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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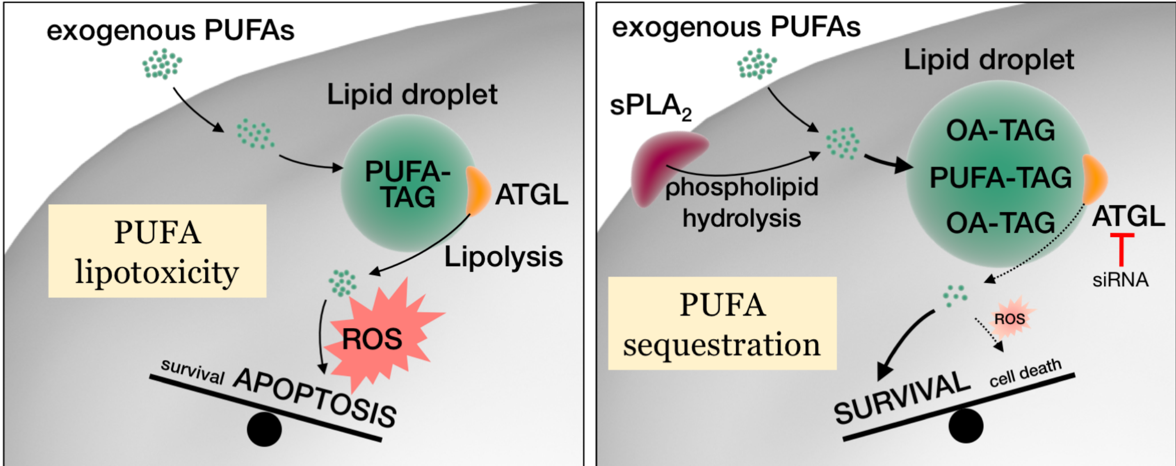
Abbreviations: AA, arachidonic acid; AMPK, AMP-dependent protein kinase; ATGL, adipose trygliceride lipase; cPLA$_2$α, cytosolic group IVA PLA$_2$; CPT1, carnitine *O*-palmitoyltransferase 1; CPT1A, the liver isoform of CPT1; DGAT1, diacylglycerol acyltransferase 1; DHA, docosahexaenoic acid; EPA, eicosapentaenoic 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.

Highlights

- PUFAs can both promote or reduce the survival of starved triple-negative breast cancer cells
- $sPLA_2$ augments PUFA-induced lipid droplet biogenesis and reduces PUFA lipotoxicity
- Suppression of lipolysis by ATGL depletion protects against PUFA lipotoxicity
- Sequestration of PUFAs in lipid droplets protects from oxidative stress and cell death
- Lipid droplets reduce nutrient and lipotoxic stress by balancing PUFA storage and use

Graphical abstract



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.

Keywords: unsaturated fatty acids, secreted phospholipase A_2, lipid droplets, adipose triglyceride lipase, breast cancer, lipotoxicity.

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 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 though 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 ºC. 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 (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 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 (1:10000 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 x 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 normalisation. 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 x 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 analyzed by selected reaction monitoring (FA: [M-H]- to [M-H]-, 0 eV). Data acquisition was done by MS Workstation (Bruker, USA). Data were normalized 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 (1000 *g*, 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 1,000 x *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 disolved 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 x 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 analyzed with the “Lipid Data Analyzer 1.6.2” software [57]. Data were normalized 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 300 *g* 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 (Figs. 1B and 1C 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 (Figs. 1D and 1E). 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. Figs. 1D and 1E). 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 more than 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 $sPLA_2$.

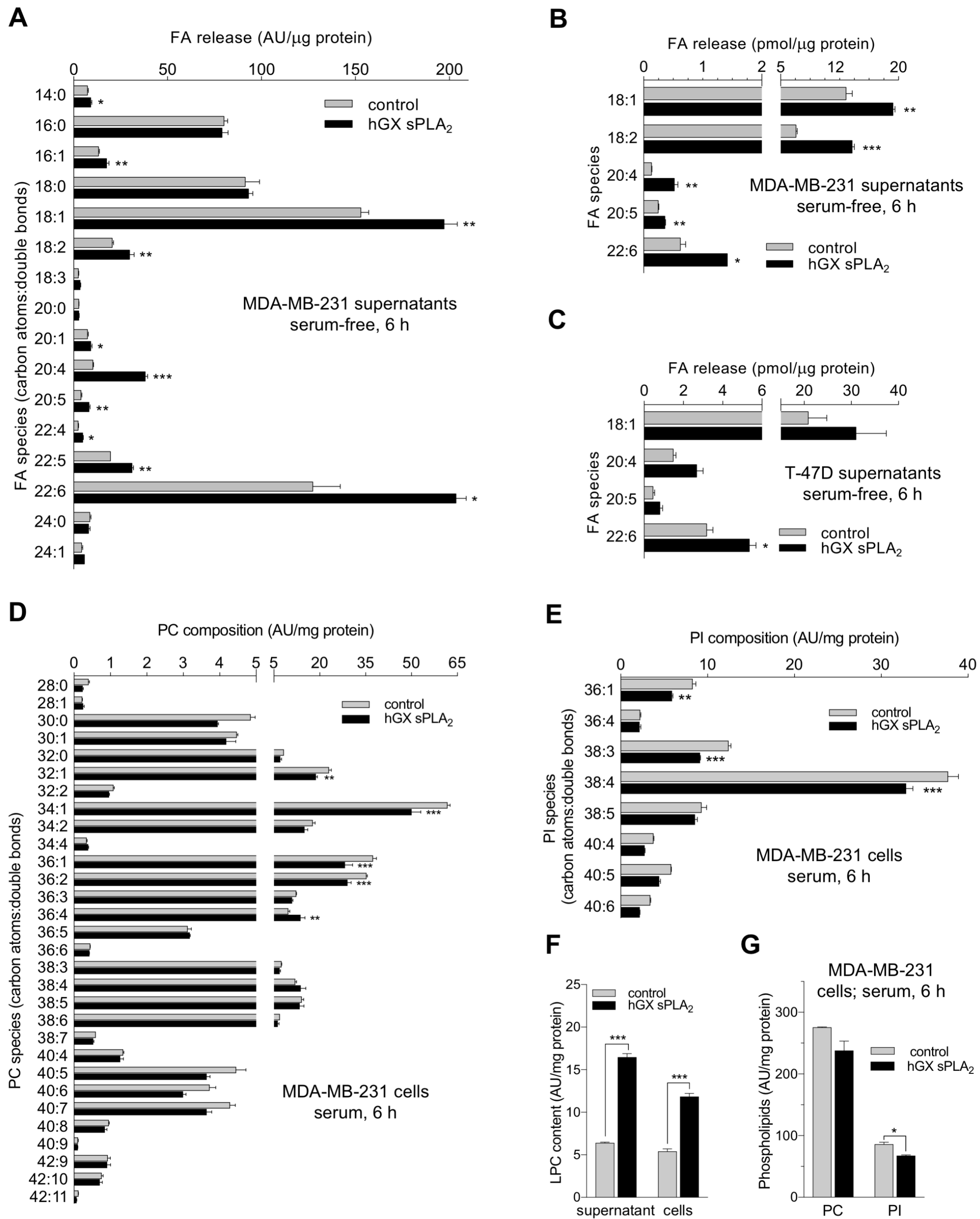


Figure 1. hGX sPLA$_2$ releases unsaturated FAs, including ω-3 and ω-6 PUFAs, from breast cancer cells. MDA-MB-231 (A, B) and T-47D (C) cells were serum-starved for 24 h and incubated with 10 nM hGX sPLA$_2$ in the presence of 1% EFAF-BSA for 6 h; non-esterified FAs were extracted from supernatants and 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.

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 (Figs. 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 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 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.

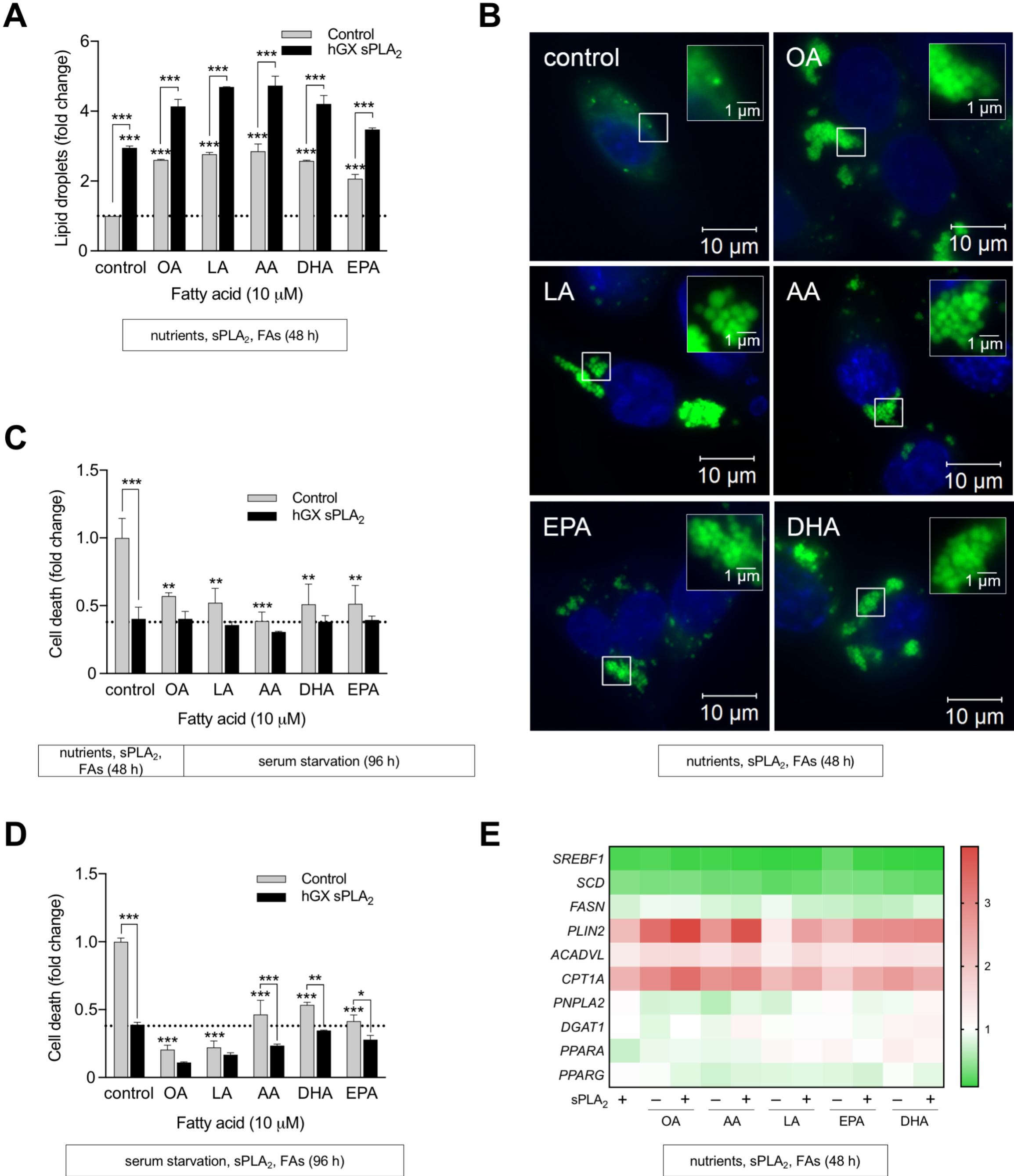


Figure 2. Unsaturated FAs induce LD formation and protect TNBC cells from starvation-induced cell death. (A–C, E) Proliferating cells grown in complete medium were treated with 10 nM hGX sPLA$_2$ and/or 10 μM exogenous FAs for 48 h (A, B, E) and starved for additional 96 h (C) in the absence of serum, 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). 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).

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 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 (Figs. 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 (Figs. 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 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 (Figs. 3H and I and Supp. Figs. 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.

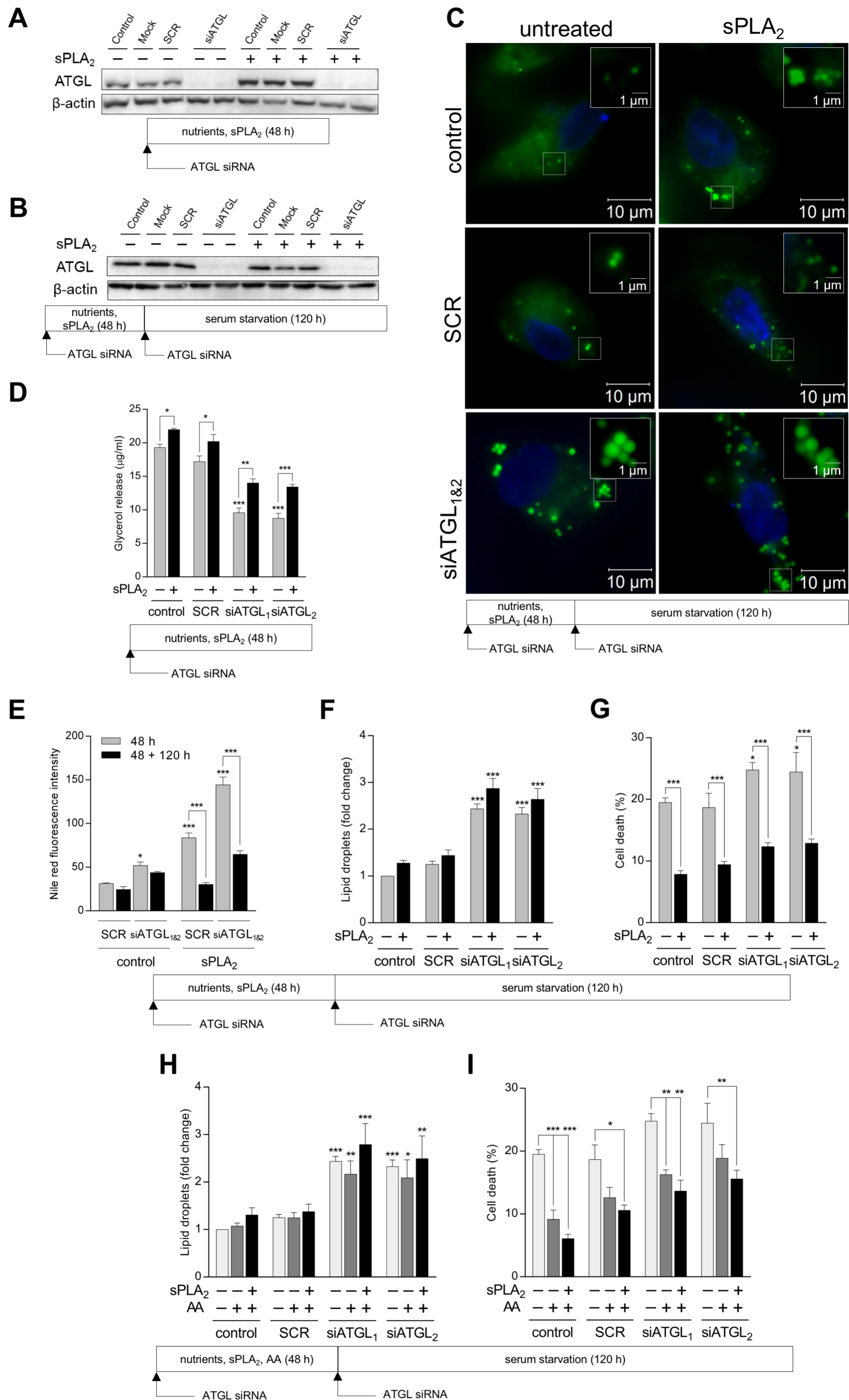

A
Control
Mock
SCR
siATGL
sPLA2
ATGL
β-actin
nutrients, sPLA2 (48 h)
ATGL siRNA
B
serum starvation (120 h)
C
untreated
sPLA2
control
SCR
siATGL1&2
1 µm
10 µm
D
Glycerol release (µg/ml)
siATGL1
siATGL2
E
Nile red fluorescence intensity
48 h
48 + 120 h
siATGL1&2
F
Lipid droplets (fold change)
G
Cell death (%)
H
AA
nutrients, sPLA2, AA (48 h)
I

Figure 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)).

We have previously shown 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.

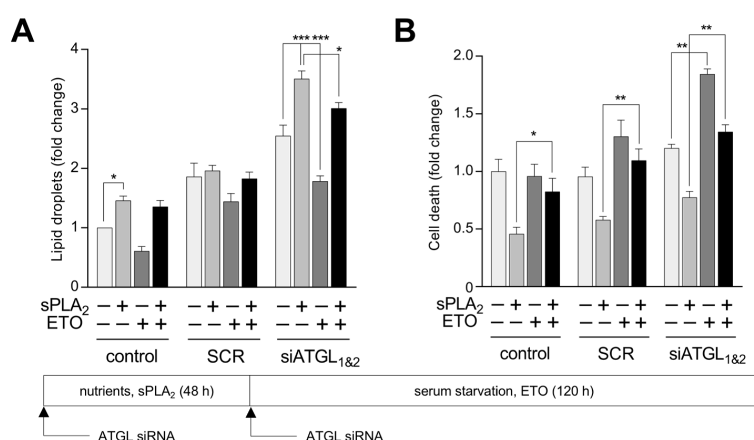


Figure 4. β-oxidation is important for cell survival during starvation. MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNAs (siATGL) or non-targeting scrambled siRNA (SCR), treated with 10 nM hGX $sPLA_2$ for 48 h in complete medium, forward transfected for the second time and serum-starved 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).

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.

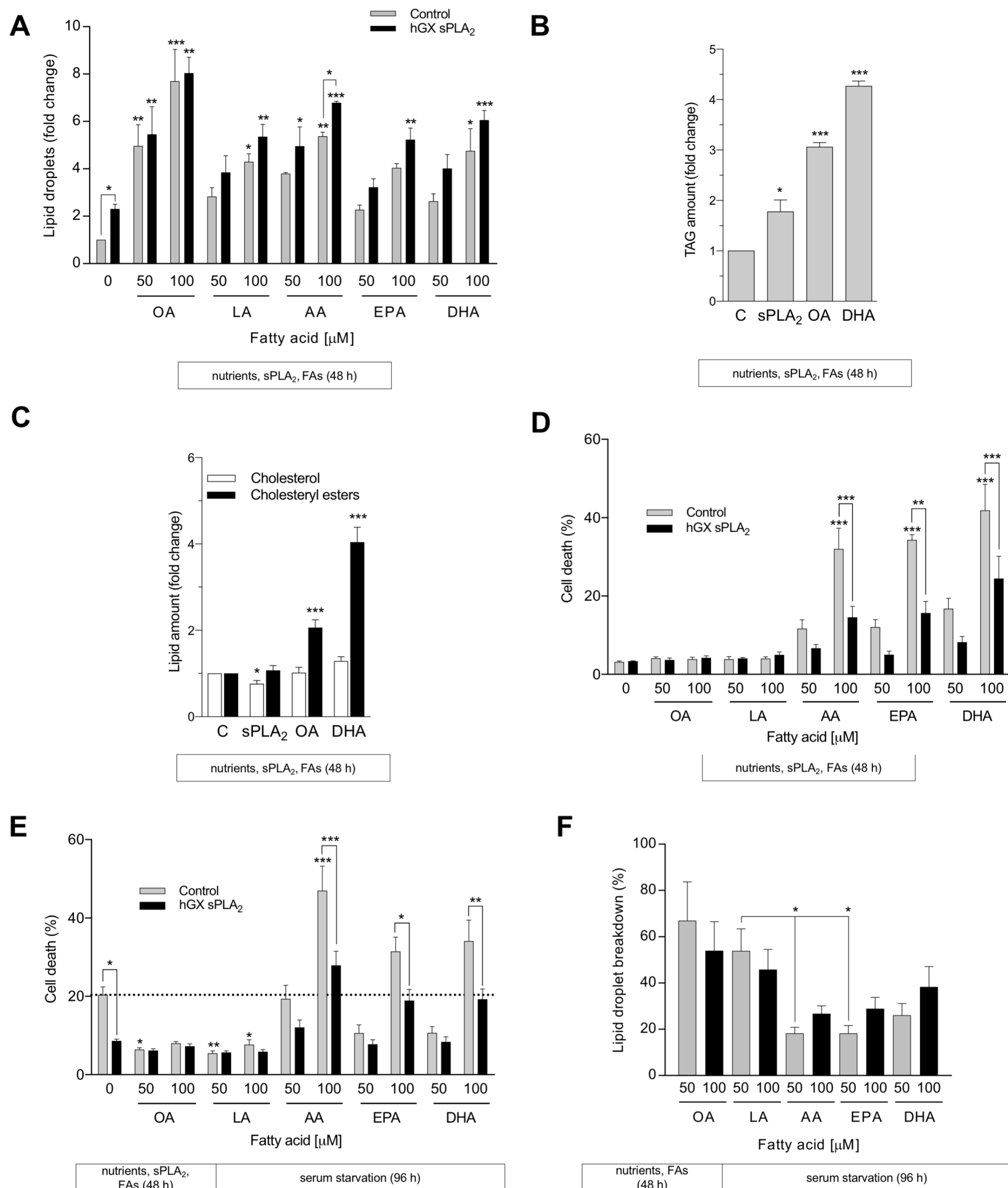


Figure 5. hGX sPLA$_2$ potentiates PUFA-induced LD formation and suppresses PUFA lipotoxicity in proliferating and in starved cells. (A, D) MDA-MB-231 cells were grown in complete medium and treated with 10 nM hGX sPLA$_2$ and/or 50–100 μM FAs for 48 h. (B, C) Cells were grown in complete medium and 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).

We next asked 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 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. Figs. 5C and 5D). 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. Figs. 6A and B). Furthermore, prolonged starvation of cells loaded with DHA resulted in a marked elevation of ROS and cell death (Figs. 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 (Figs. 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 $sPLA_2$.

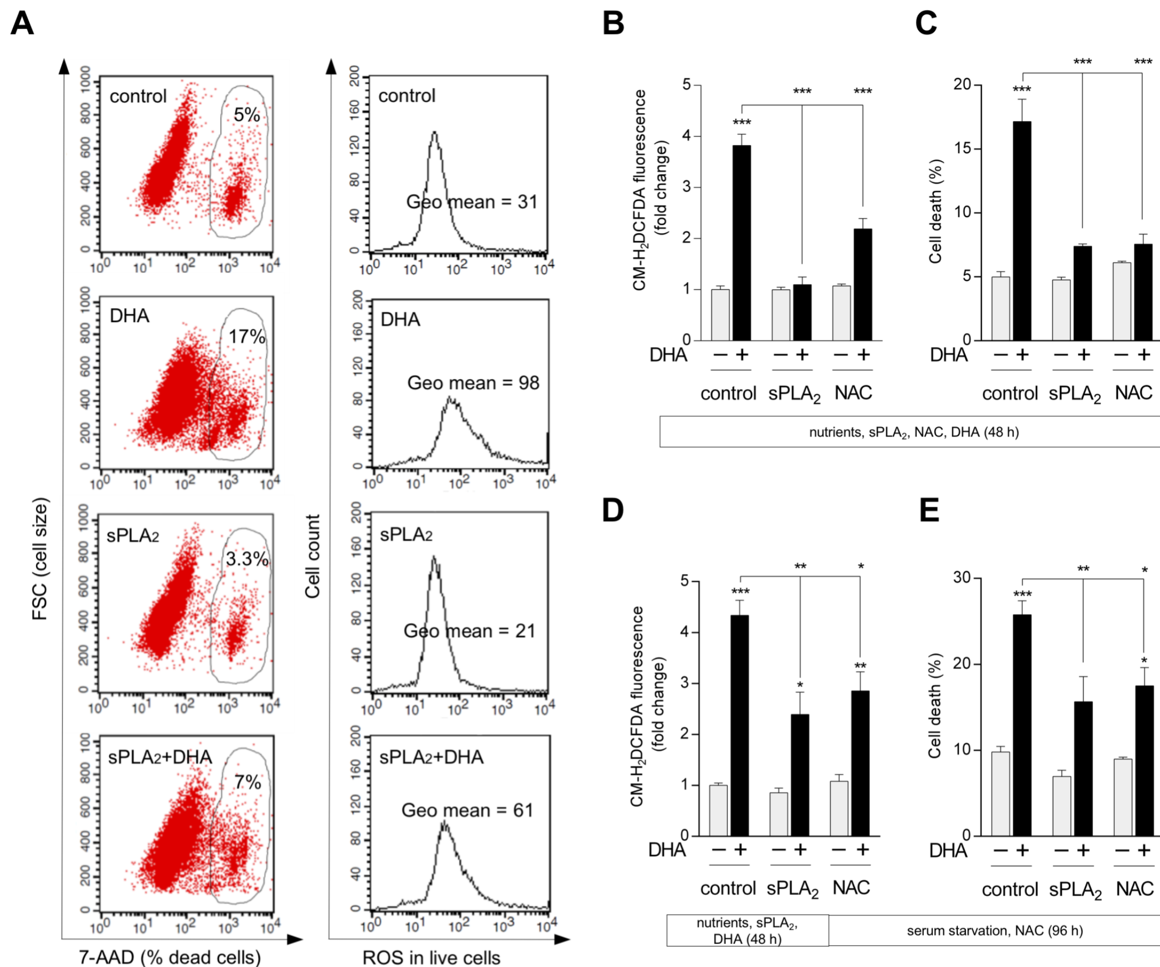


Figure 6. Breakdown of DHA-induced LDs is associated with elevated lipotoxicity and oxidative stress, which are both suppressed by hGX sPLA$_2$. (A, B, C) MDA-MB-231 cells were treated with combinations of 10 nM hGX sPLA$_2$, 100 μM DHA and 5 mM NAC in complete medium for 48 h. (D, E) Cells treated with 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$_2$DCFDA 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$_2$DCFDA 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).

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. Figs. 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 (Figs. 7A and B) and a decrease in cell death and oxidative stress in comparison with cells transfected with control siRNA and treated with DHA (Figs. 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 (Figs. 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 (Figs. 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.

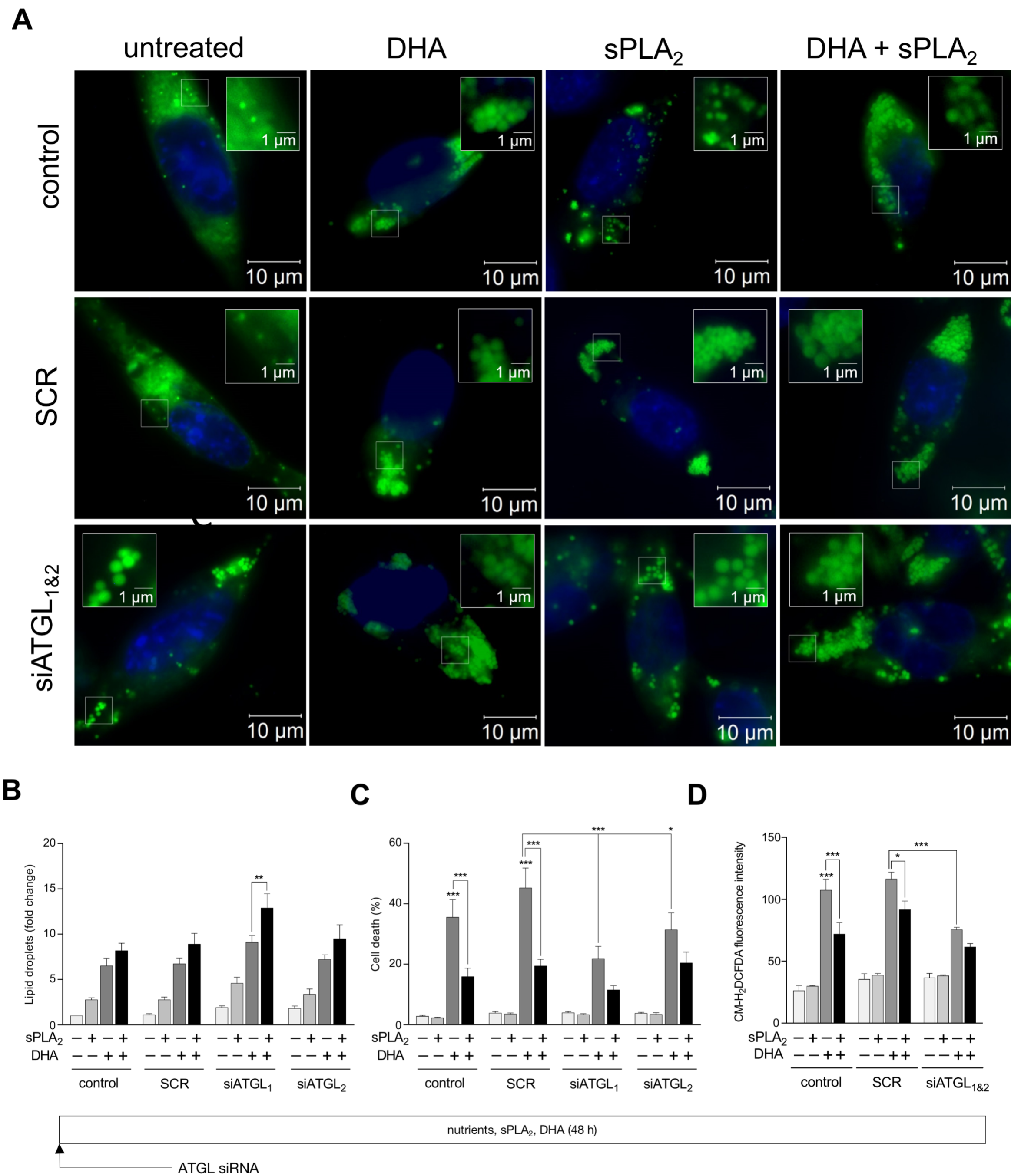


Figure 7. Suppressing ATGL-mediated lipolysis reduces DHA-induced oxidative stress and lipotoxicity. (A–D) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNAs (siATGL) or non-targeting scrambled siRNA (SCR) and treated with 10 nM hGX $sPLA_2$ and/or 100 μM DHA for 48 h in complete medium. (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).

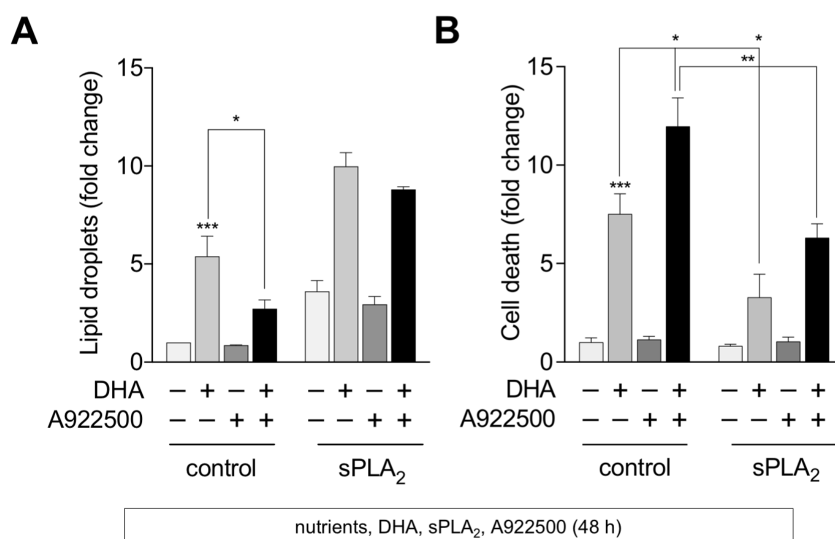


Figure 8. Inhibition of TAG synthesis augments DHA-induced lipotoxicity. (A, B) MDA-MB-231 cells were grown in complete medium and treated with 10 nM hGX $sPLA_2$ and/or the DGAT1 inhibitor A922500 (1 μM). Neutral lipids and cell death were quantified by flow cytometry using Nile Red (A) and TMRM/YO-PRO-1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

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 highly unsaturated TAG species (PUFA-TAGs) (Fig. 9A). In particular, there was a striking increase in the proportion of PUFA-TAGs containing more than 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 (Figs. 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 less than 3 and with 3–7 double bonds (including 54:2, 56:6, 56:7 and 58:7) and reduces PUFA-TAG species with more than 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% (Figs. 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% (Figs. 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.

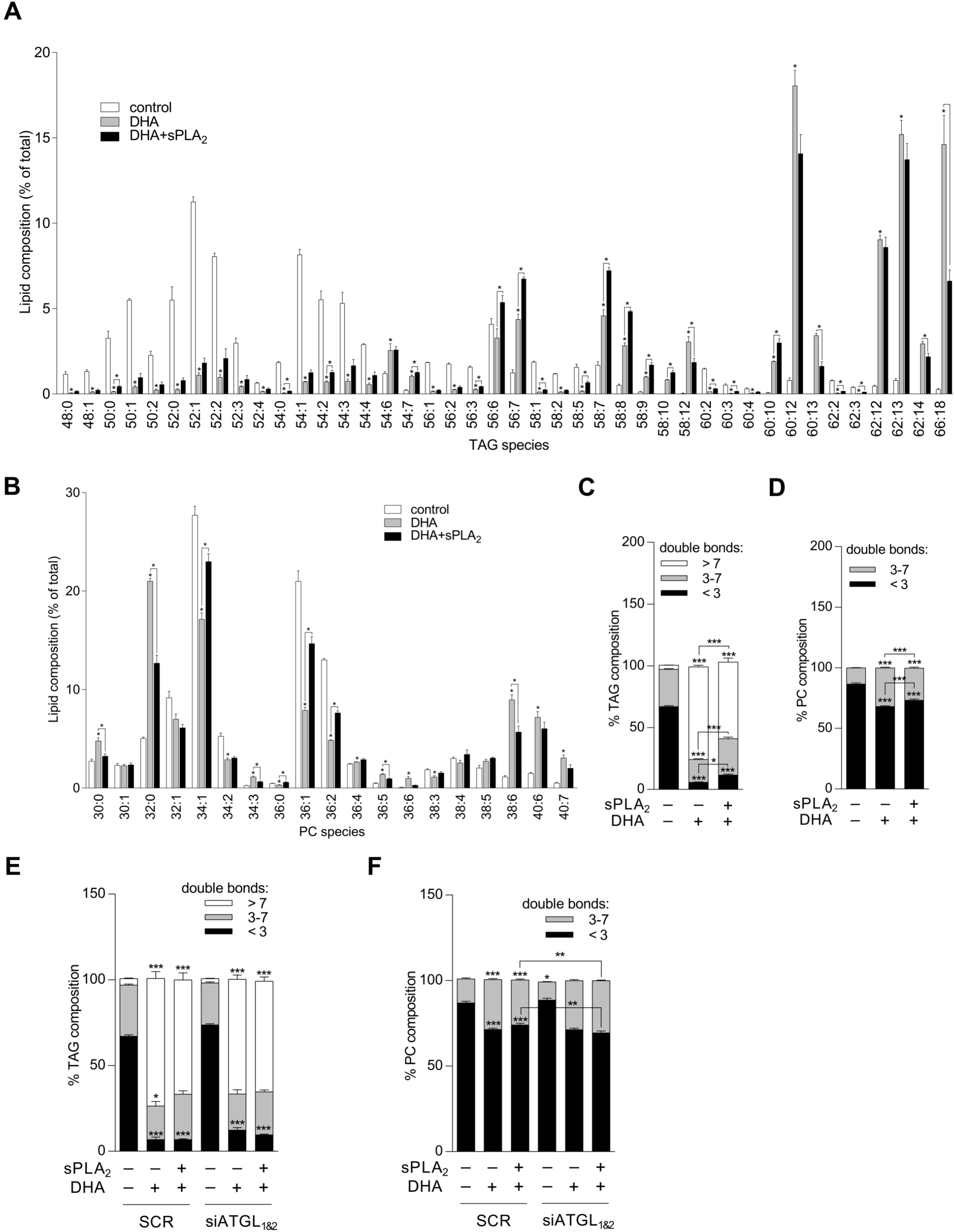


Figure 9. hGX sPLA$_2$ reduces the proportion of highly unsaturated PUFA-TAGs in DHA-treated cells. (A–D) MDA-MB-231 cells were grown in complete medium and treated with 10 nM hGX sPLA$_2$ and/or 100 μM DHA for 48 h. (E, F) MDA-MB-231 cells were reverse transfected with ATGL-targeting siRNA (siATGL) 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).

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. Figs. 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 (Figs. 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 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 nutrient and lipotoxic stress.

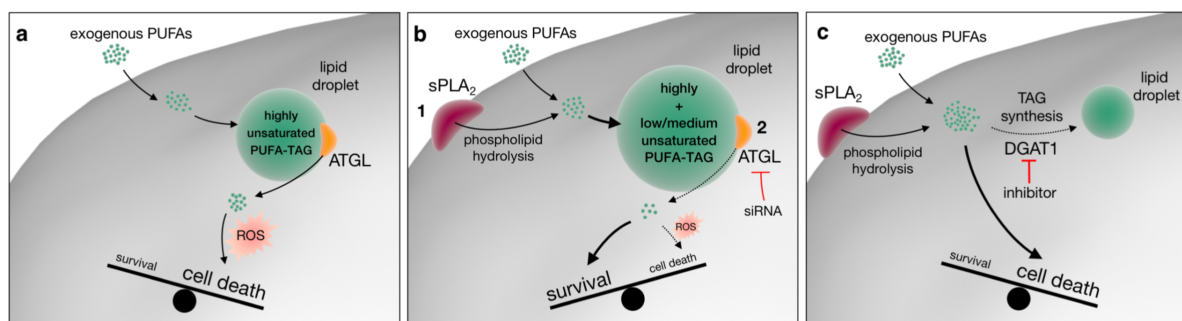


Figure 10. Antioxidant roles of hGX sPLA$_2$ and LDs in breast cancer cells exposed to a surplus of exogenous PUFAs. Ras-driven TNBC cells exposed to excess PUFAs accumulate LDs containing highly unsaturated PUFA-TAG species. (a) Starvation-induced breakdown of these LDs is associated with ATGL-dependent 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.

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 (Figs. 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 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 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 (Pucer, 2013), 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.

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