Triglycerides Promote Lipid Homeostasis during Hypoxic Stress by Balancing Fatty Acid Saturation

Graphical Abstract

Highlights
- Hypoxia influences the FA composition of TGs more than other lipid classes
- TGs maintain the cell's FA saturation index by active oleate exchange
- Release of TG-resident oleate during hypoxia prevents production of toxic lipids
- Disrupting TG synthesis compromises tumor growth

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In Brief
Tumors frequently experience hypoxia and serum limitation, which cause a harmful increase in fatty acid saturation. Studying kidney cancer, Ackerman et al. describe a protective role of lipid droplet-resident triglycerides: buffering of the cellular lipid saturation through exchange of mono-unsaturated fatty acids. Inhibiting triglyceride synthesis compromises solid tumor growth.
Triglycerides Promote Lipid Homeostasis during Hypoxic Stress by Balancing Fatty Acid Saturation

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SUMMARY

Lipid droplets, which store triglycerides and cholesterol esters, are a prominent feature of clear cell renal cell carcinoma (ccRCC). Although their presence in ccRCC is critical for sustained tumorigenesis, their contribution to lipid homeostasis and tumor cell viability is incompletely understood. Here we show that disrupting triglyceride synthesis compromises the growth of both ccRCC tumors and ccRCC cells exposed to tumor-like conditions. Functionally, hypoxia leads to increased fatty acid saturation through inhibition of the oxygen-dependent stearoyl-CoA desaturase (SCD) enzyme. Triglycerides counter a toxic buildup of saturated lipids, primarily by releasing the unsaturated fatty acid oleate (the principal product of SCD activity) from lipid droplets into phospholipid pools. Disrupting this process derails lipid homeostasis, causing overproduction of toxic saturated ceramides and acyl-carnitines as well as activation of the NF-κB transcription factor. Our work demonstrates that triglycerides promote homeostasis by “buffering” specific fatty acids.

INTRODUCTION

Proliferating cancer cells exhibit an increased dependence on biosynthetic intermediates (Vander Heiden and DeBerardinis, 2017), including fatty acids (FAs) that support the construction of organelle and plasma membranes. To meet the demand for elevated FA levels, FA synthase (FASN) overexpression is commonly observed in multiple cancers (Menendez and Lupu, 2007; Ricoult et al., 2016). Palmitate, the product of FASN enzymatic activity, can be further modified by elongation and desaturation, where double bonds between carbon atoms are introduced into long-chain FAs. Stearoyl-coenzyme A (CoA) desaturase (SCD), the principal enzyme responsible for desaturation, is critical for sustained viability of a variety of tumor cell types (Igal, 2016). By introducing a double bond into the saturated FA stearate, SCD produces monounsaturated oleate, typically the most abundant intracellular FA. Although clearly important for cell survival, activity of the oxygen (O2)-dependent SCD enzyme can be constrained by tumor hypoxia (Figure 1A). Periods of O2 starvation, therefore, lead to a buildup of saturated FA precursors, causing disruption of endoplasmic reticulum (ER) membranes and apoptosis (Kamphorst et al., 2013; Young et al., 2013). Saturated FA-induced toxicity can be alleviated by supplying exogenous unsaturated lipids (for instance, by increasing the availability of serum FAs), indicating that lipid uptake is an important mechanism for maintaining homeostasis in hypoxic cancer cells (Young et al., 2013).

Accumulated neutral lipids in large lipid droplets (LDs) is observed in a subset of tumor types, particularly clear cell renal cell carcinoma (ccRCC). In ccRCC, this phenotype has been linked to genetic loss of the von Hippel-Lindau (VHL) tumor suppressor, which causes constitutive hypoxia inducible factor-α (HIF2α) stabilization regardless of O2 availability. We have previously shown that induction of HIF2α specifically promotes lipid accumulation through upregulation of PLIN2, the gene encoding the LD coat protein perilipin-2 (Qiu et al., 2015). PLIN2 loss significantly represses tumor growth, indicating that LD formation may be driven by HIF2α stabilization and serves a cytoprotective role in ccRCC. In a separate study, HIF-dependent repression of FA β-oxidation has also been demonstrated to contribute to LD accumulation (Du et al., 2017).

LDs are primarily composed of cholesterol esters (CEs) and triglycerides (TGs), and lipidomic analyses of ccRCC samples revealed high levels of both in tumors compared with normal kidney (Saito et al., 2016; Sundelin et al., 2012). TGs consist of a glycerol backbone and three FAs (Figure 1E), with a significant diversity in FA chain length and number of double bonds. Their synthesis requires the activity of the diglyceride acyltransferase (DGAT) enzymes DGAT1 and DGAT2, which catalyze the condensation of fatty acyl-CoA with a diglyceride (DG) to form TG. The two human DGAT enzymes share no homology and have dissimilar expression patterns (Yen et al., 2008). TGs are synthesized in the ER, but DGAT2 can also be found on the surface of LDs and may generate TGs in growing LDs in situ (Wilfling
**Figure A**

A metabolic pathway diagram showing the conversion of Glutamine and Glucose to acetyl-CoA, which further leads to the synthesis of Lipid droplets through the processes involving ACC, FASN, LPA, PA, DG, TG, and Phospholipids.

**Figure B**

A graph showing the relative tumor volume over treatment duration (days) for Control Chow and Doxycycline Chow. The graph includes error bars indicating variability.

**Figure C**

A bar chart representing tumor weight (mg) for Control Chow, Doxycycline Chow, Control DGAT shRNA, and Control Doxy-cycline. The chart includes error bars showing the standard deviation.

**Figure D**

Images of Cleaved Caspase3 and Ki67 expression under vehicle and doxycycline conditions. The figures include relevant cellular markers.

**Figure E**

An example of triglyceride structure, illustrating SFA (Saturated Fatty Acid) and UFA (Unsaturated Fatty Acid) with the total triglyceride abundance under Vehicle and DGAT shRNA conditions.

**Figure F**

A fold change analysis of triglyceride levels (DV) under different conditions, showing significant differences and error bars indicating variability.
et al., 2013). To mobilize lipid stores to provide FAs, TGs are broken down by a series of lipases, and the released FAs can, in principle, be used for incorporation into other lipid types, such as phospholipids (PLs), or for mitochondrial oxidation. Although the protective function of LDs and TG turnover have been identified in a number of contexts (Bailey et al., 2015; Bensaad et al., 2014), the full scope of TG synthesis and catabolism in tumor cells remains unclear.

Here we evaluated the consequences of limiting TG synthesis in ccRCC. We found that concurrent inhibition of DGAT1 and DGAT2 severely compromised in vivo tumor growth because of increased cell death. This was replicated in cultured cells exposed to low O2 and serum, mimicking a stressful tumor microenvironment. Mechanistically, TGs sequester exogenous unsaturated FAs, particularly oleate, when in ample supply. When oleate availability becomes limited during O2 and serum deprivation, however, oleate is instead released into other lipid pools. This prevents the buildup of fully saturated, toxic lipids in cellular compartments outside of LDs. Our work reveals a dynamic mechanism by which TGs act as buffers for cellular lipid homeostasis, especially under the tumor-relevant conditions of O2 and nutrient limitation.

RESULTS AND DISCUSSION

Disruption of TG Synthesis Compromises ccRCC Tumor Growth

Although the functional roles of CEs in cancer have been interrogated to some extent (Yue et al., 2014), TGs have so far remained considerably less well studied. We investigated how direct disruption of TG synthesis by loss of DGAT enzymes affects lipid homeostasis. DGATs appear to carry out mutually redundant functions in the storage of both endogenously synthesized and exogenously derived FAs (Figure 1A). We confirmed their redundancy in A498 ccRCC cells by examining the induction of neutral lipid stores upon administration of oleic acid conjugated with BSA versus BSA alone. Although a combination of CRISPR/Cas9-mediated DGAT2 deletion and DGAT1 pharmacological inhibition fully abrogated this, loss of neither DGAT individually was sufficient (Figure S1A). This approach provides the opportunity to precisely control the timing of DGAT inhibition by adding the DGAT1 inhibitor T863 (DGAT1i) to cells with DGAT2 deletion. Importantly, DGAT deficiency was complemented with a CRISPR-resistant DGAT2 cDNA, restoring neutral lipid deposition (Figure S1A). To study the consequences of TG synthesis inhibition in vivo (and employ a complementary approach), we generated A498 cells expressing both DGAT1 and DGAT2 short hairpin RNAs (shRNAs) under the control of a Tet-inducible promoter and confirmed that these constructs effectively reduce DGAT mRNA and protein levels upon doxycycline treatment (Figures S1B and S1C). After implanting these cells subcutaneously in immunocompromised recipients and allowing tumors to grow to an average size of 300 mm3, mice were fed with either control or doxycycline-containing chow. A substantial reduction in both DGAT1 and DGAT2 transcript levels (Figure S1D) and significantly reduced tumor volume and weight was observed (Figures 1B and 1C). Immunohistochemical staining of tumor sections revealed increased numbers of apoptotic cells based on cleaved caspase-3 staining and decreased numbers of actively dividing Ki67+ cells (Figure 1D). As anticipated, TG levels were lower in DGAT-deficient tumors compared with controls (Figure 1E; Table S1), although the observed differences failed to reach statistical significance. These results likely reflect the inherent heterogeneity between cells within solid tumors with regard to TG synthesis and turnover, in addition to variable O2 and nutrient availability. Nevertheless, a pronounced increase in TGs containing one or more saturated FAs (≥1 saturated FAs [SFAs]; Figure 1F), but not those exclusively carrying unsaturated FAs (0 SFAs; Figure 1F) was observed. Thus, DGAT silencing disrupts TG FA composition and causes both increased apoptosis and reduced proliferation of ccRCC tumor cells in vivo.

DGAT Loss Compromises ccRCC Viability in Low O2 and Serum

We next sought to establish exactly how TG metabolism promotes tumor growth. Because solid tumors are notoriously poorly perfused and hypoxic (Frantz et al., 2010), we specifically focused on how serum lipid and O2 limitation results in DGAT dependency. Of note, combined serum and O2 limitation led to deteriorated cell viability upon DGAT knockdown (Figure 2A). Because hypoxia limits SCD activity (Figure S2A) and reduces cell viability in the absence of exogenous lipid supply (Kamphorst et al., 2013; Qiu et al., 2015), we investigated whether enhanced sensitivity of DGAT-deficient cells to these conditions is indeed mediated by reduced SCD function. Cells were exposed to the SCD inhibitor CAY10566, which phenocopied the effect of O2 deprivation (Figure 2B).

To understand the relationship between serum levels and TG metabolism, a lipidomic comparison of A498 cells cultured in either high (5%) or low (0.5%) serum-containing medium was

Figure 1. DGAT Loss Reduces Tumor Growth and Alters Lipid Composition In Vivo

(A) Diagram of fatty acid and lipid synthesis and the influence of O2 and exogenous lipid.

(B) Growth curves for A498 xenograft tumors with induced (doxycycline chow) and un-induced (control chow) DGAT1 and DGAT2 shRNAs (hereafter called DGAT shRNA).

(C) Tumor weights after necropsy.

(D) Immunohistochemistry for cleaved caspase-3 and Ki67 in xenograft tumors collected on day 5 of treatment, with accompanying quantification.

(E) Total TG abundance derived from summing individual TG species abundance after liquid chromatography-mass spectrometry (LC-MS) quantification.

(F) TG species binned according to the number of fully saturated FA chains present and the abundance of each category summed and displayed as a ratio of doxycycline-treated versus control groups.

All results are means of n = 10 tumors (2 tumors per mouse) per arm; error bars represent ± SEM (C). Statistical significance by t test or ANOVA, as appropriate: *p < 0.05, **p < 0.01, and ***p < 0.001. ACC, acetyl-CoA carboxylase; CE, cholesterol ester; DG, diglyceride; DGAT, diglyceride acyltransferase; FASN, fatty acid synthase; ns, non-significant; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PS, phosphatidylserine; SCD(i), stearoyl-CoA desaturase (inhibitor); SFA, saturated FA; TG, triglyceride; UFA, unsaturated FA. See also Figure S1.
Figure 2. TGs Promote Cell Viability in Low O2 and Serum by Absorbing FA Saturation

(A) Viability of A498 cells expressing inducible shRNA against DGAT1 and DGAT2 mRNAs (DGAT shRNA), assessed after 72 hr under the indicated conditions (hypoxia = 0.5% O2; serum deprivation = low serum, 0.5% fetal bovine serum [FBS]) by Annexin-propidium iodide (PI) flow cytometry assay.

(B) Viability of cells expressing inducible DGAT shRNAs after 72 hr under the indicated conditions (SCDi, 1 μM CAY10566) by Annexin-PI assay using flow cytometry. 

(C) Volcano plot showing fold change and significance of alterations in the lipidome of A498 cells cultured in low (0.5%) versus high (5%) serum. Lipids with ≥ 1.5 fold change and p ≤ 0.05 are displayed in color to denote lipid class.

(D) Changes in FA composition or saturation of TGs, calculated by aggregating TG abundances for species containing 0, 1, or 2+ SFA chains separately. Values are normalized to control conditions (5% serum).

(E) Lipid class-specific saturation indices (defined by (palmitate + stearate) / oleate) for A498 cells cultured under hypoxic (0.5% O2) versus normoxic conditions (both in low serum).

(F) As (E) but with pharmacological SCD inhibition (1 μM CAY10566) instead of hypoxia.

(G) Effect of serum deprivation and DGAT shRNA on total TG abundances.

(H) Changes in FA makeup of TGs following DGAT knockdown; values were calculated by aggregating TG abundances for species containing 0, 1, or 2+ SFA chains separately. Values were normalized to the control condition (vehicle [Veh] treatment).

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performed, revealing substantial remodeling of the intracellular lipid composition. Among the most pronounced changes were significant reductions in CEAs as well as in TGs (Figure 2C). Limiting serum in vitro led to large decreases in the abundance of unsaturated TGs (Figure 2C) and a shift toward TG saturation (Figure 2D), as noted for solid tumors (Figures 1E and 1F). A striking depletion in neutral lipid stores was also confirmed by boron-dipeyrromethene (BODIPY) imaging (Figure S2B), in line with earlier observations (Bensaad et al., 2014). This indicates that, in addition to HIF signaling, availability of exogenous serum lipids is critical for maintaining abundant lipid stores. Increased TG storage observed under hypoxia appears to be cytotoxic and renders cells more resistant to subsequent hypoxia and hypoxia-reoxygenation-mediated cytotoxicity (Bensaad et al., 2014). Moreover, TGs may also harbor polyunsaturated FAs to protect them against peroxidation (Bailey et al., 2015). To establish whether lipid stores “buffer” against lipid saturation in our system, we determined whether any one class of lipid preferentially “absorbs” alterations in FA saturation under hypoxia. Strikingly, TG composition was affected much more profoundly than other lipid classes (Figures 2E and S2C), including a loss of TGs harboring unsaturated FAs and a shift toward increased TG saturation (Figure S2D). Moreover, these changes were only observed under low-serum conditions. When A498 cells were exposed to pharmacological SCD inhibition instead of hypoxia (Figure S2E), we observed a similar but more pronounced increase in TG saturation (Figure 2F) as well as a stronger effect on other lipid classes (especially DGs), the direct precursors for TGs. This difference is most likely due to a combination of more potent SCD inhibition by pharmacological approaches compared with hypoxia (leading to increased FA saturation) as well as a limited capacity of TGs to cope with the increased FA saturation, leading to “spill-over” into other lipid classes. Importantly, when other ccRCC cell lines (786-O and UMRC2) were exposed to low O2, FA saturation was similarly most pronounced in TGs, followed by the direct precursor DGs (Figures S2F and S2G). This suggests that TGs have a capacity to promote cell viability by balancing the availability of specific FAs.

Serum deprivation reduced intracellular TG abundance (Figure 2G), in keeping with the BODIPY imaging depicted in Figure S2B. As demonstrated with in vivo tumor growth (Figures 1E and 1F), DGAT silencing in vitro also caused a further decrease in TG abundance (Figure 2G) and selective depletion of unsaturated TGs (Figure 2H). Given that both hypoxia and DGAT depletion cause increased saturation of the TG pool under low-serum conditions, we asked whether combination of the two leads to a cumulative effect; i.e., an even more saturated TG pool (Figures 2I and 2J). These results highlight a protective role of LDs under serum- and O2-limited conditions through buffering cellular FA saturation.

**TGs Neutralize Excess Fatty Acid Saturation through Release of Stored Oleate**

Although the aforementioned protective role of TGs could simply be due to their ability to sequester excess saturated FAs into TGs, TG synthesis by DGAT enzymes is more efficient when the substrates are unsaturated rather than saturated (Listenberger et al., 2003). LDs could alternatively protect cells by preferentially releasing unsaturated FAs from stored TGs for use in the production of cytosolic and membrane-associated lipids. Because monounsaturated oleate (C18:1) is the single most abundant FA in TG pools (Figure S3A), its mobilization during periods of unsaturated lipid deprivation should ameliorate stress by preventing the synthesis of fully saturated, potentially toxic lipids. A498 cells experiencing stringent conditions of low serum and pharmacological SCD inhibition exhibited reduced total TG levels (Figure 3A), supporting this hypothesis. To further test this, we assessed the protective potential of oleate, which is efficiently incorporated into TGs and, consequently, the most abundant TG FA. A498 cells were pre-treated with oleate under low-serum conditions before being exposed to SCD inhibition. Oleate pre-treatment indeed promoted cell viability under these conditions (Figure 3B), which was largely abolished upon DGAT silencing, suggesting that it occurs through TGs.

To study the cellular fate of oleate in more detail, we designed a washout labeling approach (Figure 3C; see STAR Methods for information about lipid tracing). Serum-starved and, hence, LD-depleted A498 cells were first exposed to labeled [U-13C]-oleate in the “loading” phase. We employed cells with DGAT2 loss induced through CRISPR/Cas9-mediated mutagenesis (Figure S1A) to again precisely time DGAT inhibition by adding DGAT1i. Following loading, the [U-13C]-oleate tracer was removed, and [U-13C]-oleate in the TG pool was left to “wash out,” allowing its fate (such as re-incorporation into other lipid classes) to be determined. Mass spectrometry analysis of TGs post-loading but pre-washout demonstrated that oleate was avidly incorporated into TGs, mostly producing TGs with three oleate FAs (Figure 3D; i.e., 3×[U-13C]-18:1 versus 2× or 1×[U-13C]-18:1). Similarly, BODIPY imaging of neutral lipid stores was used to confirm that oleate loading led to abundant TG accumulation, largely prevented by DGAT inhibition (Figure 3E). Moreover, a non-negligible amount of [U-13C]-oleate (C18:1) was elongated to [13C18:2]-20:1 (i.e., a C20:1 FA with 18 13C or 18 15N) prior to its incorporation into TGs like TG 66:3 (Figure 3D; data not shown). As expected, DGAT inhibition severely limited the incorporation of labeled oleate into TGs.

Based on our data, we reasoned that, under conditions of saturated FA excess, TG oleate mobilization and subsequent re-esterification into other lipid classes enables continued production of lipids with at least one unsaturated FA, preventing

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(1) TG saturation indices for the indicated conditions. Values are relative to normoxic untreated cells.

(2) As (1) but with pharmacological SCD inhibition (1 μM CAY10566). Values are relative to the untreated vehicle control.

Data are means of 3 (A, B, and D–J) or 5 (C) replicate wells and were confirmed in independent experiments; error bars represent SD. Statistical significance by t test or ANOVA, as appropriate. **p < 0.05, and ***p < 0.005. See also Figure S2.
Figure 3. $^{13}$C-Oleate Tracing Reveals a Critical Buffering Role for TG-Resident Unsaturated FAs

(A) Effect of SCDi on total TG abundances as measured by LC-MS.

(B) Effect of oleate pre-loading with or without DGAT shRNA on subsequent A498 cell survival (by Annexin-PI) during serum limitation and SCD inhibition.

(C) Schematic of the experimental workflow. DGAT2 knockout cells were serum-starved for 24 hr and then loaded for 24 hr with 10 μM [U$^{13}$C]-oleate (C18:1) ± DGAT1 inhibitor (T863, 2 μM). The medium was then replaced and the tracer removed, and cells were subjected to a 48-hr washout.

(D) TG labeling patterns after 24-hr loading with [U$^{13}$C]-oleate with or without DGATi, where numbers of mono-unsaturated FA (MUFA) and FA carbons are indicated. 1, 2, and 3 indicate whether TGs have one, two, or three oleates (includes [13C18]-20:1) conjugated to their glycerol backbones.

(E) BODIPY and DAPI staining directly after [U$^{13}$C]-oleate loading with or without DGATi.

(F) Labeling patterns as assessed by incorporation of the $^{13}$C label in 18:1 and 20:1 FAs in TG, DG, PC, and PE species.

(G) Replete conditions (presence of serum and oleate) vs. Serum limited conditions, cellular lipids hydrolysis, HSL.

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the synthesis of harmful, fully saturated lipids. To evaluate the dynamics of oleate redistribution to other lipid species, changes in bulk [U\(^\text{13C}_{18}\)]-18:1 and [U\(^\text{13C}_{18}\)]-20:1 in TGs, DGs, and membrane phospholipids, phosphatidylcholines (PCs) and phosphati
diy ethanolamines (PES), were analyzed after a 48-hr washout in low serum (Figure 3F). In accordance with total TG levels (Fig-
ure 3A) and BODIPY imaging (Figure 3E), cells loaded with labeled oleate (0 hr, −DGAT1i) exhibited a strong reduction in
TG FA labeling over the course of the washout (48 hr). Cells
treated with DGAT1i during loading had lower labeled FA levels to
start, which were almost entirely depleted during washout
(Figure 3F). Analysis of DG, PC, and PE lipids indicated that
[U\(^\text{13C}_{18}\) ]- mono-unsaturated FAs (MUFAs) were incorporated
into all three classes during loading. Because the exogenous
[U\(^\text{13C}\) ]-oleate tracer was removed at the start of washout, we ex-
pected levels of labeled FAs in these lipids (as for TGs) to
diminish because of continuous turnover. This was evidently
the case for the DGAT1i-treated cells because at least a 2-fold
reduction in labeling was observed. Strikingly, this reduction
was not detected in untreated cells, most likely because oleate
originally loaded in the TG pool is subsequently feeding into
gD, PC, and PE lipid pools during washout. By reducing the
TG pool, DGAT inhibition prevents the subsequent flow of oleate
from TGs to other lipid classes during periods of unsaturated
lipid deprivation

Adipose triglyceride lipase (ATGL) and monoacylglycerol
lipase (MAGL) have been shown previously to play supportive
roles in cancer progression (Nomura et al., 2010; Zagani et al.,
2015), suggesting that mobilization of TG stores is critical for
tumor cell metabolism. We investigated the outcome of pharma-
cological inhibition of TG hydrolysis (Figure S3B) and did not
observe appreciable effects of disrupting ATGL or MAGL activity
on the flow of labeled oleate from TG into other lipid types (see
STAR Methods for experimental details). In contrast, inhibition
of hormone-sensitive lipase (HSL) led to accumulation of labeled
DG, in accordance with its ascribed function as a DG lipase (Fig-
ure S3B). HSL inhibition also substantially reduced the washout
of TG labeling and caused more abundant PC labeling than in
untreated cells. The effects of HSL inhibition were confirmed in
two additional cCRC cell lines (Figure S3C), consistent with
gD’s role as a substrate for PL synthesis. Of note, in 786-O cells,
almost no labeled FAs were left in the TG pool at the end of
the washout experiment, suggesting that the FA buffering capacity
in this cell line is somewhat more limited. This is consistent
with the more pronounced saturation of the DG pool upon

(G) Model of the metabolic mechanism by which TGs alleviate the saturation of certain lipid classes (e.g., PCs) under conditions of unsaturated lipid deprivation by releasing stored oleate.

Data are means of triplicate wells confirmed in independent experiments (A, B, and D) or means of three independent experiments each conducted in triplicate (F); error bars represent SD. Statistical significance by t test or ANOVA, as appropriate. *p < 0.05, **p < 0.05, and ***p < 0.005. See also Figure S3.
Figure 4. DGAT Loss Modifies Lipid Homeostasis, Elevates Ceramide, Acyl-ceramide, and Acyl-carnitine Levels, and Activates NF-κB Target Gene Expression

(A) Effect of SCD and DGAT inhibition on ceramide levels in serum-deprived A498 cells in vitro.
(B) Effect of DGAT loss on ceramides in vivo (i.e., A498 xenografts).
(C) Effect of DGAT loss on acyl-ceramides in vivo (i.e., A498 xenografts).
(D) Effect of hypoxia on the FA composition of acyl-carnitines (CARs) on serum-deprived A498 cells in vitro.
(E) Effect of DGAT loss on the FA composition of acyl-carnitines (CARs) on serum-deprived A498 cells in vitro.
(F) Effect of DGAT loss on the FA composition of acyl-CARs in A498 xenograft tumors.

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engagement was inhibited by the specific NF-κB inhibitor PS-1145 (Figure 4H) as well as the proteasome inhibitor ixazomib. Of note, ixazomib inhibits NF-κB by stabilizing its negative regulator inhibitor of κB (IκB) (Mujtaba and Dou, 2011). Serum deprivation also led to increased NF-κB reporter activity, which was fully inhibited by administration of both PS-1145 and ixazomib (Figure 4I). Collectively, our data suggest that DGAT deficiency leads to broad disruption of lipid homeostasis, resulting in unsaturated FA depletion and accumulation of saturated FAs and, consequently, ceramides, acyl-ceramides, and acyl-carnitines (Figure 4J). This is accompanied by activation of NF-κB signaling, previously documented to respond to increased saturated FAs. Future work will delineate whether NF-κB signaling contributes to balancing FA availability during stressful conditions as a prosurvival mechanism.

Altered TG Saturation in ccRCC Patient Samples

Given the requirement for DGAT activity to survive conditions of excess lipid saturation in our experiments, we determined whether this is a common feature of human tumor biology. We therefore re-analyzed published data comparing the TG composition of ccRCC patient samples and normal kidney tissues (Saito et al., 2016). As expected, TG levels were strongly elevated in ccRCC, as documented previously (Sundelin et al., 2012). However, the fold change was particularly pronounced for TGs containing one or more SFAs, demonstrating an increase in TG saturation (Figure 4K). This increase in ccRCC TG saturation is consistent with TG regulation of FA composition in lipid pools, as observed in our in vitro experimental system (Figures 3E and 3F). We therefore suggest that increased FA saturation (and its buffering by TGs) occurs in tumor samples derived from ccRCC patients.

Conclusion

Although oncogenic signaling alters cellular metabolism to promote the synthesis of macromolecules, malignant cells are also programmed to withstand nutrient scarcity (Boroughs and DeBerardinis, 2015). This metabolic flexibility can involve the induction of scavenging pathways, such as autophagy in RAS- or BRAF-driven cells (Yang et al., 2011) or macropinocytosis in KRAS-driven cells deprived of glutamine (Commissio et al., 2013). Both KRAS and hypoxia promote the uptake of extracellular unsaturated lipids with similar consequences: cells become more resistant to O2 deprivation and its associated inhibition of cellular unsaturated lipids with similar consequences: cells become more resistant to O2 deprivation and its associated inhibition of cellular unsaturated lipids with similar consequences: cells become more resistant to O2 deprivation and its associated inhibition of cellular unsaturated lipids.

We therefore propose that unsaturated FA storage in TGs contributes to ccRCC metabolic plasticity and that its inhibition may effectively target cancer cells residing in ischemic tumor domains (Figure 4J).

STAR METHODS

Detailed methods are provided in the online version of this paper and include the following:

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SUPPLEMENTAL INFORMATION

Supplemental Information includes five figures and one table and can be found with this article online at https://doi.org/10.1016/j.celrep.2018.08.015.

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DECLARATION OF INTERESTS
The authors declare no competing interests.

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# STAR★METHODS

## KEY RESOURCE TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| DGAT1               | Abcam  | ab54037; RRID: AB_869453 |
| V5                  | Life Technologies | R960-25; RRID: AB_2556564 |
| K67                 | Abcam  | ab15580; RRID: AB_443209 |
| Cleaved Caspase3    | Cell Signaling | 9661; RRID: 2341188 |
| Calnexin            | Cell Signaling | 2679; RRID: 10827903 |
| **Chemicals, Peptides, and Recombinant Proteins** |        |            |
| DMEM                | Life Technologies | 11965-084 |
| Pen/Strep           | Life Technologies | 15140-122 |
| Standard FBS        | Gemini  | 900-108 |
| [U^{13}C]-oleate    | Sigma   | 490431 |
| T863 DGAT1i         | Sigma   | SML039-5MG |
| Matrigel Basement Membrane Matrix | Corning | 356234 |
| 200mg/kg doxycycline chow | Harlan Labs | TD04104 |
| oleic acid- BSA mix | Sigma   | O3008 |
| Butylated hydroxytoluene (BHT) | Sigma | W218405 |
| SPLASH lipidmix internal standard mix | Avanti Polar Lipids | 330707 |
| Atglistatin         | Sigma   | SML1075 |
| CAY10499            | Cayman chemicals | 10007875 |
| JJKK048             | Tocris  | 5206 |
| **Critical Commercial Assays** |        |            |
| Volupac             | Sartorius | 11729265 |
| RNAeasy purification kit | QIAGEN | 74106 |
| High Capacity RNA-to-cDNA master mix | Life Technologies | 4387406 |
| TBP Taqman assay    | Life Technologies | HS00427620_M1 |
| ACTB Taqman assay   | Life Technologies | HS01060665_G1 |
| DGAT1 Taqman assay  | Life Technologies | HS01017541_M1 |
| DGAT2 Taqman assay  | Life Technologies | HS01045913_M1 |
| QiaPrep Miniprep kit | QIAGEN | 27104 |
| BODIPY 493/503      | Life Technologies | D3922 |
| FITC–Annexin V, PI Kit | BD Biosciences | 556547 |
| Annexin-V binding buffer | BD Biosciences | 556454 |
| **Deposited Data**  |        |            |
| *In vivo* microarray study | NCBI GEO | GSE117774 |
| *In vitro* microarray study | NCBI GEO | GSE117775 |
| **Experimental Models: Organisms/Strains** |        |            |
| NIH-III nude mice (female) 4-6 weeks old | Charles River | #201 |
| **Oligonucleotides** |        |            |
| DGAT2 Crispr1       | This paper | N/A |
| Forward: caccgTGTGCTCTACTTCACTTGGC |        |          |
| Reverse: aaacGCAAAGTGAGTAGAGCACCA |        |          |

(Continued on next page)
CONTACT FOR REAGENT AND RESOURCE SHARING

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Jurre Kamphorst (jurre.kamphorst@glasgow.ac.uk).

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Mice
Subcutaneous xenograft experiments were approved by the Animal Care and Use Committee at the University of Pennsylvania. 10 Female NIH-III nude mice (Charles River; 4–6 weeks old) were injected subcutaneously in both flanks. The injection mix contained 5 million cells in PBS, mixed 1:1 with Matrigel Basement Membrane Matrix (Corning). Tumor volume was monitored by caliper measurements. After tumors reached 300 mm³, mice were split into cohorts of 5 mice receiving doxycycline chow (200 mg/kg; Harlan Labs) or control chow (Harlan Labs) ad libitum. After experiment completion, animals were sacrificed by CO₂ inhalation, and xenograft tumors were dissected for downstream analyses.

Cell Lines and cell culture conditions
Authenticated (short tandem repeat profiling) human cell lines HK-2, 786-O, 769-P, A498, RCC4, RCC10, UOK101, and UMRC2 were obtained from the American Type Culture Collection. Cell lines were routinely passaged in DMEM (GIBCO) with 25 mM glucose and 2 mM L-glutamine with 5% (v/v) fetal bovine serum (FBS, GIBCO) at 37°C and 5% CO₂. Cells were split at 80% confluency. All cell lines described in this study were verified mycoplasma-negative. Experiments were performed in DMEM supplemented with 10 mM glucose, 2 mM L-glutamine and indicated levels of FBS (Sigma). Hypoxic conditions were maintained at 0.5% O₂, 37°C and 5% CO₂ in the InVivo2 Hypoxia Workstation with a Ruskinn Gas mixer Q (Baker Co.). Cell number was assessed using the Countess Cell Counter (ThermoFisher) or estimated using packed cell volume (PCV, Sartorius Volupac), as appropriate.
METHOD DETAILS

\[ ^{13}\text{C}-\text{FA tracing} \]

For the \[^{13}\text{C}\]-oleate washout experiment, DGAT2 knockout A498 cells were seeded in 6-well plates and serum starved in DMEM containing 0.5% dialyzed FBS for 24h. The medium was then replaced with new medium containing 10 \( \mu \text{M} \) \[^{13}\text{C}\]-oleate (Sigma) with or without DGAT1i (T863, 1 \( \mu \text{M} \)) and incubated for 24h. The medium containing \[^{13}\text{C}\]-oleate was replaced with fresh medium (0.5% dFBS). Cells were incubated for 48h in these conditions followed by lipid extraction as described in Lipid extraction and liquid chromatography - mass spectrometry (LC-MS) analysis, below. The tracing experiment was repeated three times independently with each condition conducted in triplicate.

\[ \text{qRT-PCR studies} \]

Total RNA was isolated using the RNeasy purification kit (Qiagen). cDNA was synthesized using the Applied Biosystems High Capacity RNA-to-cDNA master mix. qRT-PCR was performed on a Viia7 Real Time PCR systems from Applied Biosystems. Pre-designed Taqman primers were obtained from Life Technologies for the following genes: TBP (HS01060665_G1), ACTB (HS01060665_G1), DGAT1 (HS01017541_M1), and DGAT2 (HS01045913_M1).

\[ \text{DGAT mutant and knockdown lines} \]

DGAT1 and DGAT2 shRNA (specified as DGAT shRNA unless stated otherwise) was achieved by expressing Dox-inducible shDGAT2_5 (TRCN0000005195) using the Tet-pLKO-puro plasmid and shDGAT1_1 (TRCN0000036151) using the Tet-pLKO-neo. Lentivirus was generated for each plasmid in HEK293T cells and used to infect the relevant cell line. After selection with puromycin and G418, the knockdown of both DGAT1 and DGAT2 transcripts was confirmed by qRT-PCR (Tagman probes; ThermoFisher) DGAT2 knockout cell lines were generated by cloning sgRNA sequences \( 5'-\text{TGTGCTCTACCTTGACCTGC-3'} \) and \( 5'-\text{GTACATTAGGATGCCACTTCG-3'} \) into the lentiviral vector lentCrisprv2 (Addgene), generating lentivirus in HEK293T cells and transducing ccRCC cell lines with 25\( \mu \text{M} \) of un-concentrated supernatant. After puromycin selection, single cell clones were generated by limiting dilutions in 96 well plates. Single-cell clones were expanded and genomic DNA was isolated from a portion of the expanded cell population. PCR of the DGAT2 locus was performed using DA182 (TCCTCTTGCCCAGGAATCTG) forward and DA184 (TACTGGGAGTGGCaTGCAGTGCCAT) as well as DA199 and DA201 (GTTTCTgcggccgcTCAATGGTGATGGTGATGATG) as reverse primers. PCR products were TOPO-cloned using the Zero Blunt PCR cloning kit (ThermoFisher) and transformed into competent E. coli. For each clone, 3-6 colonies were picked and grown overnight in LB-Ampicillin. Plasmid DNA was isolated using the Qiaprep Miniprep kit (Qiagen) and sequenced using a nested primer DA183 (GAATCTGCTCC). Clones containing mutations in both alleles were tested phenotypically by Bodipy neutral lipid staining, and LC/MS confirmed reduced TG production (data not shown), as expected. Cells were incubated with 1:50 dilution of oleic acid-BSA mix (Sigma; 2 mole OA/mole albumin; 100mg/ml albumin) for 16h with and without the presence of 2 \( \mu \text{M} \) DGAT1i (T863, Sigma). Clonal lines with full loss of DGAT2 activity loss had complete abrogation of neutral lipid storage by oleic acid stimulation when DGAT1 was inhibited. Rescue of DGAT2 loss was performed by cloning DGAT2 cDNA from the pcDNA3.1 vector (GeneCopoeia) into the pCDH lentiviral expression plasmid pCDH-CMV-MCS-EF1-Neo using the primers DA199 (GGCTTCTgtagcATGAGAACCCTCATAGCCG), DA200 (GTTTCTgcggccgTCAATGGTGATGGTGATG) as well as DA199 and DA201 (ggtctgGCGGCCGctcaGTCTACCTTGACCTGCAG) for expression with and without V5 and Histags. RNA was then mutated to prevent cutting by Cas9 protein expressed in the mutant cell lines. Synonymous mutations were introduced using DA187 (TACTGGGAGTGGCaTGCAGTGCCAT) and DA188 (ATGGGACTGCAaTGCAGTGCCAT).

\[ \text{Flow cytometry assays} \]

For experiments, cells were seeded in 6-well plates 24 hours before the experiment at a cell density that led to 80% confluency at the end of the experiment. BODIPY 493/503 (Life Technologies) forward and DA184 (TACTGGGAGTGGCaTGCAGTGCCAT) as reverse primers. qRT-PCR was performed on a Viia7 Real Time PCR systems from Applied Biosystems. Pre-designed Taqman primers were obtained from Life Technologies for the following genes: TBP (HS01060665_G1), ACTB (HS01060665_G1), DGAT1 (HS01017541_M1), and DGAT2 (HS01045913_M1).

\[ \text{Lipid droplet imaging} \]

Cells were seeded on round glass coverslips of 24-well plates and supplemented with 1 mL of medium and exposed to the indicated conditions. The medium was then aspirated, cells washed once with 1 mL room temperature PBS, fixed with 0.5 mL of 4% formaldehyde (Sigma) for 30 min after which excess was removed and cells washed 3x with 1 mL PBS. Cells then were incubated with 0.3 mL of 1 \( \mu \text{M} \) BODIPY 493/503 (Life Technologies) (excitation wavelength 480nm, emission maximum 515 nm) for 15 min in the dark, washed 2x with 1 mL PBS, incubated with 0.3 mL of 1 \( \mu \text{g/mL} \) DAPI (Sigma) for 15 min in the dark and washed 2x with 1 mL PBS. Thereafter, the coverslips were mounted on glass slides using Dako Fluorescent Mounting Medium (Dako). Z stack images were acquired using Olympus FV1000 confocal laser scanning microscope (405 nm laser for DAPI and 488 nm laser for BODIPY) and processed with ImageJ software.
**Lipid extraction and liquid chromatography - mass spectrometry (LC-MS) analysis**

For cultured cells the medium was aspirated and cells washed 2x with 1 mL room temperature PBS. The cells were then scraped into glass tubes (Fisher Scientific), 0.5 mL chloroform at −20 °C and 50 μL of 1 mg/mL methanol/butylated hydroxytoluene (BHT, Sigma) added, followed by addition of SPLASH lipidomix internal standard mix (Avanti Polar Lipids) at 1 μL per 10^5 cells. This was vortexed for 1 min and centrifuged at 500 g for 10 min. The chloroform layer was transferred to a new glass vial, dried under nitrogen gas and stored at −20 °C for further LC-MS analysis. Samples were reconstituted in chloroform/methanol (1:1 v/v) at 50 μL per 10^5 cells prior to the LC-MS analysis.

For extraction of tumor tissues, 10-35 mg was transferred to ice-cold Precells lysing tubes, 0.75 mL of methanol/PBS (1:1, v/v) at −20 °C and 50 μL of 1 mg/mL BHT in methanol added, and homogenized using pre-cooled Precellys Tissue Homogenizer at −10 °C. The homogenization program included 3 cycles of 30 s of shaking at 5,000 rpm and 15 s pause per cycle. Further sample treatment was as for cultured cells, with addition of 0.5 mL chloroform at −20 °C and internal standard mix at 10 μL per 10 mg tissue. Samples were reconstituted at a concentration of 200 μL per 10 mg of tissue in chloroform/methanol (1:1 v/v) prior to the LC-MS analysis.

Lipidomic analysis was performed using a Q Exactive orbitrap mass spectrometer coupled to a Dionex UltiMate 3000 LC system (Thermo Scientific). The LC parameters were as follows: 4 μL of sample was injected onto a 1.7 μm particle 100 × 2.1 mm ID Waters Acquity CSH C18 column (Waters) which was kept at 50 °C. A gradient of (A) water/acetonitrile (40:60, v/v) with 10 mM ammonium formate and (B) acetonitrile/2-propanol (10:90, v/v) with 10 mM ammonium formate at a flow rate of 0.3 mL/min was used. The gradient ran from 0% to 40% B over 6 min, then from 40% to 100% B in the next 24 min, followed by 100% B for 4 min, and then returned to 0% B in 2 min where it was kept for 4 min (40 min total). Lipids were analyzed in both positive and negative mode. The electrospray and mass spec settings were as follows: spray voltage 3 kV (positive mode) and 3.5 kV (negative mode), capillary temperature 300 °C, sheath gas flow 50 (arbitrary units), auxiliary gas flow 7 (arbitrary units) and sweep gas flow 5 (arbitrary units). The mass spec analysis was performed in a full MS and data dependent MS2 (Top 10) mode, with a full scan range of 300-1200 m/z, resolution 70,000, automatic gain control at 1x10^6 with a maximum injection time of 250 ms. MS2 parameters were: resolution 17,500, automatic gain control was set at 1x10^5 with a maximum injection time of 120ms.

**Lipase inhibitor assays**

A498, 786-O or UMRC2 cells were grown in 0.5% serum (low serum) for 24h, then loaded with 10 μM U-13C olate for 24h incubation under low serum. Labeled oleate was washed out by growing cells under low serum for 48h with or without addition of a lipase inhibitor. ATGL inhibitor atglistatin (Sigma SML1075) was used at 50 μM, HSL inhibitor CAY10499 (Cayman chemicals) was used at 50 μM and MAGL inhibitor JKK048 (Tocris) was used at 50 μM. Cells were then counted and harvested for lipidomic analysis.

**Microarray experiments**

For in vivo analysis of gene expression following inducible DGAT knockdown, mice bearing 300 mm3 tumors from subcutaneously injected A498 DGAT shRNA cells were fed either doxycycline- or control- chow for 5 days. The animals were then sacrificed, the tumors harvested and RNA was extracted using the RNEasy kit (QIAGEN). RNA was then deposited with the University of Pennsylvania Molecular Profiling core facility for processing, microarray analysis using the Affymetrix HTA 2.0 Chip and analysis.

**Suitability of using 13C-labeled FAs to study lipid metabolism**

FAs supplied to cells can be used for oxidation to generate energy, can directly be incorporated into lipids, or can first be further matured (i.e., elongated, desaturated) prior to lipid assembly. Oxidation of 13C-labeled FAs leads to generation of 13C-acetyl-CoA, which in turn can be used for the synthesis of new FAs. This would lead to complex labeling distributions that would complicate interpretation of labeling patterns and hence lipid metabolic events. To determine the feasibility of using 13C-FAs to study lipid metabolism, A498 cells were incubated for 6 hours with 25 μM [U-13C]-stearate (C18:0) and labeling of triglycerides was assessed (Figure S5A). This short time span was sufficient to generate extensively labeled TGs. Notably, after correcting for natural 13C occurrence, the majority of TG isotopologs observed were the unlabeled (M0), the M+18 isotope corresponding to the incorporation of one [U-13C]-C18:0, as well as M+36 and M+54 that result from the incorporation of 2 and 3 labeled FAs, respectively. Some minor odd-labeled isotopes (M+19, M+37, M+55) were observed; these are most likely caused by imperfect corrections for 13C-natural abundance by the algorithm. Importantly, no significant partial labeling was observed, demonstrating that FA synthesis from 13C-acetyl-CoA due to FA oxidation is not detectable and does not complicate FA tracing experiments.

Fragmentation spectra of labeled TGs further confirmed that labeled FAs shorter than 18 carbons do not occur. This is arguably best demonstrated by the MS2 pattern of TG(48:0) M+18 (one labeled FA, Figure S5B). While one could assume that TG(48:0) is primarily made up of 3x C16:0 (palmitate), the fragmentation pattern actually reveals a mixture of FA compositions, which each combination totaling 48 carbons (16:0/16:0/16:0, 16:0/18:0/14:0, 12:0/18:0/18:0). This means that these TGs have the same mass and do not separate by LC-MS. While this should be kept in mind, it does not affect interpretation of the labeling pattern. Importantly, in TG(48:0) M+18 and other TGs (data not shown) only [U-13C]-C18:0 is observed and no shorter 13C-FAs, further demonstrating that partial oxidation of labeled FAs does not occur in these cells.
We did find that Labeled stearate is desaturated and elongated leading to FAs such as [U\(^{13}\)C]-18:1 and [\(^{13}\)C\(_1\)]-20:0 as well as longer chain FAs, as evidenced by the direct observation of their acylium ions in MS\(^2\) (FAs are observed as their acylium ions in positive mode MS\(^2\), Figure S5C). We therefore concluded that tracing with \(^{13}\)C-labeled FAs is suitable for investigating the dynamics of lipid metabolism.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Lipidomic data processing**

Peak detection, peak area quantification, lipid identification, and alignment were performed using LipidSearch (Thermo Fisher Scientific/Mitsui Knowledge Industries) with standard settings for Q Exactive Product Search. Data was then exported to Excel and lipid peak areas were normalized to the peak area of the corresponding lipid internal standard using an in-house R script. The normalized peak areas of identified lipids were used for plotting.

Volcano plots were generated using `ggplot2` R package by plotting \(\log\) Fold change (n = 5 for each condition) against \(-\log\) P value (Wickham, 2009). Significant changes with \(\geq\) 1.5-fold and \(p \leq 0.05\) are indicated in color according to the figure legend.

Saturation indices for different lipid classes are represented as a ratio of total palmitate and stearate to oleate. The total level of palmitate in individual lipid class was calculated by summing up the intensities of each palmitate-containing lipid multiplied by the number of palmitate moieties in each lipid (i.e., total palmitate in TG = \(\Sigma (1\times\text{TG}(16:0/18:0/18:1) + 2\times\text{TG}(16:0/16:0/18:1) + 3\times\text{TG}(16:0/16:0/16:0))\)). The same principle was used for calculation of total stearate and oleate.

For stable isotope tracing experiments MAVEN software was used. A total \(^{13}\)C-FA incorporation value for each lipid class was calculated by summing up the labeling intensities for those lipids of that class that were most intensely labeled and together contained \(\geq 80\%\) of total label. A labeling intensity per lipid was calculated by summing up the intensities for each labeled isotope multiplied by the number of labeled FAs for that particular isotope (i.e., total labeling = \(\Sigma (1\times\text{M+18} + 2\times\text{M+36} + 3\times\text{M+54})\)).

**Statistical Analysis**

For bar plot, the height of the bar represents the mean of all replicates and error bars represent \(\pm\) SD or \(\pm\) SEM, as indicated in the Figure Legends and Supplemental Figure Legends. Replicate numbers, statistical tests used and explanations for error bars are indicated in the Figure Legends and Supplemental Figure Legends. Statistical significance was derived using R or GraphPad Prism 7.0 by t test or ANOVA, as appropriate; \(p < 0.05\), \(**p < 0.01\) and \(***p < 0.001\).

**DATA AND SOFTWARE AVAILABILITY**

The A498 DGAT shRNA in vivo microarray experiment and the A498 DGAT shRNA in vitro microarray experiment data were deposited at NCBI GEO ([https://www.ncbi.nlm.nih.gov/geo/](https://www.ncbi.nlm.nih.gov/geo/)) under accession numbers GEO: GSE117774 and GSE117775 respectively.