HILPDA Regulates Lipid Metabolism, Lipid Droplet Abundance, and Response to Microenvironmental Stress in Solid Tumors

Matthew J. VandeKopple, Jinghai Wu, Erich N. Auer, Amato J. Giaccia, Nicholas C. Denko, and Ioanna Papandreou

Abstract

Accumulation of lipid droplets has been observed in an increasing range of tumors. However, the molecular determinants of this phenotype and the impact of the tumor microenvironment on lipid droplet dynamics are not well defined. The hypoxia-inducible and lipid droplet associated protein HILPDA is known to regulate lipid storage and physiologic responses to feeding conditions in mice, and was recently shown to promote hypoxic lipid droplet formation through inhibition of the rate-limiting lipase adipose triglyceride lipase (ATGL). Here, we identify fatty acid loading and nutrient deprivation–induced autophagy as stimuli of HILPDA-dependent lipid droplet growth. Using mouse embryonic fibroblasts and human tumor cells, we found that genetic ablation of HILPDA compromised hypoxia–fatty acid– and starvation-induced lipid droplet formation and triglyceride storage. Nutrient deprivation upregulated HILPDA protein posttranscriptionally by a mechanism requiring autophagic flux and lipid droplet turnover, independent of HIF1 transactivation. Mechanistically, loss of HILPDA led to elevated lipolysis, which could be corrected by inhibition of ATGL. Lipidomic analysis revealed not only quantitative but also qualitative differences in the glycerolipid and phospholipid profile of HILPDA wild-type and knockout cells, indicating additional HILPDA functions affecting lipid metabolism. Deletion studies of HILPDA mutants identified the N-terminal hydropathic domain as sufficient for targeting to lipid droplets and restoration of triglyceride storage. In vivo, HILPDA-ablated cells showed decreased intratumoral triglyceride levels and impaired xenograft tumor growth associated with elevated levels of apoptosis.

Implications: Tumor microenvironmental stresses induce changes in lipid droplet dynamics via HILPDA. Regulation of triglyceride hydrolysis is crucial for cell homeostasis and tumor growth.

Introduction

Metabolic networks respond to fluctuations in the supply of substrates in order to maintain homeostasis. Microenvironmental stress conditions commonly found in solid tumors, such as nutrient and oxygen deprivation, have well-recognized effects on metabolic pathways, including but not limited to promotion of glycolysis, inhibition of mitochondrial function, and induction of autophagic recycling (1). In parallel, changes in lipid metabolism also serve the tumors’ requirements for energy production, membrane biosynthesis, redox balance, and protection from lipotoxicity (2). Hypoxia increases the fraction of glutamine-derived carbons used for citrate production and de novo lipogenesis, enhances the uptake of lysophospholipids, and can promote lipid droplet formation by mechanisms involving fatty acid uptake through the fatty acid binding proteins FABP3 and FABP7, induction of the phosphatidate phosphatase Lipin1, and repression of the carnitine palmitoyltransferase CPT1A (3–7).

Formation of lipid droplets has been observed in an increasing number of physiologic and pathologic conditions and virtually all tested cell types. Lipid droplets are not only sites of neutral lipid storage, but also dynamic hubs of signal transduction, lipid metabolism, viral replication, and protein trafficking, with significant impact on normal physiology and metabolic diseases (8). Their neutral lipid core consists mostly of triglycerides, diglycerides, and cholesteryl esters and to lesser degrees other esterified lipids; the core is enclosed in a phospholipid monolayer and proteins decorate the periphery (9). The lipid droplet proteome encompasses lipid metabolic enzymes and an increasing number of regulatory proteins which are either constitutively bound or present in distinct spatiotemporal patterns. The lipid content is protected from hydrolysis by proteins that block the access to lipolytic enzymes. The majority of our knowledge on lipolytic enzyme sequestration on the lipid droplet surface involves the perilipin family (PLIN), particularly Plin1. Plin1, which is abundantly expressed in adipocytes, protects lipid droplets from hydrolysis under basal conditions and responds to beta adrenergic signals through a phosphorylation cascade resulting in recruitment of ATGL/PNPLA2 (adipose triglyceride lipase), interaction with its coactivator CGI-58, recruitment of HSL (hormone-sensitive lipase) and stimulation of lipolysis (10).
Interestingly, lipid droplet formation is observed not only in lipid-rich conditions but also during nutrient starvation and induction of autophagy. Nutrient deprivation–induced autophagy recycles intracellular components to generate primary substrates for energy production and survival. In the absence of extracellular nutrients, preexisting phospholipids are broken down by group IVA phospholipase A_2 (cPLA_2) and are stored in lipid droplets as triglycerides before they get oxidized in the mitochondria (11). Starvation-induced lipid droplet turnover requires the formation of triglycerides by diacylglycerol acyltransferase 1 (DGAT1), which are subsequently hydrolyzed by ATGL (12, 13). The seemingly counterintuitive storage of lipid under nutrient deplete conditions is proposed to regulate fatty acid entry to the mitochondria and protect from lipotoxicity, by mechanisms that are not completely understood (12, 13).

The biological significance of lipid accumulation in malignant progression is becoming increasingly appreciated. Lipid droplet formation appears to be genetically programmed in response to tumor suppressor loss (14, 15) or to be part of a stress response to cytotoxic therapies (16–18). Inhibition of lipid storage has been shown to reduce tumor growth (14–16, 19, 20), although some tumor types favor lipid oxidation over esterification (21). HILPDA (formerly known as HIG2) expression is induced under hypoxic conditions and the protein localizes to lipid droplets (22, 23); however, its mechanistic function remains poorly defined. At the organismal level, we and others have shown that mouse HILPDA plays physiologic roles in lipid storage, systemic glucose metabolism, and body temperature defense, and that its expression response to both hypoxia and hypoxia-independent stimuli, such as PPAR and cAMP signaling (24–29). HILPDA mRNA is highly overexpressed in lipid-rich tumor types, including colorectal and renal clear cell carcinomas, and positively regulates model tumor growth (23, 30, 31). Recently, interaction of HILPDA with ATGL was reported, along with evidence that this binding decreases ATGL hydrolytic activity and promotes adaptation to hypoxia (31, 32). Here, we asked what nutritional stress responses, in addition to hypoxia, may depend on HILPDA for lipid droplet formation, if there is specificity in lipid classes stored under these conditions, and how these metabolic perturbations impact tumor growth in vivo.

Materials and Methods

Cell culture and media manipulations

Transformed MEFs from HILPDA WT and KO embryos were established by our group (29). HIF1α WT and KO MEFs have been described previously (33). HCT116 cells were obtained from ATCC in 2013 and have not been authenticated since then. All cell lines were maintained in DMEM supplemented with 10% FBS, and stocks were kept in culture for up to 4 months. Samples were routinely assayed for Mycoplasma contamination by Hoechst 33258 staining. Ear's buffered salt solution (Fisher) CQ (20 μmol/L, Sigma-Aldrich), ATGLi (25 μmol/L, Sigma-Aldrich), triacsin C (5 μmol/L, Abcam), DGAT1i (10 μmol/L, Sigma-Aldrich), phospholipase inhibitor (20 μmol/L, Abcam), and oleate/oleate (30 μmol/L each, Sigma-Aldrich) were used as indicated. Hypoxia was produced using a Whitley H35 Hypoxystation set at 1% O_2.

Fluorescence microscopy

Cells were grown on glass coverslips, treated as required, and fixed with 4% paraformaldehyde. Lipid droplets were visualized by staining with 0.1 μg/mL Nile red (Santa Cruz Biotechnology) or HCS LipidTox Green (1:200, Invitrogen H34475). For costaining, cells were fixed with 4% paraformaldehyde, blocked for 1 hour in IF solution (PBS, 2% BSA, 0.1% Triton-X100), and incubated overnight with mouse a- myc (1:250, Cell Signaling Technology 2276S), followed by donkey anti-mouse Alexa Fluor 594 secondary (1:400, Invitrogen R37115), donkey anti-rabbit Alexa Fluor 488 (1:400, Invitrogen A21206) and LipidTox green (1:200, Invitrogen H34475) for 1 hour at room temperature. Finally, cells were incubated with Hoechst 33342 nuclear stain for 5 minutes, and mounted with SlowFade Diamond antifade mounting media (Life Technology S36968). Slides were imaged on an Olympus FY000 confocal microscope or a Zeiss AxioSkope wide field microscope. Lipid droplet numbers and sizes were quantified with ImageJ and the particle counting plugin.

Flow cytometry

Cells grown in 6-well plates were treated as required, harvested using trypsin-EDTA, and resuspended in DMEM with 2 μmol/L BODIPY 493/503 (Fisher). Following a 20-minute incubation at 37°C in the dark, cells were pelleted, resuspended in PBS, and run on a BD FACScalibur cell analyzer at the OSUCCC analytical cytometry core.

Lipid measurements

Triglycerides were measured using the Triglyceride Quantification Assay Kit (Abcam, ab65336). Glycerol was quantified in media with the Glycerol Assay Kit (MAK117-1KT, Sigma-Aldrich). For thin layer chromatography (TLC) analysis, lipids from 6 million cells were extracted with hexane/isopropanol (3:2), dried under a nitrogen stream, and resuspended in 20 μL chloroform. Ten μL of lipid extract were spotted on 20 × 20 cm silica gel plates (Analtech, P11011) along with lipid standards (glycerol trioleate Sigma-Aldrich T7140, Cholesterol oleate Sigma-Aldrich C9253) and developed in 80:10:1 cyclohexane:ethyl acetate:acetic acid mixture. Dried plates were sprayed with 0.2% primuline dissolved in 80:20 acetone:water and imaged on a ProteinSimple FluorChem E gel imaging system. Glycolipid mass spectrometric analysis was performed at the University of California San Diego lipidomics core essentially as described previously (34). Briefly, 400,000 cells were supplemented with internal standards and extracted with methanol and dichloromethane according to the Bligh and Dyer method. The extracts were brought to dryness under Argon, the extracted lipids were dissolved in 50 μL hexane/MTBE (95:5)/4.5/5. v/v) and analyzed by mass spectrometry (Sciex 6500 Qtrap). For quantitative measurement of phospholipid classes, the samples were spiked with internal standards, and dried lipid extracts were resuspended in 59/40/1 IPA/HEX/H_2O with 10 mmol/L NH_4OAc followed by LC/MS.

Western blotting

Proteins were extracted in RIPA buffer supplemented with 100× Halt protease inhibitor cocktail (Thermo) and 1 mmol/L PMSF (Boston BioProducts). Lysates were sonicated, cleared by centrifugation, and the protein concentration measured with a BCA kit (Thermo). Thirty μg of protein/lane were resolved in 12.5% or 14% acrylamide gels and transferred onto the PVDF membrane. Primary antibodies used were custom-made rabbit...
anti-Hilpda 1:250 (29), rabbit a-Perilipin2 (1:1,000, Origene TA321279), rabbit a-SCD1 (Cell Signaling Technology 2438), rabbit a-G0s2 (Epitek E05229), rabbit a-ELOVL6 (Millipore Sigma AB5456), rabbit a-LC3 (1:1,000, MBL International PM036), rabbit a-p62 (1:1,000, Santa Cruz Biotechnology sc-28359), mouse a-tubulin (1:1,000, Invitrogen PIM516308), rabbit a-FLAG (1:1,000, Sigma F7425), mouse a-myc (1:1,000, Cell Signaling Technology 2276). Primary antibodies were detected using LI-COR goat anti-mouse (1:3,000, LI-COR 926-68070), or goat anti-rabbit (1:3,000, LI-COR 926-32211) secondary antibodies, and visualized with a LI-COR Odyssey CLx imager.

Molecular biology methods

Human HILPDA truncation mutants were subcloned from the pCDNA3.1 + /C-KDYR (HsHILPDA) ORF clone (GenScript OHu08913). Primers with a 5’ reverse linker sequences (restriction sites underlined) were used to amplify truncated coding sequences by PCR (Platinum PFX polymerase, Life Technologies 68070), or goat anti-rabbit (1:3,000, LI-COR 926-32211) secondary antibodies, and visualized with a LI-COR Odyssey CLx imager.

Statistical analysis

Data were analyzed in GraphPad Prism 7. Comparison of >2 groups was done using two-way ANOVA followed by Bonferroni, Dunnet, or Holm–Sidak multiple comparisons tests. Student t test was used for 2 group comparisons. P < 0.05 was considered statistically significant.

Results

Hypoxia and exogenous fatty acids induce HILPDA-dependent LD formation

By using whole body genetic ablation of HILPDA in mice, we recently reported that HILPDA participates in thermoregulation under stress in vivo (29). In order to delineate the function of HILPDA at the cellular level, we isolated MEFs from HILPDA WT and KO embryos (hereafter referred to as WT and KOs), immortalized them with SV40 T-antigen and transformed them with H-ras (29). Based on the reported lipid droplet localization of HILPDA (23), we hypothesized that its loss may affect lipid droplet number and/or size. Cells were either kept under basal culture conditions, incubated in hypoxia for 72 hours, or supplemented with 60 μM oleate and linoleate complexed with BSA for 24 hours (FA loading), and lipid droplets were visualized by Nile red staining and fluorescent microscopy (Fig. 1A) or BODIPY 493/503 staining and FACS (Supplementary Fig. S1A). Loss of HILPDA severely blunted visible LD formation under hypoxia and FA loading, and the changes in lipid storage were paralleled by upregulation of the HILPDA protein (Fig. 1B).

In vivo xenograft growth

All animal work was approved by OSU’s Institutional Animal Care and Use Committee. Five million cells in PBS were injected subcutaneously on the back of 7- to 9-week-old female nude mice (Charles River Laboratories), and tumor growth was measured weekly using calipers.

Excised tumors were snap-frozen in OCT medium, and cryosections were prepared at the OSUCCC Comparative Pathology and Mouse Phenotypic core. For IHC detection of Ki67 and cleaved caspase-3, slides were equilibrated at room temperature for 20 minutes, fixed with 4% paraformaldehyde, and blocked for 1 hour in IF solution (PBS, 2% BSA, 0.1% Triton-X100). Slides were then incubated overnight with rabbit anti-Ki67 (1:250, Abcam ab15580) or rabbit anti-cleaved caspase-3 (1:250, Cell Signaling Technology 9664S), followed by donkey anti-rabbit 488 (1:400, Life Technologies A21206) or goat anti-rabbit 594 (1:400, Life Technologies A11012), respectively, for 1 hour at room temperature. Finally, nuclei stained with Hoechst 33342 and mounted with SlowFade Diamond antifade mounting media (Life Technology S36968). Slides were imaged with a Zeiss Axioscope microscope equipped with a CCD camera.
Figure 1.
HILPDA-dependent lipid droplet formation. A, HILPDA WT and KO MEFs were kept in complete media (CM), incubated in 1% O2 for 72 hours or supplemented with 60 μmol/L oleate/linoleate/BSA complexes for 24 hours (FA), and lipid droplets were stained with Nile red. B, HILPDA protein levels in WT and KO MEFs treated as in A. C, Quantification of LDs per cell in WT and KO MEFs in complete media or supplemented with FA for 24 hours. D, LD diameter distribution in WT and KO MEFs. Floating bars, minimum to maximum, vertical line at the median. E, Restoration of HILPDA expression in KO MEFs. Cells were transfected with either pLenti-Hilpda-C-Myc-DDK-IRES-neo (KO + Hilpda) or empty vector (KO) and stable transfectants were selected by G418 resistance. Black arrow: endogenous HILPDA, gray arrow: HILPDA-myc-Flag. Asterisk: nonspecific. F, Nile red staining of KO MEFs and pool of reintroduced clones following treatment with oleate/linoleate for 24 hours. G, Genetic manipulations in HCT116 cells. Knockout lines were generated by the nickase CRISPR technology and four independent KO clones after puromycin-resistance selection were pooled together. H, Quantification of LD per cell in complete media and after FA loading. I, TLC of hexane/isopropanol extracted lipids separated in cyclohexane:diethyl acetate:acetic acid and stained with primuline. Lipid class standards: CE, cholesteryl esters; TAG, triglycerols; FA, fatty acid. ****, P < 0.0001; N.S., not significant by one-way ANOVA and Sidak post hoc test.
expression in the KO by a C-terminus Myc/Flag-tagged construct, which was able to restore LDs (Fig. 1E and F). Next, we extended these findings in a diverse cell system of human colorectal cancer in order to rule out murine-specific effects. HILPDA was knocked out using CRISPR technology in HCT116 cells, followed by puromycin selection. In agreement with the MEF findings, HILPDA was upregulated by hypoxia and fatty acid loading (Fig. 1G), and HILPDA KO cells contained fewer Nile red positive lipid droplets after FA loading (mean difference: at baseline 1.49, after FA loading 18.5; Fig. 1H) or BODIPY 493/503 staining (Supplementary Fig. S1C–S1D).

Next, we performed TLC of hexane/isopropanol extracted non-polar components to identify lipid classes regulated by HILPDA (Fig. 1I). In agreement with the LD staining data, the KO MEF and KO HCT116 contained lower levels of triglycerides under basal conditions and when supplemented with fatty acids, whereas cholesteryl esters showed no difference.

**HILPDA loss causes qualitative and quantitative changes in glycerolipids and phospholipids**

In order to get a more comprehensive picture of HILPDA’s impact on triglyceride levels, we measured relative di- and triglyceride isobaric species abundance by LC/MS in WT and KO HCT116 cells under normoxia, hypoxia, or fatty acid loading (Fig. 2). In agreement with the previous approaches, triglyceride steady-state levels were lower in the HILPDA ablated cells, and their ability to elevate them after a hypoxic stimulus or fatty acid feeding was significantly impaired (Fig. 2A and B). Interestingly, the normoxic KO cells contained detectable levels of diglycerides, which were undetectable in the WT, but they were nonetheless impaired in their stimulus-induced accumulation (Supplementary Fig. S2A). Hypoxia is known to decrease the relative abundance of desaturated lipids by inhibiting the oxygen-consuming enzyme Δ9 stearoyl-CoA desaturase (SCD1; refs. 7, 35) so we asked if HILPDA preferentially regulates the storage of triglycerides with saturated or unsaturated fatty acid chains (Supplementary Fig. S2B–S2C). Because the LC/MS detection does not distinguish isobaric species, all triglycerides with at least one double bond were assigned to the unsaturated list. In agreement with the reported decrease of the fatty acid desaturation index under hypoxia, accumulation of triglycerides under hypoxia was more pronounced for saturated than unsaturated species (6.2-fold vs. 1.7-fold). HILPDA-ablated cells were impaired in the hypoxia-induced storage of all triglycerides. Under all tested conditions, the majority of TAGs were unsaturated; hypoxia decreased their relative abundance, while supplementation of oleic and linoleic acid mixture (C18:1 and C18:2, respectively) increased it (Fig. 2C). The HILPDA KO cells contained a lower fraction of unsaturated TAGs in normoxia but moderately higher fraction under hypoxia. In addition, HILPDA loss altered the distribution of TAG species toward longer carbon chain lengths under stress (Fig. 2D–F). Under normoxia, the most abundant TAG species had a total carbon length of 52 (C52), followed by species with 50C atoms regardless of HILPDA status. Hypoxia overall shifted the distribution toward shorter C lengths; however, the KO cells had fewer species with C<50 and more with C>50. Fatty acid loading (C18) led to the formation of longer species and, consistent with the hypoxic data, HILPDA loss shifted the distribution frequencies to the right. Overall, the data suggest that HILPDA is a positive regulator of TAG accumulation and that, via either primary or secondary mechanisms, it can affect fatty acid metabolic processes such as desaturation and elongation.

Given the strong biochemical and biological connections between triglyceride and phospholipid metabolism (36), we asked if HILPDA loss also perturbs phospholipid abundance under stress conditions in vitro. Quantitative analysis of phospholipid classes by LC/MS detected phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI), and lysophosphatidylcholine (LPC). Phosphatidylserine, phosphatic and lysosphosphatic acid, and other lysosphospholipid classes were below detection levels (data not shown). Under normoxia, HILPDA WT and KO HCT116 cells had very similar amounts of PC, PE, and PI. Interestingly, under hypoxia, but not fatty acid loading, the KO cells contained more PC, PE, and PI by 21.7%, 21%, and 39.5%, compared with WT, respectively (Fig. 2G–I). LPC levels were not regulated by HILPDA status (data not shown). To test whether these HILPDA-dependent processes affect gene expression, we measured transcript or protein levels of select lipid metabolic enzymes and LD-localized proteins (Fig. 2J–L). Because of the lipidomic results, we checked the levels of long-chain fatty acid elongases ELOVL1, ELOVL6, and ELOVL7, whose activity is the highest for saturated and mono-unsaturated substrates. Neither transcript nor protein levels showed changes by the HILPDA status. Similarly, protein expression of the LD protein Plin3 or the ATGL inhibitor G0S2 was HILPDA-independent under these conditions. Interestingly, FA-induced Plin2 and hypoxia-induced fatty acid desaturase SCD1 were diminished in the absence of HILPDA. These results support the notion that HILPDA lies at a crucial node of hypoxic lipid metabolism, and its loss triggers widespread primary and secondary alterations in lipid profiles and protein expression.

An established function of LDs is the storage of lipids for generation of secondary messengers and inflammatory mediators including prostaglandin E2 (PGE2; refs. 27, 37), so we measured PGE2 secretion in the genetically matched HCT116 WT and KO cells (Fig. 2M). Consistent with the positive association between LD abundance and eicosanoid synthesis, PGE2 secretion was significantly higher in the presence of HILPDA after FA loading. In normoxia and hypoxia there was a trend toward decreased PGE2 secretion by the HILPDA KO but it did not reach statistical significance.

**Accumulation of LDs during starvation requires HILPDA**

Nutrient starvation elicits a number of metabolic responses that coordinately limit energy-consuming processes such as protein translation, and mobilize intracellular nutrients through autophagic recycling. Interestingly, nutrient deprivation also induces LD biogenesis to regulate the flux of recycling-generated fatty acids (38). Because HILPDA positively regulated hypoxia- and fatty acid–induced LD growth, we hypothesized that it may also mediate the starvation response. Incubation in Earle’s balanced salt solution (EBSS) for 24 hours increased the abundance of LDs in both MEFs and HCT116 in a HILPDA-dependent manner (Fig. 3A), and was associated with upregulation of the HILPDA protein (Fig. 3B). The loss of LD expansion in the absence of HILPDA was not due to a possible failure to activate the autophagic response, as LC3 processing and SQSTM1/p62 clearance after treatment were rescued by 20 μM/L of the autophagy inhibitor chloroquine to similar degrees independently of HILPDA status (Fig. 3C). This was confirmed by densitometric

HILPDA Controls Lipid Metabolism Under Stress

www.aacrjournals.org Mol Cancer Res; 17(10) October 2019 2093

Downloaded from mcr.aacrjournals.org on February 3, 2021. © 2019 American Association for Cancer Research.
Figure 2. Lipid metabolism in HCT116 WT and HILPDA KO. Cells were kept in complete media (21%), incubated in 1% O₂ for 72 hours (1%) or supplemented with 60 μmol/L oleate/linolate/BSA (FA) for 24 hours. A–F, Lipid extracts were analyzed by LC/MS and isobaric species measured against internal standards. G–I, For phospholipid measurement, the samples were spiked with internal standards. J and K, Transcript levels of ELOVL1 and ELOVL7, respectively, measured by qRT-PCR after normoxic (21%) and hypoxic (1%) incubation. L, Western blotting detection of lipid metabolic proteins in HILPDA WT and KO HCT116. M, PGE2 secretion under the same conditions as in L. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001; N.S., not significant by two-way ANOVA and Holm–Sidak test; N = 3, error bars, SEM.
quantiﬁcation of LC3II/I ratios or relative p62 protein expression (indicated by the numbers underneath each lane). Autophagic recycling has been shown to contribute to LD formation (12, 13); therefore, it was possible that defective autophagy-generated LDs was responsible for the HILPDA KO phenotype (Fig. 3D). After 24 hours incubation in EBSS media, careful quantiﬁcation showed that the KO cells contained fewer LDs than the WT cells; cotreatment with chloroquine to inhibit autophagic ﬂux decreased LD numbers in both genotypes but did not equalize their abundance, suggesting that autophagy-independent mechanisms are dysregulated in the absence of HILPDA. TAG quantitation conﬁrmed that nutrient deprivation results in their accumulation in a HILPDA-dependent manner in MEF and HCT116 cells (Fig. 3E).

LD biogenesis can posttranscriptionally stabilize HILPDA independently of HIF1

Hypoxia is known to transcriptionally induce HILPDA mRNA through binding of the HIF1 transcription factor to hypoxia responsive elements of the HILPDA promoter (22, 23). We also observed upregulation of HILPDA protein by fatty acids and starvation by as yet unknown mechanisms. To determine if transcriptional or posttranscriptional mechanisms were responsible for this latter upregulation, we measured mRNA abundance by qRT-PCR (Fig. 4A). Incubation of MEF and HCT116 at 1% O2 increased the transcript levels of HILPDA as well as PDHK1 (pyruvate dehydrogenase kinase 1), another well-established HIF1 target gene. In contrast, FA or EBSS failed to increase the mRNA of either HILPDA or PDHK1, showing that HILPDA induction under these conditions is posttranscriptional.

To rule out indirect HIF1 effects in our systems, we used immortalized Hif1α WT and KO MEF and subjected them to LD-promoting conditions. HILPDA protein was induced under hypoxia in a Hif1α-dependent manner, whereas the increase by fatty acids was Hif1 independent (3-fold over the respective baseline levels; Fig. 4B). Neutral lipid staining correlated with HILPDA levels and was lower in the Hif1α KO cells under hypoxia (Fig. 4C) but not after normoxic stresses (Fig. 4D). Based on published reports showing upregulation of lipid droplet proteins by posttranslational mechanisms after lipid loading (39), we asked if starvation-induced lipid metabolism is required for HILPDA upregulation. Indeed, pharmacologic blockade of lipid

Figure 3.
Decreased starvation-induced lipid droplet accumulation in the absence of HILPDA. A, HILPDA WT and KO MEFs and HCT116 were incubated in complete media (CM) or EBSS for 24 hours. Fixed cells were stained with LipidTox and Hoechst and imaged by confocal fluorescent microscopy. Scale bar, 10 μm. B, HILPDA protein levels after similar treatment. C, Protein levels of autophagic ﬂux markers LC3 and p62 in full media and EBSS in the absence or presence of 20 μmol/L chloroquine (CQ) for 24 hours. Relative LC3II/LC3I ratios and p62 protein levels were calculated from 3 independent experiments. Top row, average values; bottom row, SEM. D, LD quantiﬁcation in HILPDA WT and KO MEFs after 24 hours of EBSS treatment ±20 μmol/L chloroquine (CQ). E, Quantiﬁcation of glycerolipids after 24 hours in complete media (DMEM) or EBSS in MEF (left) and HCT116 (right); N = 5–6. *** P < 0.001; **** P < 0.0001; N.S., not signiﬁcant by one-way ANOVA and Holm–Sidak multiple comparisons test; error bars, SEM.
droplet formation through inhibition of either: (i) autophagy by chloroquine, (ii) phospholipase A2 by oleyloxyethylphosphocholine (OOEPC), (iii) ACSL (long-chain-acyl-CoA ligase) by triacsin C, or (iv) DGAT1 by A922500 suppressed starvation-induced HILPDA accumulation (Fig. 4E). Nile red LD staining confirmed that inhibition of these steps of triglyceride turnover blocked LD accumulation under starvation (Supplementary Fig. S3).

The N-terminal hydrophobic domain of HILPDA is sufficient for LD targeting and function

The HILPDA protein does not contain functionally annotated domains and shares only limited similarity with known proteins. In order to identify protein fragments with biological activity, we performed a structure-function analysis and asked which domain is sufficient for proper localization and for LD growth. A series of human HILPDA truncation mutants (Fig. 5A) were cloned into the bicistronic pLenti-C-Myc-DDK-IRES-neo vector, transfected into the HCT116 HILPDA KO background and stable expressors selected by G418 resistance. For each construct, drug-resistant colonies were screened for expression of the transgene and then select clones were pooled together in order to achieve similar expression levels among mutants (Supplementary Fig. S3).

Figure 4.
Posttranscriptional control of HILPDA upregulation. A, Relative levels of the HIF targets HILPDA and PDHK1 by RT-qPCR in MEF and HCT116. Samples were treated for 24 hours with: normoxia (21%), hypoxia (1%), loaded with 60 μmol/L oleate/linoleate/BSA (FA) or incubated in EBSS. Shown are results from a representative of 2 independent experiments in 3 technical replicates. B, HILPDA protein levels after 24 hours treatments in HIF1α WT and KO MEF. C and D, LipidTox staining of lipid droplets in MEF following the indicated incubations for either 72 hours (hypoxia) or 24 hours (all normoxic stresses). E, Impact of lipid turnover inhibitors on HILPDA protein in HCT116 cells. Samples were kept either in complete media (CM) or starved in EBSS in the absence or presence of 20 μmol/L chloroquine (CQ), 20 μmol/L OOEPC, 5 μmol/L Triacsin C, or 10 μmol/L A922500 for 24 hours.

HILDA inhibits ATGL activity

The reduced triglyceride storage under microenvironmental stresses after HILPDA loss could potentially derive from either decreased synthesis or elevated hydrolysis. As a surrogate for triglyceride breakdown, we measured glycerol release during autophagy in MEF (Fig. 6A) and found that after 24 hours of EBSS treatment the KO cells had released 3 times more glycerol than the WT. The first and rate-limiting step in the breakdown of
Figure 5. HILPDA truncation mutants restore lipid storage. A, Diagram of truncation mutations. B, Expression of truncation mutants in fatty acid-loaded cell lysates. Stable cell lines were generated by transfection of myc-Flag-HILPDA transgenes or empty vector (Neo) and selected by antibiotic resistance. Note that only the full length peptide is recognized by the rabbit anti-HILPDA antibody raised against the C-terminus. C, Lipid droplet localization of mutants in the HILPDA KO HCT116 background. The numbers refer to amino acid length, with H63 being the full-length peptide. Samples were loaded with 60 μmol/L oleate/linoleate/BSA for 24 hours, fixed and stained with mouse anti-myc followed by anti-mouse-Alexa594 antibodies. Lipid droplets were stained with LipidTox and nuclei with Hoechst. Samples imaged with an Olympus FV3000 confocal microscope. Bar, 10 μm. D, Triglyceride levels in the various mutants. *, P < 0.05; **, P < 0.01; ***, P < 0.001; N.S., not significant by two-way ANOVA and Dunnet test. N = 3–6, error bars, SEM.

triglycerides is catalyzed by ATGL (40); hence, we tested the small-molecule inhibitor ATGListatin (ATGLi; ref. 41) for its ability to restore triglyceride storage in the absence of HILPDA. ATGListatin blocked the elevated glycerol release of the KO cells (Fig. 6B) and restored starvation-induced LD formation (Fig. 6C). It is possible that ATGL inhibition could increase triglyceride levels independently of HILPDA. To confirm that ATGL activity is regulated by HILPDA, we first analyzed the effect of ATGLi on total neutral lipids by BODIPY 493/503 staining and flow cytometry. EBSS-treated KO MEFs showed lower staining than the WTs, whereas inhibition of ATGL accumulated lipids in both types to the same maximum levels (Supplementary Fig. S4A). To investigate a potential mechanistic interaction between HILPDA and ATGL in vivo, we confirmed HILPDA's and ATGL's localization to LDs during stress. Our custom-made polyclonal anti-HILPDA antibody is not suitable for immunofluorescence (data not shown); therefore, we used the HCT116 KO + H63 HILPDA-myc-FLAG cells described in Fig. 5. Samples were grown in complete media, EBSS, or FA-supplemented media for 24 hours, fixed and stained with mouse anti-myc and rabbit anti-ATGL antibodies (Supplementary Fig. S4B). Under all conditions, there was colocalization of exogenous HILPDA and endogenous ATGL at dot-like structures consistent with LDs.

Next, we measured LD number and diameter under starvation during physiologic or inhibited ATGL activity (Fig. 6D and E; Supplementary Fig. S4C). As also shown in Fig. 3D, HILPDA was required for maximal EBSS-induced LD size and number, with the KO cells containing fewer LDs (mean difference 8.43) and smaller LDs (mean rank difference 219.7). The calculated LD surface area per cell was 82 μm² and 34.5 μm² in the WT and KO MEFs, respectively. When ATGL activity is inhibited during starvation with ATGListatin, both genotypes accumulated fewer but larger LDs and the differences caused by HILPDA ablation were eliminated (LD number mean difference −5.05, size mean rank difference 56.7, LD surface area 116.2 vs. 135.4 μm²). Finally, we measured triglyceride levels in the presence and absence of ATGLi (Fig. 6F). Under all tested conditions, ATGL inhibition was sufficient to restore triglyceride in the HILPDA KO to the levels of the WT. Collectively, these findings support that ATGL activity is elevated in the absence of HILPDA.

HILPDA ablation impairs tumor growth and TAG storage in tumors in vivo

Next, we asked if regulation of lipolysis by HILPDA has biological consequences in tumor cell growth in vivo and in vitro. Cell proliferation in complete media under normoxic or hypoxic (1% O₂) for up to 4 days was not significantly slower in the KO cells, showing that under these conditions HILPDA is dispensable for growth (Fig. 7A). Next, HCT116 HILPDA WT and KO cells were grown as xenografted tumors in nude mice and growth was monitored over time. The KO tumors grew significantly slower (Fig. 7B), and KO tumor explants’ triglyceride content was reduced by 60% (Fig. 7C). In accordance with the low triglyceride levels, after Nile red staining of tumor tissue sections the lipid droplet-positive areas were more pronounced in the HILPDA-expressing tumors (Fig. 7D, top). The lipid droplet-containing cells were frequently seen in perinecrotic areas, which may represent hypoxic regions, further confirming the stress inducibility of HILPDA dependent of lipid storage. The mechanism for the slow growth of the KO tumors did not involve reduced proliferation, as positivity for Ki67 was similar between the groups (Fig. 7D, middle and data not shown). Rather, higher positivity
of cleaved caspase-3 in the KO samples suggests that they are sensitive to apoptosis in vivo (Fig. 7D, bottom; Fig. 7E).

Discussion

Here, we provide phenotypic and mechanistic data showing that HILPDA interacts with lipid droplets via its amphipathic N-terminus domain to decrease triglyceride hydrolysis in response to different microenvironmental conditions. These adaptive functions of HILPDA support the growth of model tumors. HILPDA has been implicated in lipid turnover in mice (24–29), and recently two independent reports provided evidence of physical interaction between HILPDA and the rate-limiting triglyceride hydrolase ATGL (31, 32), which appears to be responsible for its protumorigenic activity (31).

HILPDA is a much weaker ATGL inhibitor than the structurally related G0S2 protein in enzymatic assays with purified proteins (32); and we and others have shown that it does not directly regulate lipolysis in adipocytes (24, 26, 29). It is thus probable that specific upstream stimuli are required for HILPDA activation and/or its weak ATGL inhibitory activity becomes necessary with respect to other lipases and their inhibitors. Various regulatory pathways for ATGL control in addition to HILPDA have been reported, such as activation by CGI-58/ABHD5 (42), and inhibition by nonphosphorylated Perilipin1 (43, 44), by G0S2 (45), or by FSP27/Cidec (46, 47). The existence of multiple regulatory points may reflect the variety of upstream lipolytic signals, tissue specificity, and the presence of separate pools of ATGL activity.

Our work now identifies a novel HILPDA-dependent stress response during starvation-induced lipid droplet formation. These new findings complement works showing that in the absence of extracellular nutrients, autophagy-generated fatty acids are esterified and are transiently stored in lipid droplets before their hydrolysis and eventual mitochondrial oxidation (11–13). As part of the autophagic recycling programs, triglycerides and cholesteryl esters are hydrolyzed for the provision of fatty acids and free cholesterol (48, 49). Discrete and interrelated mechanisms of lipid droplet turnover have been described during times of nutrient scarcity. These mechanisms ultimately lead to lipid hydrolysis, which can be broadly categorized as neutral or acidic hydrolysis. A specialized form of autophagy, termed lipophagy, was originally described in hepatocytes and mouse liver and later identified in multiple cell types (50). Lipid droplets are tagged for autophagosomal engulfment in part by binding of LC3 onto the lipid droplet surface (51). Clearance of Plin2 and Plin3 by chaperone-mediated autophagy also participates in autophagic lipid droplet mobilization, by selectively removing part of the surface protein coating and permitting access to cytosolic lipases (52). On the other hand, lipases positively control autophagy, by supplying the building blocks for autophagosome expansion (53); and by ATGL promoting Sirt1-dependent increase in autophagy genes and lipophagy (54). We found that autophagy activation was not defective after HILPDA loss. Additionally, inhibition of autophagy by chloroquine decreased starvation-induced LD formation; however, this did not equalize LD formation.
abundance in the presence or absence of HILPDA. These data point to the existence of separate pathways of LD turnover in starvation. Mechanistically, our findings indicate enhanced ATGL activity under starvation in the absence of HILPDA, because chemical inhibition of ATGL decreased glycerol release during autophagy and permitted the growth of large LDs in the KO cells.

Another finding of this report is the HIF1-independent increase in HILPDA protein in states of high fatty acid mobilization, as inhibition of autophagic lipid droplet turnover at different stages of the cascade diminished HILPDA upregulation. These HIF-independent mechanisms of protein stabilization possibly explain the effects of HILPDA on normoxic signaling pathways (30). We hypothesize that such protein accumulation acts as a brake against surges of fatty acid hydrolysis. Further work is needed to determine if HILPDA protein stability is controlled similarly to other lipid droplet proteins, such as by proteasomal degradation or posttranslational modifications (55–57).

Protein trafficking to and from the lipid droplets involves multiple mechanisms of varied specificity. A recent model proposes that cytoplasmic proteins are targeted to the lipid droplet surface through amphipathic helix motifs which bind to packing defective areas of the lipid droplet membrane monolayer (58). Consistent with this model, our structure-function analysis of HILPDA truncation mutants revealed that a short 28aa fragment rich in hydrophobic aa residues is able to localize to the lipid droplet surface and promote triglyceride accumulation. Interestingly, the presence of hydrophobic residues 29–31 (GLL in human HILPDA) increased protein steady-state levels. We hypothesize that the increased hydrophobicity of the longer peptide improves the binding affinity for lipid droplet surfaces which may protect it from cytoplasmic degradation. Our finding that protein expression of Plin2 is decreased in the HILPDA KO

Figure 7.

HILPDA promotes tumor growth in vivo. A, In vitro cell growth of HCT116 WT and HILPDA KO cells grown in normoxia (21% O2) or hypoxia (1% O2). B, Volumes of HCT116 WT and KO subcutaneous tumors in female nude mice (WT N = 8, KO N = 12). C, Quantification of triglycerides in tumor explants after 3 weeks of in vivo growth (N = 7). D, Staining of tumor cryosections with Nile red (top) and immunofluorescent detection of the proliferation marker Ki67 and apoptosis marker cleaved caspase-3. E, Quantification of cleaved caspase-3-positive cells on HILPDA WT and KO tumor sections (WT N = 8, KO N = 10, each point is the average of two nonconsecutive tumor sections). *P < 0.05; **P < 0.01; ***P < 0.0001; in B by two-way ANOVA and Sidak post hoc test, in C and E by Student t test; error bars, SEM.
cells is in agreement with the model of molecular crowding on the LD surface can determine LD protein composition (59). The smaller surface area of the HILPDA KO LDs may force proteins to be displaced and degraded. In support of this hypothesis, FA-stabilized Plin2 has been shown to be ubiquitinated and degraded by the proteasome upon FA removal (55), a condition that favors lipolysis.

Maximal model tumor growth required HILPDA and was associated with lower levels of apoptosis, as was also reported by Zhang and colleagues (31). Sequestration of excess fatty acids in lipid droplets has been shown to mitigate oxidative damage and protect membranous structures such as the ER and mitochondria from lipotoxicity (60), and elevated ROS and lipid peroxidation were detected in HILPDA KO tumors (31). Our data open up additional mechanisms of vulnerability to microenvironmental stress by HILPDA loss: In addition to controlling triglyceride abundance, HILPDA, HILPDA, HILPDA affected fatty acid chain length and saturation and also amounts of major phospholipid classes. Levels of long-chain fatty acid elongases were not altered in the KO cells, and SCD1 expression did not correlate with global desaturation indexes. It is possible that deregulated FA flux perturbs allosteric regulators of such enzymes, such as reducing equivalents necessary for steps of the elongation cycle or oxygen required for desaturation reactions. Such secondary or noncanonical functions of lipid droplet proteins have been reported in the literature. For example, loss of the Arf/COPI protein complex, which is responsible for the trafficking of some enzymes to the lipid droplet, increased the levels of PC and PE on the lipid droplet surface and altered the kinetics of target enzyme recruitment (61). Our observed differences in lipid species may indicate differences in total membrane content and composition, which could affect its fluidity, homeostasis, and metabolism of phospholipid intermediates. Further work is needed to identify how HILPDA affects these processes and to what extent they contribute to HILPDA’s tumor-promoting function.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: I. Papandreou, A.J. Giaccia, N.C. Denko
Development of methodology: I. Papandreou, M.J. VandeKopple, J. Wu, N.C. Denko
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): I. Papandreou, M.J. VandeKopple, J. Wu, E.N. Auer
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): I. Papandreou, M.J. VandeKopple, J. Wu, N.C. Denko
Writing, review, and/or revision of the manuscript: I. Papandreou, M.J. VandeKopple, N.C. Denko
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): E.N. Auer
Study supervision: I. Papandreou

Acknowledgments
We wish to thank Soumendrakrishna Karmakaputra for technical assistance. This work was supported by NCI awards CA191653 (I. Papandreou) and CA197713 (A.J. Giaccia). The OSUCCC shared resources are supported by Cancer Center Support Grant CA016058. NIH had no role in the study design, data generation, the writing of this report, or the decision to submit it for publication.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received December 19, 2018; revised May 24, 2019; accepted July 10, 2019; published first July 15, 2019.

References
4. Bensaad K, Favaro E, Lewis CA, Peck B, Lord S, Collins JM, et al. Fatty acid stabilization of Plin2 has been shown to be ubiquitinated and degraded. In support of this hypothesis, FA-stabilized Plin2 has been shown to be ubiquitinated and degraded by the proteasome upon FA removal (55), a condition that favors lipolysis.

Downloaded from mcr.aacrjournals.org on February 3, 2021. © 2019 American Association for Cancer Research.


HILPDA Regulates Lipid Metabolism, Lipid Droplet Abundance, and Response to Microenvironmental Stress in Solid Tumors

Matthew J. VandeKopple, Jinghai Wu, Erich N. Auer, et al.