs and promoting resolvin D1 production [139,140]. The group X $sPLA_2$ is the most potent mammalian $sPLA_2$ in hydrolysing phosphatidylcholine-rich membranes [66,122,123,141–143]. It releases a mixture of unsaturated FAs, including ω-3 PUFAs, from different cells *in vitro* and murine tissues *in vivo* and protects against inflammation, atherosclerosis and obesity [66,144–146]. In the lung, it affects allergen response and asthma by stimulating the production of cysteinyl leukotrienes [147]. In the intestines, the group X $sPLA_2$ modulates inflammation and tumourigenesis by regulating Wnt signalling and eicosanoid production [142,148]. Additionally, although the major physiological role of the most well-studied group IIA enzyme, the prototypical "inflammatory" $sPLA_2$, is now accepted to be anti-microbial defence, this $sPLA_2$ also acts on microparticles, including externalized mitochondria, thereby promoting the production of pro-inflammatory eicosanoids and exacerbating inflammation [149]. In summary, besides $cPLA_2\alpha$, at least several other intracellular $cPLA_2$ and $iPLA_2$ enzymes, as well as members of the primarily extracellularly-acting $sPLA_2$ family, contribute to the production of lipid mediators in various settings. Clearly, we are still far from understanding the complexity of the regulation and the variety of sources of PUFA precursor availability for lipid mediator production by different PLA_2s. Given that the biological functions of many PLA_2s are still unknown, and, as discussed in the following section, a number of PLA_2s have recently been associated with lipid droplet metabolism, it will not be surprising to discover that many of the PLA_2-mediated pathways of lipid mediator production also include lipid droplet turnover and various forms of trafficking between phospholipid and TAG pools.

4.3. PLA_2s and lipid droplet metabolism

Numerous PLA_2s have also been implicated in the regulation of lipid droplet metabolism [150]. $sPLA_2$s may provide FAs for TAG synthesis by hydrolysing lipoprotein phospholipids and the plasma membrane of different cells [143,151–155], while some intracellular PLA_2s, such as $iPLA_2\beta$ [156], may support TAG synthesis by releasing FAs from intracellular membranes. $cPLA_2\gamma$ (encoded by the *PLA2G4C* gene) promotes lipid droplet formation via its enzymatic activity in hepatocytes exposed to exogenous FAs and to hepatitis C virus infection [157]. $cPLA_2\alpha$ plays a regulatory role in the formation of lipid droplets in nutrient deprived cells, likely by regulating ER membrane curvature at the lipid droplet nucleation site [150,158,159]. $cPLA_2\alpha$ is also involved in lipid droplet accumulation in cell exposed to excess exogenous FAs and oxidized lipoproteins and during viral infection [150,160,161]. $cPLA_2\alpha$-deficient mice are resistant to high-fat diet induced hepatosteatosis, and have reduced levels of PGE_2, suggesting that $cPLA_2\alpha$-mediated eicosanoid production may be responsible for excessive lipid droplet biogenesis and liver damage [162,163]. These mice are also resistant to obesity when fed a high-fat diet, which may be associated with the proposed role of $cPLA_2\alpha$ in adipogenesis [164].

The group X $sPLA_2$ has also been implicated in the regulation of lipid droplet biogenesis, adipogenesis and other aspects of metabolism [66,122,143,146,150,165–168]. Its activity on breast cancer cells leads to the release of a mixture of unsaturated and polyunsaturated FAs, which are incorporated into newly formed lipid droplets that in turn provide long-term protection against nutrient and oxidative stress (Fig. 3B) [66,143]. The group X $sPLA_2$-induced lipid droplet biogenesis is also associated with the activation of metabolic and inflammatory pathways, including AMP-activated protein kinase (AMPK) and COX-2 signalling [143]. Interestingly, visceral adipose tissue inflammation has been associated with an elevated expression of the group X $sPLA_2$ and of eicosanoid synthesis enzymes, along with increased levels of arachidonic acid and long-chain PUFA-containing TAGs (PUFA-TAGs) [169]. It is thus possible that the enzyme is involved in the trafficking between membrane phospholipids and TAGs, thereby modulating the acyl composition of both lipid pools and controlling the availability of PUFAs for lipid mediator production. Intriguingly, the ability of the group X $sPLA_2$ to promote eicosanoid production in peripheral blood eosinophils has been associated with translocation of the enzyme

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to lipid droplets upon cell activation and with the activation of cPLA$_2\alpha$ [170]. Therefore, PLA$_2$s contribute to lipid droplet metabolism in numerous ways and play important roles as moderators of metabolic fluxes and lipid mediator signalling.

4.4. Endocannabinoids as sources of arachidonic acid for lipid mediator production

Endocannabinoids, such as 2-arachidonoylglycerol (2-AG), are a potent group of signalling lipids derived from membrane lipid precursors, which act through their cell surface cannabinoid (CB) receptors, affect various physiological processes and generally display anti-inflammatory properties [171]. The bioactive pool of endocannabinoids is regulated by their removal through hydrolysis by MAGL [21,114,118,172]. Intriguingly, the breakdown of the anti-inflammatory 2-AG by MAGL also regulates the cellular levels of free arachidonic acid available for the synthesis of pro-inflammatory eicosanoids. In fact, in the brain and several other tissues, MAGL, but not cPLA$_2\alpha$, was found to be responsible for the production of various bioactive lipids, including lysophospholipids and eicosanoids, thereby exacerbating inflammation and tumourigenesis [116–118]. Through this dual function, MAGL finely tunes the inflammatory response by simultaneously affecting two major lipid mediator pathways with opposing physiological roles. Intriguingly, a tissue-specific segregation of function was found for MAGL and cPLA$_2\alpha$, whereby the former plays a predominant role in eicosanoid production in the brain, liver and lung, whereas the latter seems to be important in the gut and spleen [117]. However, in the heart and kidney, neither enzyme was responsible for eicosanoid production, suggesting the involvement of other (phospho)lipases. Although MAGL is also the terminal enzyme in the TAG lipolysis pathway, current evidence does not support a role of TAGs as sources for 2-AG and other endocannabinoids, which are predominantly derived from membrane glycerophospholipids and extracellular lipoprotein lipolysis [6,21]. The major pathway for the production of 2-AG seems to be the sequential membrane phospholipid hydrolysis by phospholipase C (PLC) and DAG lipase (DAGL) (Fig. 3D) [21,173]. Accordingly, DAGLβ has been shown to promote eicosanoid production and complement the activity of cPLA$_2\alpha$ in immune cells [173,174]. Thus, the availability of arachidonic acid for the production of eicosanoids is controlled by distinct and complementary pathways involving various lipases and phospholipases, whose contribution to the process may be stimulus-, cell- and tissue-specific.

5. Lipid droplets as sources of lipid mediators in immune cells

5.1. Lipid droplets are formed during immune cell activation and maturation

Lipid droplets were observed by electron microscopy in immune cells already in the 1970s [175,176], but they first came into focus in 1983 when Dvorak et al. [177] studied lipid droplets in macrophages and mast cells. Later, they were also found in eosinophils, basophils, neutrophils and other immune cells under a variety of inflammatory conditions and diseases, including rheumatoid arthritis, atherosclerosis, allergies, lung disease, intestinal inflammation, obesity and cancer [27,69,104,178,179]. Lipid droplet biogenesis is rapidly induced in immune cells in response to various inflammatory stimuli and infection with viruses, bacteria and intracellular parasites [27–30,180–183]. Several major characteristics of lipid droplets, including number, size, lipid and protein composition are dynamically altered during immune cell activation and maturation [27–29,45,71,184]. The functions of lipid droplets in immune cells are expanding in recent years, ranging from energy supply for pathogen survival, viral assembly and interferon responses, to immunometabolic regulation, antigen cross-presentation, immune evasion and lipid mediator production [29,47,182,184–186].

5.2. Lipid droplets are often enriched with arachidonic acid and contain eicosanoid synthesis enzymes

Lipid droplets have long been known to dynamically respond to inflammatory stimuli, to store arachidonic acid, in both phospholipid and triglyceride pools, and harbour important elements of the eicosanoid synthesis machinery, thereby suggesting that they act as specialized sites for the synthesis of eicosanoids during the inflammatory response. The association between lipid droplets and arachidonic acid metabolism in immune cells was recognized early on, when exogenous radiolabelled arachidonic acid was found predominantly incorporated in lipid droplets in several types of immune cells and conditions [63,177,187]. A remodelling of arachidonic acid from phospholipid to triglyceride pools was observed in inflammatory cells migrating into tissue or reaching maturation, which suggested a dynamic, stimuli-dependent enrichment of lipid droplets with arachidonic acid that is likely important for lipid mediator production in activated inflammatory cells [45]. The emerging hypothesis that lipid droplets participate in the production of eicosanoids [46,188] was strengthened with reports suggesting that numerous enzymes involved in eicosanoid production, including cPLA$_2\alpha$ and several COXs and LOXs, localize to lipid droplets in macrophages and other immune, epithelial and cancer cells [47,178,179,189–192]. Immunolocalization of endogenous cPLA$_2\alpha$ and visualization of ectopically expressed cPLA$_2\alpha$ fused with fluorescent proteins have suggested that the protein is dynamically translocated to lipid droplets following stimulation with ionophores, arachidonic acid or other unsaturated FAs, but also that some cancer cells contain lipid droplet-localized cPLA$_2\alpha$ under unstimulated conditions [189,191,192]. *In situ* immunolocalization of PGE$_2$ and other eicosanoids further suggested that lipid droplets are sites of intracellular eicosanoid production in immune and other cell types (reviewed in Ref. [47]).

5.3. Is the lipid droplet phospholipid pool a source of arachidonic acid?

These studies indicated, on the basis of inhibitor and localization studies, that cPLA$_2\alpha$ is likely responsible for the release of arachidonic acid from lipid droplet phospholipid pools (Fig. 3A). However, the ability of cPLA$_2\alpha$ to hydrolyse phospholipid molecules at the lipid droplet surface has not been demonstrated yet. In this scenario, triglycerides could serve merely as reservoirs for replenishment of the phospholipid pool with arachidonic acid [47]. The enrichment of triglycerides with arachidonic acid in immune cells has also been suggested to serve as a sink to limit the phospholipid-resident pool of arachidonic acid in order to reduce the inflammatory response [45,63,64]. However, the enzymatic pathways and regulatory mechanisms governing the remodelling of the phospholipid and triglyceride pools have not been identified and the possible functional consequences of lipid mediator production at the lipid droplet surface have not been delineated. Moreover, given that cPLA$_2\alpha$ and other cPLA$_2$ isoforms, such as cPLA$_2\gamma$, are also involved in lipid droplet biogenesis, their localization to lipid droplets may also be associated with other functions not related to eicosanoid production [157,158]. A series of studies have shown that the activation of cPLA$_2\alpha$ is necessary for lipid droplet biogenesis in stressed cells, whereby it most likely participates in the remodelling of the ER phospholipid membrane to facilitate lipid droplet expansion [150,156,158,180]. It is also possible that the translocation of cPLA$_2\alpha$ to the lipid droplet surface and its involvement in phospholipid deacylation/reacylation reactions enables its participation in both lipid droplet biogenesis and arachidonic release for lipid mediator production [150]. Finally, the contribution of the triglyceride pool and neutral lipases to eicosanoid production has not been investigated until recently, as described in the next section.

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5.4. ATGL-mediated lipolysis drives eicosanoid production in mast cells

In comparison with various circulating inflammatory cells (monocytes, neutrophils, eosinophils and platelets), mast cells, along with macrophages, contain a large portion of their arachidonic acid esterified in the TAG lipid droplet pool [45]. Mast cells are known as major effector cells in the acute allergic immune response, but are also involved in tissue remodelling and long-term changes that accompany chronic inflammatory conditions, including asthma, rheumatoid arthritis, obesity, atherosclerosis and cancer [193]. Along with dendritic cells, they form the primary defence line close to external body surfaces and guard against invading pathogens and allergens. Their immature precursors, derived from hematopoietic stem cells, circulate the blood and undergo terminal differentiation upon migration to target peripheral tissues. Mast cells reside in tissues in their quiescent form and store secretory granules filled with histamine, cytokines and growth factors. These are released within seconds of cell activation, which is most commonly triggered by antigen binding to IgE and cross-linking of its cell surface receptor FcεRI, and may promote or reduce inflammation and affect tissue function in a variety of ways [193] Activated mast cells also initiate *de novo* synthesis of lipid mediators, including eicosanoids, which requires a regulated supply of precursors and activation of the synthesis machinery. Eicosanoids may be released in the immediate or delayed phase of the response to further modulate the allergic and inflammatory reaction. The most common bioactive lipid mediators in mast cells include prostaglandin D_2, leukotrienes B_4 and C_4, which complement and even significantly surpass the potency of histamine under certain conditions [194].

During maturation of human peripheral blood-derived $CD34^+$ progenitors into connective-tissue mast cells, the number and size of lipid droplets increases significantly [28]. The addition of exogenous arachidonic acid further elevates lipid droplet levels and they incorporate the exogenous FA predominantly into their TAG pool. Dichlberger et al. [40] analysed the contribution of TAG lipolysis to the production of eicosanoids in activated human primary mast cells. Silencing of ATGL led to lipid droplet accumulation and a significant reduction in the production of prostaglandin D_2 and leukotriene C_4 in the acute phase of the response immediately following cell activation, suggesting that the enzyme provides TAG-derived FAs for downstream oxidative reactions by COX and LOX enzymes. Interestingly, this activity was reduced to similar levels by silencing either ATGL, cPLA$_2\alpha$, or both, suggesting that both enzymes contribute to eicosanoid production [40]. This also indicates that the role of the TAG pool as a source of arachidonic acid is of a comparatively similar importance as the various phospholipid pools that may be targeted by cPLA$_2\alpha$. It also opens the possibility that ATGL and cPLA$_2\alpha$ cooperate in the provision of arachidonic acid in various ways (Fig. 3). Arachidonic acid could be first released from TAGs by ATGL and 1) directly used by COX/LOX enzymes or 2) reacylated into phospholipids, either at the lipid droplet monolayer or other membranes, only to be subsequently released by cPLA$_2\alpha$ [194]. Both enzymes may also work independently on their own respective target lipids and feeding arachidonic acid to their respective proximal eicosanoid synthesis machineries. The role of the TAG pool in eicosanoid production as a direct source or as a reservoir of precursors for phospholipid reacylation in immune (and other) cells has yet to be firmly established.

5.5. ATGL enables eicosanoid production in neutrophils

Moving closer to understanding the role of TAGs as sources of precursors for lipid mediator production, a recent study by Schlager et al. [41] employed both global and myeloid-specific ATGL-deficient mice to study the role of TAG lipolysis in immune cell function and eicosanoid production. They found that ATGL-deficient mice display a pronounced increase in lipid droplet accumulation in neutrophils isolated from peripheral blood, bone marrow and naive lavages. Under inflammatory conditions, there was also an increased abundance of lipid droplets and elevated TAG accumulation in neutrophils, macrophages and monocytes isolated from peritoneal lavages of ATGL-deficient mice. ATGL is thus important for TAG lipolysis in several types of blood and tissue-resident immune cell types in mice. Furthermore, ATGL-deficient neutrophils contained increased levels of PUFA-containing TAGs, whereas the levels of TAG species containing predominantly saturated and monounsaturated FAs were reduced. Notably, pharmacological inhibition of ATGL by Atglistatin in neutrophils collected under inflammatory conditions from wild-type mice, resulted in a significantly reduced release of several PUFAs, including linoleic and arachidonic acid, and a reduced production of a number of lipid mediators derived from COX, LOX and cytochrome P450 pathways. Importantly, the impact of ATGL deficiency on eicosanoid production was also shown *in vivo*. Namely, peritoneal exudates collected under inflammatory conditions from ATGL-deficient mice contained reduced levels of most major PUFA species and of eicosanoids derived from all three enzymatic pathways in comparison with wild-type littermates. Although this study did not address the involvement of PLA$_2$s in eicosanoid production, the fact that ATGL-deficient neutrophils contained elevated levels of PUFA-TAG species, while no significant changes in PUFA acyl chain composition in phospholipids were observed, speaks in favour of a direct ATGL-mediated provision of TAG-derived PUFAs into eicosanoid production pathways [41]. These results suggest that ATGL-mediated TAG hydrolysis is a major and physiologically relevant source of arachidonic acid and other PUFAs that feed into all three enzymatic pathways of lipid mediator synthesis in murine neutrophils and probably other immune cells [41]. Nevertheless, the possibility that phospholipid pools along with cPLA$_2\alpha$ (or other PLA$_2$ isoforms) complement or contribute to the ATGL-mediated precursor release for eicosanoid production still remains andand requires further examination. Interestingly, Schlager et al. [41] observed that not all lipid mediators were affected by inhibition or depletion of ATGL, suggesting that these lipid mediators are produced in an ATGL-independent manner, likely from other subcellular compartments. It could also be envisaged that the relative importance of the PUFA-TAG pool and ATGL in lipid mediator production depends on the cell type and state, specific environmental conditions and the tissue in question.

5.6. Lysosomal lipolysis is a source of fatty acids for eicosanoid production

Lysosomal neutral lipid lipolysis has been recently found to drive the synthesis of lipid mediators in immune cells. Neutral lipid hydrolysis by LAL is involved in the catabolism of endocytosed lipoproteins and in the breakdown of lipid droplets by lipophagy (Fig. 3C) [24]. LAL displays a broad substrate specificity and it hydrolyses various neutral lipids, including TAGs, DAGs and cholesteryl esters. Its deficiency leads to severe lysosomal storage disorders in humans associated with elevated accumulation of lipids in multiple tissues, dyslipidemia, liver dysfunction and atherosclerosis. LAL-deficient mice accumulate neutral lipids in various immune cells under basal and inflammatory conditions [48]. Macrophages depleted in LAL were found to accumulate neutral lipids within lysosomes, predominantly cholesteryl esters, which were enriched with arachidonic and linoleic acids, both precursors of pro-inflammatory lipid mediators [48]. The levels and lipidomic profile of triglycerides, activities of neutral lipases and expression of the lipid droplet marker perilipin 2 remained unchanged, suggesting that LAL-deficiency does not affect triglyceride and lipid droplet metabolism under these conditions. Importantly, specific pharmacological inhibition of LAL in wild-type macrophages reduced the release of numerous lipid mediators derived from COX, LOX and CYP biosyn-

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thetic pathways, without affecting neutral TAG or cholesteryl ester hydrolase activity in the cells [48]. Thus, lysosomal cholesteryl ester catabolism by LAL is an additional source of precursors for bioactive lipid synthesis in macrophages. It remains to be investigated whether lysosomal lipolysis contributes to lipid mediator generation in other immune and non-immune cells and to examine the relative contributions of the endosomal vs. autophagosomal pathways of lipid delivery to lysosomes in the process. It will be interesting to find out whether lipophagy has a role in supplying neutral lipids for LAL-mediated eicosanoid production under certain conditions.

6. Lipid droplets and inflammatory lipid mediators in adipose tissue

6.1. Lipid mediators and inflammation in adipose tissue

Lipid droplet metabolism, lipid mediators and inflammatory signalling are also intricately linked in adipose tissue. This is particularly important in obesity, which has been characterized as a low-grade chronic inflammatory state fuelled by the continuous nutrient surplus and metabolic imbalance [35,36]. This chronic state of inflammation is initiated by metabolic cells, which release increasing levels of inflammatory cytokines that regulate both inflammatory responses and metabolic processes, and is further amplified and sustained in the long-term by the recruitment of immune cells and their impaired clearance. Besides the release of cytokines and adipokines, adipose tissue has long been recognized for its ability to generate bioactive lipid mediators that affect both metabolic and immune function [35,36,39,195]. In fact, being derived from nutrient FA precursors that are frequently dysregulated in obesity and other metabolic diseases, lipid mediators are likely essential for the development of chronic inflammation. Elevated saturated FA concentrations in obesity lead to activation of multiple inflammatory signalling pathways, including the production of prostaglandins, which promote immune cell recruitment and function, but they also disrupt neutrophil clearance and macrophage phagocytosis thereby impairing the resolution of inflammation [36,196,197]. Eicosanoids affect major aspects of adipose tissue biology by overstimulating or inhibiting their cognate cell surface GPCRs, TLRs and nuclear PPAR receptors [35,198]. Eicosanoid production via the COX-2 pathway has been implicated in adipogenesis, cold-induced browning, adipose tissue inflammation, obesity and insulin resistance [199–202]. Lipid mediators generated by the 5-LOX and 12/15-LOX pathways also play an important role in adipose tissue inflammation and insulin resistance associated with obesity [197,203,204]. In addition to the elevated and continued generation of pro-inflammatory lipid mediators driven by nutrient excess in obese adipose tissue, the low-grade chronic inflammatory state is also perpetuated by the decreased synthesis of anti-inflammatory and pro-resolving lipid mediators leading to a reduced resolution capacity [197,205].

6.2. Lipid mediators affect lipid droplet metabolism in adipose tissue

Although it is clear that PUFAs and their oxygenated metabolites are important for both metabolic and inflammatory aspects of adipose tissue biology, there are many contrasting reports and controversy regarding the effects of particular lipid mediator species/pathways on lipogenesis and lipolysis [35,206–209]. Arachidonic acid has been shown to inhibit adipogenesis *in vitro* via COX-dependent prostaglandin signalling leading to suppression of *de novo* FA synthesis and DGAT activity [208]. However, arachidonic acid signalling has also been suggested to promote adipogenesis [210] and restrict the phenotypic conversion of white into brite adipocytes [207]. Moreover, the prototypical arachidonic acid-releasing enzyme cPLA$_2\alpha$ is involved in early adipocyte differentiation [164]. The adipose-specific group XVI PLA$_2$ (also called AdPLA and PLA2G16; encoded by *PLA2G16*) regulates adipose tissue lipolysis through the production of PGE$_2$ [209]. The deletion of AdPLA$_2$ in mice led to a reduction in adipose PGE$_2$ levels, an increase in lipolysis, fat oxidation and resistance to diet-induced obesity. The increase in lipolysis was dependent on elevated levels of cAMP and phosphorylation of HSL, suggesting that PGE$_2$ inhibits lipolysis by reducing signalling through the cAMP/PKA/HSL cascade (Fig. 4) [209]. Among the four PGE$_2$ receptors, EP2 and EP4 activate cAMP signalling via Gα_s protein-coupled receptor signalling pathways, while the EP3 receptor, which is the most abundant EP receptor in adipose tissue, primarily acts via Gα_i-proteins to inhibit adenylyl cyclase and reduce cAMP levels. A specific antagonist of EP3 inhibited the antilipolytic effect of PGE$_2$, suggesting that PGE$_2$ acts via the inhibitory Gα_i-coupled EP3 receptor to decrease cAMP levels and inhibit lipolysis [209]. Intriguingly, studies in EP3-deficient mice suggest that the EP3 receptor may regulate both adipogenesis and lipolysis in adipose tissue [201]. The lack of EP3 signalling was associated with the facilitation of adipogenesis via cAMP/PKA-dependent activation of the major adipogenic transcription factor PPAR-γ and stimulation of lipolysis via the cAMP/PKA/HSL pathway [201]. Interestingly, the prostaglandin EP4 receptor has recently been implicated in the regulation of lipid droplet remodelling and mitochondrial metabolism in subcutaneous white adipose tissue [211]. Its depletion in mice resulted in the appearance of smaller, multilocular lipid droplets and elevated mitochondrial biogenesis and respiration following β-adrenergic stimulation. These effects were independent of uncoupling protein 1 (UCP1), but were rather associated with elevated AMPK signalling and reduced expression of fat-specific protein of 27 kDa (FSP27; also called CIDEC), which is essential for lipid droplet growth and fusion [212]. This study thus suggests that prostaglandin signaling via EP4 maintains lipid droplet size, reduces TAG remodelling and mitochondrial activity by suppressing AMPK-dependent FSP27 protein clearance in white adipose tissue (Fig. 4) [211]. Clearly, the pathways of bioactive lipid-mediated regulation of lipid droplet metabolism, adipogenesis, inflammation and obesity require further investigation.

6.3. Adipose tissue lipolysis regulates local and distant inflammation

The findings presented above suggest the importance of eicosanoid signalling for adipose tissue function, but do not reveal much regarding the sources and regulation of their production and, in particular, how elevated lipid accumulation leads to increased lipid mediator production. In fact, despite the well-accepted concept that obesity-related inflammation is initiated by the dysregulated function of metabolic cells [35,213], we know surprisingly little about the mechanisms promoting bioactive lipid mediator production and the possibility that lipid droplets are sources of lipid mediators in these cells. Recent studies have reinforced the idea that lipolysis promotes inflammation and have also provided evidence that it stimulates the production of bioactive lipid mediators in adipose tissue [42,89,214,215]. Adipose tissue lipolysis by both ATGL and HSL has been implicated in the activation of inflammatory pathways in adipose and other tissues. For instance, acute activation of β-adrenergic signalling triggers the expression of pro-inflammatory genes, including IL-6, through a HSL-dependent activation of sphingosine kinase 1 and JNK signalling [214,215]. Since adipose tissue ATGL regulates the provision of FAs for other tissues, dysregulation of its activity may affect both local and distant tissue inflammation. In accordance, ATGL activity is elevated in inflamed adipose tissue and strongly affects hepatic insulin resistance and inflammation through the regulation of hepatic acetyl-CoA levels [216]. Moreover, adipocyte-specific deletion of ATGL protects against hepatic inflammation and insulin resistance, and reduces immune cell recruitment in adipose tissue; however, it does not ameliorate chronic adipose tissue inflammation due to diet-induced obesity [89,217,218]. On the other hand, pharmacological inhibition of ATGL in mice, which

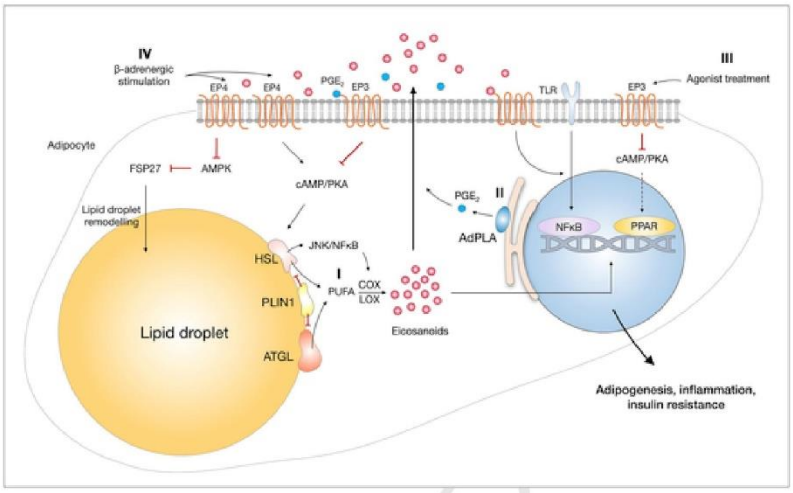

Fig. 4. Adipose ATGL/HSL lipolytic activity drives lipid mediator production and inflammatory signalling. (I) In adipocytes, emerging evidence suggests that adipose triglyceride lipase (ATGL)- and hormone sensitive lipase (HSL)-mediated lipolysis are both involved in the production of lipid mediators. ATGL and HSL likely provide precursors for eicosanoid synthesis by releasing polyunsaturated fatty acids (PUFAs) from lipid droplets. The lipid droplet protein perilipin 1 (PLIN1) limits the production of eicosanoids by negatively regulating ATGL- and HSL-mediated lipolysis. HSL activity may also activate the *c*-Jun N-terminal kinase (JNK)/nuclear factor κB (NF-κB) inflammatory pathway leading to elevated expression of cyclooxygenase-2 (COX-2) and prostaglandin E_2 (PGE_2) production. Eicosanoids are released from the cell and bind to cell surface receptors in autocrine and paracrine manner or directly bind to peroxisome proliferator-activated receptor (PPAR) transcription factors. Elevated eicosanoid signalling in adipocytes through their cognate cell surface G protein-coupled receptors (GPCRs), such as the PGE_2 receptors 3 and 4 (EP3/4), or through their effects on Toll-like receptor (TLR) signalling, leads to overstimulation or inhibition of major downstream signalling pathways, including those mediated by AMP-activated protein kinase (AMPK), protein kinase A (PKA) and the NF-κB transcription factor, which together with PPAR receptors modulate lipid droplet metabolism, adipogenesis, inflammatory responses and insulin resistance. (II) The adipose-specific group XVI phospholipase A_2 ($AdPLA_2$) modulates lipolysis through the production of PGE_2, which acts via its EP3 receptor to reduce cAMP/PKA signalling and inhibit HSL-mediated lipolysis. (III) EP3 receptors, which signal via inhibitory $G\alpha_i$ proteins, may also supress adipogenesis by reducing cAMP/PKA/PPAR-γ signalling. (IV) In cells exposed to β-adrenergic stimulation, EP4 receptors, which typically act via $G\alpha_s$ proteins to increase cAMP levels, supress AMPK-mediated clearance of fat-specific protein 27 (FSP27; CIDEC), thereby leading to lipid droplet remodelling and elevated mitochondrial oxidative metabolism. LOX, lipoxygenase.

likely targets both adipocyte and immune cell ATGL, leads to reduced FA release, inflammatory marker gene expression and cytokine production in adipose tissue [94]. In line with the possibility that some of these effects are mediated by inhibition of ATGL in immune cells, deletion of ATGL in macrophages restricts the ATP supply for efficient phagocytosis, leads to reduced expression of pro-inflammatory cytokines and impairs cell migration, thereby effectively reducing the immune response [219,220]. Since ATGL integrates numerous metabolic processes and signalling pathways that affect metabolism, inflammation and immunity in a variety of cells, its role as a promotor or suppressor of inflammation in particular pathophysiological contexts likely depends on the balance between its expression and activity in multiple cell types and tissues.

6.4. ATGL/HSL lipolytic activity drives lipid mediator production in adipocytes

Apart from several recent studies, the potential connection between lipolysis and the production of bioactive lipid mediators in adipose tissue has been poorly investigated. Stimulation of cAMP-mediated lipolysis in adipocytes leads to arachidonic acid release, $cPLA_2\alpha$ activation and production of PGE_2, which has been suggested to promote the accumulation of anti-inflammatory macrophages in adipose tissue of fasted mice [221]. Conditioned media from adipocytes induced macrophage migration, which was blocked by inhibition of COX-2 and an EP4 antagonist. However, this study did not address the possibility that TAG lipolysis may be the source of arachidonic acid for the production of PGE_2. More recently, it was shown in 3T3-L1 adipocytes that β-adrenergic stimulation of lipolysis activates JNK/NF-κB signalling leading to upregulation of COX-2 expression, elevated cytokine and eicosanoid synthesis [42]. Interestingly, the observed increase in the production of numerous eicosanoid species, including COX, LOX and epoxygenase products, was abrogated in cells pre-treated with a selective HSL inhibitor. Pharmacological inhibition of HSL also prevented the elevation of COX-2 activity in response to β-adrenergic stimulation *in vivo*, suggesting that lipolysis activates COX-2-mediated eicosanoid production. Furthermore, COX-2 inhibitors reduced macrophage/monocyte cell infiltration into adipose tissue under lipolytic conditions, indicating that its products may be involved in immune cell recruitment and exacerbation of inflammation [42]. This study thus suggests that HSL-mediated lipolysis provides signals and/or FA precursors for lipid mediator production and provoke an inflammatory response in adipose tissue. Recently, the regulation of lipolysis by perilipins has been implicated in adipose tissue inflammation. Perilipin 1 (PLIN1) is a lipid droplet-coating protein highly expressed in adipocytes and one of the principal negative regulators of ATGL activity in these cells [24]. A recent study using PLIN1-deficient mice has shown that its ability to restrict lipolysis is required to limit the production of pro-inflammatory eicosanoids, reduce macrophage infiltration and prevent the development of insulin resistance in adipose tissue [43]. The effect was dependent on ATGL- and HSL-mediated lipolysis and COX-2 activity. Intriguingly, adipose tissue inflammation in obesity has been recently associated with the activation of autophagy leading to selective degradation of PLIN1 and elevated lipolysis [222]. These studies suggest a positive feedback regulatory loop between adipose tissue inflammation and ATGL/HSL lipolysis, whereby elevated cytokine production in

obese adipose tissue leads to increased PLIN1 clearance and elevated lipid mobilization, which in turn promotes the production of eicosanoids that may further exacerbate inflammation. Therefore, adipose tissue lipolysis via both ATGL and HSL provides signals or precursors for the production of bioactive lipid mediators that modulate adipose tissue inflammation. The nature of these signals and precursors and the molecular mechanisms involved remain to be discovered in future studies.

7. Lipolysis contributes to the production of eicosanoids in endothelial cells

Eicosanoids have an important role in the regulation of vascular physiology, inflammation and atherogenesis [33,223]. Recent studies have revealed that lipid droplets are actively involved in the regulation of FA metabolism and transport in endothelial cells [224]. It was shown that endothelial lipid droplet turnover is dynamically regulated by DGAT1 and ATGL to control postprandial nutrient metabolism, reduce lipotoxicity and provide FAs to neighbouring cells [224]. In accordance, mice deficient in ATGL display a severe endothelial dysfunction [225]. Silencing of the *PNPLA2* gene in human aortic endothelial cells exacerbates inflammatory signalling and induces monocyte recruitment, suggesting that ATGL activity may be necessary for anti-inflammatory signalling and prevention of atherogenesis [226]. Namely, ATGL loss led to an increase in TNFα-induced expression of intercellular adhesion molecule-1 (ICAM-1), which was associated with elevated levels of NF-κB and PKC signalling. Recently, endothelial ATGL activity has been linked to the production of prostaglandin I_2 (PGI_2, or prostacyclin), which is the principal anti-inflammatory and anti-thrombotic product of arachidonic acid metabolism in the endothelium [44,227]. Endothelial cells deficient in caveolin 1 (Cav-1), a caveolae coat protein that has been implicated in lipid droplet metabolism, display increased lipid droplet breakdown, which was attributed to elevated cAMP/protein kinase A signalling, HSL phosphorylation and enhanced lipolysis [44]. Atglistatin restored lipid droplet levels, suggesting that enhanced ATGL/HSL-mediated lipolysis is responsible for the observed elevated lipid droplet breakdown. Interestingly, Cav-1-depleted endothelial cells also produced more PGI_2, which acted in an autocrine manner through its cognate GPCR to stimulate cAMP-mediated lipolysis, indicating that Cav-1 deficiency upregulates lipolysis at least in part via the production of PGI_2. Furthermore, a recent analysis of the effects of ATGL silencing on the subcellular distribution of radiolabelled arachidonic acid in human aortic endothelial cells found a decrease in the amounts of arachidonic acid in phospholipid pools, which coincided with a decrease in the production and release of PGI_2 [227]. This suggests that ATGL-mediated TAG lipolysis provides arachidonic acid for membrane phospholipid synthesis and PGI_2 production in endothelial cells. Thus, ATGL may be involved in an autocrine feedback regulatory mechanisms that includes ATGL-mediated PGI_2 production that in turn affects cAMP-mediated regulation of lipolysis in endothelial cells. It still remains to be determined whether endothelial ATGL contributes to eicosanoid synthesis by providing TAG-derived arachidonic acid directly to COX/LOX enzymes or instead acts to replenish phospholipid pools for subsequent release by PLA_2s, such as $cPLA_2\alpha$ [228].

8. Conclusions

Despite the well-accepted concept that elevated lipid accumulation and chronic inflammation in adipose and non-adipose tissues are hallmarks of many metabolic disorders and diseases, we know surprisingly little about the mechanisms that connect neutral lipids with the production of inflammatory lipid mediators. Recent studies have reinforced the idea that lipolysis modulates inflammation at least in part via the production of bioactive lipid mediators in several tissues, but much remains to be elucidated in future studies. We are only beginning to unveil the contribution of lipid droplet-associated lipases and the neutral lipid pool to the regulation of the availability of FA precursors for lipid mediator production. The potential roles of neutral lipids in lipid mediator production as a direct source or as a reservoir of precursors for phospholipid reacylation has to be established. Emerging studies suggest that depending on the stimuli, cell and tissue type in question numerous phospholipases and lipases acting on different cellular compartments may contribute to lipid mediator production. Currently, we know very little about the possible cooperative and/or complementary roles of lipid droplet-associated lipases and phospholipases in lipid droplet function and lipid mediator production. The involvement of an increasing number of phospholipase A_2 enzymes in the generation of lipid mediators and their emerging roles in lipid droplet metabolism provides exciting prospects for new discoveries integrating metabolism, inflammation and immunity. Moreover, it will be fascinating to discover how lipid droplets fit into the bigger picture of the regulation of FA trafficking and lipid mediator production in different cells and tissues. Future studies will provide answers to whether and how different lipid sources that drive lipid droplet biogenesis (e.g., exogenous lipid uptake, *de novo* synthesis, autophagy) contribute to lipid droplet-dependent lipid mediator generation. They will also uncover the mechanisms and conditions in which lipid droplet biogenesis serves as a sequestration mechanism that disrupts cellular signalling pathways. Furthermore, it remains to be understood how the regulation of lipolysis at the cellular level and at the lipid droplet surface is coordinated with lipolytic signalling and lipid mediator production and whether lipid droplet breakdown via lipophagy also contributes to the process. It is also paramount to aim at elucidating how the dynamic lipid composition and protein ensemble of these organelles affects their function in the context of various cellular stress responses. The pleiotropic roles of lipid droplets in managing the trafficking of specific lipid species, cellular stress mechanisms and inflammatory signalling will surely expand in the future as new functions and mechanistic details are elucidated. Their roles in immune cell function and inflammatory signalling in various tissues and in different pathologies will be an exciting field of discovery that may help us understand, prevent and fight disease.

Authors' contributions

E.J. and T.P. performed literature research and wrote manuscript draft; E.J. performed fact checking and prepared figures; T.P. conceptualized the study, finalized figures and manuscript.

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Publications Related to the Thesis

Journal Articles

Jarc, E., Kump, A., Malavašič, P., Eichmann, T. O., Zimmermann, R., & Petan, T. (2018). Lipid droplets induced by secreted phospholipase A_2 and unsaturated fatty acids protect breast cancer cells from nutrient and lipotoxic stress. *Biochimica et Biophysica Acta–Molecular and Cell biology of Lipids, 1863* (3): 247–265.

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Selected Conference Contributions

Jarc, E., Kump, A., Guštin, E., Pucer Janež, A., Brglez, V., Eichmann, T. O., Zimmermann, R., Gerstmeier, J., Werz, O., & Petan, T. (2019) Lipid droplets: fatty managers of stress and inflammation. In: Kisovec, M., (ed.) *Book of abstracts, 13th Meeting of Slovenian Biochemical Society with International participation*, 24–27 September 2019, Dobrna, p17. (invited lecture)

Jarc, E., Kump, A., Malavašič, P., Eichmann, T. O., Zimmermann, R., & Petan, T. (2018) Lipid droplets balance fatty acid trafficking with cell survival mechanisms and protect cancer cells from nutrient and lipotoxic stress. In: *Biogenesis and fate of lipid droplets*. 14th GERLI Lipidomics Meeting – Gerli 40th Anniversary, September 30th–October 3rd, St. Maxim la Saint-Baume, p. 40. (invited lecture)

Jarc, E., Kump, A., Malavašič, P., Pucer Janež, A., Brglez, V., Eichmann, T. O., Zimmermann, R., & Petan, T. (2018) Lipid droplets integrate metabolic and signalling pathways and are indispensable for the cellular stress response in breast cancer cells. In: *Book of abstracts. 7th European Workshop on Lipid Mediators*, September 12th–14th, Brussels, p. 40. (oral presentation)

Jarc, E., Kump, A., Malavašič, P., Pucer Janež, A., Brglez, V., Eichmann, T. O., Zimmermann, R., & Petan, T. (2018). Lipid droplets–key organelles in the protection of breast cancer cells against nutrient stress. In: *Lipid dynamics and membrane contact sites: FEBS Advanced Course 2018*, August 31–September 7, Island of Spetses, p.49. (poster presentation)

Jarc, E., Kump, A., Malavašič, P., Pucer Janež, A., Brglez, V., Eichmann, T. O., Zimmermann, R., & Petan, T. (2017) Lipid droplets are involved in eicosanoid generation and protection against nutrient stress in breast cancer cells. In: Goričar, K., (ed.), Hudler, P., (ed.). *Book of abstracts, 12th Meeting of Slovenian Biochemical Society with International participation*, September 20–23 September, Bled, p.78. (awarded poster presentation)

Biography

The author of this thesis Eva Jarc Jovičić was born on June 14, 1990 in Ljubljana, Slovenia. She finished her primary education in Kamnik and secondary education at the Gimnazija Poljane in Ljubljana. In 2012, she graduated at the Faculty of Pharmacy, University of Ljubljana and obtained her first BSc in Laboratory Biomedicine. She was awarded by the faculty for excellent grades during her BSc study. Three years later, she obtained her MSc in Laboratory Biomedicine at the Faculty of Pharmacy, University of Ljubljana. She was awarded again with a financial award by the faculty for excellent grades during her MSc study. In 2015, she was selected as a PhD candidate at the Jožef Stefan Institute at the Department of Molecular and Biomedical Sciences under the supervision of Assist. Prof. Dr. Toni Petan. She attended her PhD studies at the Jožef Stefan International Postgraduate School, Ljubljana. In 2016, she obtained her second BSc in Nursing at the Faculty of Health Sciences, University of Ljubljana. Her PhD and ongoing work is focused on lipid metabolism in cancer cells, in particular on lipid droplets. For her work, she uses *in vitro* cancer cell lines and several biochemical and molecular methods that enable her to change specific metabolic processes in cancer cells and follow subsequent consequences on cancer cell survival and growth. In collaboration with Dr. Thomas O. Eichmann and Prof. Dr. Robert Zimmermann from the University of Graz, Austria, and Dr. Jana Gerstmeier and Prof. Dr. Oliver Werz from the University of Jena, Germany, she performs state-of the art technology of lipid detection with UPLC/MS-based analysis, i.e. lipidomics. These collaborations are extremely important for studying biology of lipids in cancer and elsewhere, since lipidomic facility and methodology are still unavailable in Slovenia. During her PhD, she attended several conferences and workshops in Slovenia and abroad, and obtained two scholarships for their attendance. She is the first author of four original scientific papers and one review paper, and co-author of an original scientific paper and a review paper.