

The lipid droplet protein Hypoxia-inducible gene 2 promotes hepatic triglyceride deposition by inhibiting lipolysis*

Marina T. DiStefano¹, Laura V. Danai¹, Rachel J. Roth Flach¹, Anil Chawla¹, David J. Pedersen¹, Adilson Guilherme¹, and Michael P. Czech¹

¹From the Program in Molecular Medicine,

University of Massachusetts Medical School, Worcester, MA 01605

*Running Title: *Hig2 inhibits hepatocyte lipolysis*

To whom correspondence should be addressed: Michael P. Czech, Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA, USA, Tel: (508) 856-2254; Fax: (508) 856-1617 ; Email: Michael.Czech@umassmed.edu

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Background: Excess hepatic triglyceride accumulation is associated with metabolic disease.

Results: Hig2 localizes to hepatic lipid droplets, enhancing their lipid content, and its deficiency increases triglyceride lipolysis.

Conclusion: Hig2 is a lipid droplet protein in hepatocytes that promotes liver triglyceride deposition by reducing its rate of degradation.

Significance: Hig2 is revealed as a critical lipid droplet protein controlling liver fat.

ABSTRACT

The liver is a major site of glucose, fatty acid (FA), and triglyceride (TG) synthesis and serves as a major regulator of whole body nutrient homeostasis. Chronic exposure of humans or rodents to high-calorie diets promotes non-alcoholic fatty liver disease (NAFLD), characterized by neutral lipid accumulation in lipid droplets (LD) of hepatocytes. Here we show that the LD protein Hypoxia-inducible gene 2 (Hig2) functions to enhance lipid accumulation in hepatocytes by attenuating TG hydrolysis. Hig2 expression increased in livers of mice on a high fat diet (HFD) and during fasting, two states associated with enhanced hepatic TG content. Hig2 expressed in primary mouse hepatocytes localized to LDs and promoted LD TG deposition in the presence of oleate. Conversely, tamoxifen-inducible Hig2 deletion reduced both TG content and LD size in primary hepatocytes from mice harboring floxed alleles of Hig2 and a Cre/ERT2 transgene controlled by the ubiquitin C promoter. Hepatic TG was also decreased by liver - specific deletion of Hig2 in mice with floxed Hig2 expressing Cre controlled by the albumin promoter. Importantly, we demonstrate that Hig2 - deficient hepatocytes exhibit increased TG lipolysis, TG turnover, and fatty acid oxidation compared with controls. Interestingly, mice with liver - specific Hig2 deletion also display improved glucose tolerance. Taken together, these data indicate that Hig2 plays a major role in promoting lipid sequestration within LDs in mouse hepatocytes through a mechanism that impairs TG degradation.

INTRODUCTION

The liver is a major site of glucose, triglyceride (TG), and fatty acid (FA) synthesis and serves as a master regulator of whole body

nutrient homeostasis (1). Chronic exposure of humans or rodents to high-calorie diets can lead to a disease spectrum known as non-alcoholic fatty liver disease (NAFLD) (2). This syndrome begins with simple neutral lipid accumulation in liver,

progresses to liver inflammation, and can culminate in irreversible cirrhosis and hepatocellular carcinoma (3). Overabundance of liver lipids has also been associated with insulin resistance both in humans and rodents (4), although these conditions can also appear independently (5). Thus, understanding the molecular pathways that contribute to hepatic lipid accumulation is important in addressing therapeutic strategies for NAFLD and in understanding how it relates to metabolic disease.

In all cells including hepatocytes, neutral lipids are stored in organelles termed lipid droplets (LDs) (6). These LDs are highly dynamic and are regulated by the nutritional status of the organism (7). Two main families of LD - associated proteins are the PAT family (7), named for its three founding members Perilipin, Adipophilin, and Tip47 that have PAT domains, and the Cell death - inducing DNA fragmentation factor 45-like effector (CIDE) family (8). The PAT family has five members (Perilipin 1-5), while the CIDE family has three members (Cidea, Cideb, and Cidec/Fsp27). LDs are heterogeneous in their coats and LD proteins generally demonstrate tissue - specific distribution patterns (9). In healthy murine liver, Perilipin 2 and Perilipin 3 promote LD formation (10), while Cideb promotes VLDL lipidation (11). Although deletion of Perilipin 3 has not yet been performed, genetic deletion of Perilipin 2 or Cideb ameliorates hepatic steatosis (12,13). However, in the context of diet-induced obesity and fatty liver, Fsp27 (Cidec in humans) and Cidea are critical for LD formation (8). Both are highly upregulated in murine liver upon diet - induced obesity and genetic deletion of either also results in clearance of obesity - associated hepatic steatosis (8,14,15). Fsp27 is also relevant to human disease, as a patient with a homozygous nonsense mutation in Fsp27 displays partial lipodystrophy, fatty liver, and metabolic syndrome (16,17). As the proteome of the LD may be quite extensive (18), identifying additional members will shed new light on the mechanisms for TG deposition and potentially the basis of human disease.

Hypoxia - inducible gene 2 (Hig2) was initially identified in a screen for genes induced by oxygen deprivation in human cervical cancer cells and encodes a 7 kD protein with little apparent homology to known proteins (19). Its expression is

also increased in many cancers, particularly renal clear cell carcinoma (RCC) and it has been shown to be a target gene of both hypoxia-inducible factor 1 alpha (Hif1 α) and peroxisome proliferator-activated receptor alpha (PPAR α) (19-23). Gimm et al. demonstrated that Hig2 localized to LDs and promoted TG deposition in cancer cells *in vitro* (20). These authors also showed that Hig2 co-localized with Perilipin 2 and 3, two LD proteins essential for neutral lipid deposition in healthy liver. Due to the importance of lipid homeostasis and deposition in liver, we examined the role of Hig2 in these processes.

Here, we demonstrate that Hig2 localizes to LDs in primary mouse hepatocytes. Importantly, its deletion in hepatocytes *in vivo* causes depletion of hepatic TG, indicating that it plays a physiological role in regulating liver lipid abundance in mice. Furthermore, we show the basis for its ability to promote LD formation and TG deposition in liver is through the inhibition of TG lipolysis.

EXPERIMENTAL PROCEDURES

Animal Studies: All of the studies performed were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Massachusetts Medical School. Animals were maintained in a 12 hour light/dark cycle. Hig2 $^{fl/fl}$ animals were purchased from Jackson Laboratories (*Hilpdatm1.1Nat/J*). For metabolic studies, the animals were backcrossed onto C57Bl/6J animals for at least 6 generations. Genomic DNA was extracted from the obtained mice and subjected to PCR for genotyping using Qiagen Fast Cycling PCR Kit (Hig2 $^{fl/fl}$ primer 5'-CCGGCAGGGCCTCCTTGCTCCTG-3', 5' GTGTGTTGGCTAGCTGACCCCTCGTG-3'). Hig2 $^{fl/fl}$ animals were crossed to a tamoxifen-inducible ubiquitous cre mouse line (*B6.Cg-Tg(UBC-cre/ERT2)1Ejb/J*, Jackson Laboratories). Hig2 $^{fl/fl}$ animals were also crossed to an albumin cre mouse line (*C57BL/6-Tg(Alb-cre)21Mgn/J*, Jackson). Cre genotyping was performed according to the method of Jackson Laboratories. At 5-6 weeks of age, male C57Bl/6J, Hig2 $^{fl/fl}$, or Hig2 $^{fl/fl}$ albumin cre+ littermates animals were placed on a high fat diet (60% fat, 12492i Harlan) or fed chow (Lab Diet 5P76) for 12 or 16 weeks.

Mice were fasted 16 hours for glucose tolerance tests and 4 hours for insulin tolerance tests. Mice were injected IP with 1 g/kg of glucose or 1IU/kg of insulin, blood was drawn from the tail vein at the indicated times, and blood glucose levels were measured with a Breeze-2-glucose meter (Bayer). Mice were euthanized by CO₂ inhalation followed by bilateral pneumothorax.

Plasma and lipid analysis: Mice were fasted for 3 hours for plasma lipid analysis. Blood was taken via cardiac puncture, and EDTA - containing plasma was collected. Total serum cholesterol levels (ab65359 Abcam), serum triglyceride levels (Triglyceride Determination Kit, Sigma), serum NEFAs (Wako Diagnostics), and β - hydroxybutyrate (Sigma) were measured using calorimetric assays according to the manufacturer's instructions. Insulin levels were measured with an Insulin ELISA Kit (Millipore) according to the manufacturer's instructions.

Triglyceride and Cholesterol Extraction: Whole livers were isolated and flash frozen in liquid nitrogen. Lipids were extracted from livers or pelleted hepatocytes via the Folch method (24). Lipids were dissolved in isopropanol with 1% Triton-X100. Triglyceride (Triglyceride Determination Kit, Sigma) and cholesterol ester (ab65359 Abcam) levels were measured using calorimetric assays according to the manufacturer's instructions and normalized to liver weight or hepatocyte protein content.

Hepatocyte isolation: Male or female 8-10 week old chow - fed animals were anesthetized with an IP injection of 1:1 ketamine:xylazine and perfused with HBSS supplemented with .5M EGTA. Livers were digested with a perfusion of 50 mg/ml collagenase (Sigma, C6885) in HBSS supplemented with 1mM CaCl₂, physically dissociated, and filtered through a 100 μ m filter. Hepatocytes were washed, centrifuged at low speed, filtered through a 70 μ m filter, and plated at a density of 1 million cells/ml.

RNA Isolation and RT-qPCR: Total RNA was isolated from cells or tissues using TriPure isolation reagent (Roche) according to the manufacturer's protocol. The isolated RNA was DNase treated (DNA-free, Life Technologies),

and cDNA was synthesized using iScript cDNA synthesis kit (BioRad). RT-qPCR was performed on the BioRad CFX97 using iQ SybrGreen supermix, and 36B4 served as the reference gene. Primer sequences are as follows: 36B4 (5'-TCCAGGCTTGGGCATCA-3', 5'-CTTATCAGCTGCAC ATCACTCAGA-3'); Hig2 (5'-CATGTTGACCCTGCTTCCAT-3', 5'-GCTCTCCAGTAAGCCTCCCA-3').

Immunoblotting: Tissues and cells were lysed in a high-salt, high-SDS buffer (2% SDS, 150mM NaCl, 2mM EDTA) with 1x Halt protease and phosphatase inhibitors (Thermo Scientific). Lysates were resolved by 15% SDS-PAGE gel run in a 1x Tris-Tricine Buffer (National Diagnostics) and transferred to nitrocellulose membranes. Membranes were blotted with the following antibodies: β -Actin (A2228, Sigma), HA-Tag (2367, Cell Signaling Technology). The Hig2 antibody was directed against a 15 amino acid peptide (PPKGLPDHPSRGVGV) at the C terminus of murine Hig2 (Rockland Immunochemicals).

Cell Culture: Hepatocytes were isolated from male or female 8-10 week old Hig2^{f/f} Ubc ERT2 Cre + animals, plated in M199 adherence media (Life Technologies, 11150, supplemented with 2% FBS, 10% BSA, 1%Pen/Strep, 100 nM Insulin, and 100 nM Dexamethasone) for 3 hours, changed to M199 maintenance media (M199 supplemented with 1% Pen/Strep, 100 nM Insulin, and 100 nM Dexamethasone) and treated with ethanol vehicle or 2.5 μ M (Z)4-Hydroxytamoxifen (Sigma H7904) dissolved in filtered ethanol (5 mg/ml) for 48 hours before experiments.

For imaging experiments, hepatocytes were plated on collagen-coated Millipore 4 chambered slides (Millipore) and transfected with GFP constructs using 1.5 μ g DNA, Optimem and Lipofectamine 2000 (Life Technologies) according to manufacturer's instructions 48 hours prior to experiments. Hepatocytes were also infected with HA-tagged adenoviruses 48 hours prior to experiments. Cells were loaded with the indicated concentration of oleic acid (Sigma) dissolved in ethanol and conjugated to 10% fatty acid-free BSA dissolved in 0.1M Tris pH 8. For radiation experiments, hepatocytes were plated on collagen-coated plates in William's E Medium Adherence

Media (Life Technologies 12551, supplemented with 2% Fetal Bovine Serum, 10% BSA (Sigma A4503), 1% Pen/Strep, 100 nM Insulin, and 100 nM Dexamethasone). After three hours, media was changed to maintenance media (William's E Medium supplemented with 1% Pen/Strep, 100 nM Insulin, and 100 nM Dexamethasone).

Cell Imaging: Cells were fixed in 10% buffered formalin in PBS for 15 minutes, stained with Oil-Red-O, and mounted with Prolong Gold with DAPI (Life Technologies). Cells were imaged at room temperature with a Solamere Technology Group modified Yokogawa CSU10 Spinning Disk Confocal with a Nikon TE-2000E2 inverted microscope at 60x, and 100x. Images were acquired with MetaMorph Software, version 6.1 (Universal Imaging, Downingtown PA).

Lipid droplet analysis was performed on fixed, Oil-Red-O- and DAPI- stained cells with BioPix iQ Imaging Software (BioPix AB, Sweden). At least 90 cells were analyzed per condition.

Oleate Tracer Studies: Hepatocytes were isolated from male 8-10 week old $Hig2^{fl/fl}$ or $Hig2^{fl/fl}$ albumin cre + animals. 24 hours after isolation, cells were loaded with 1 μ Ci/ml [³H] oleic acid mixed with 100 μ M oleic acid conjugated to 0.5% fatty acid-free BSA in William's E Medium. Assays were performed as previously described (15,25).

Oleate Uptake: Cells were loaded with 100 μ M [³H] oleic acid overnight. The following day, media was removed, cells were washed, and lysed in 0.5 ml of lysis buffer (1% Triton-X100 in PBS), and lysates were placed in vials with scintillation fluid and counted using a Beckman LS 6500 scintillation counter. Counts were normalized to length of incubation time and protein content.

Total β -Oxidation: Cells were loaded with 100 μ M [³H] oleic acid overnight. The following day, media was collected, precipitated twice with perchloric acid and BSA, spun to pellet insoluble products, and the soluble fraction was removed, placed in vials with scintillation fluid, and counted. Counts were normalized to length of incubation time and protein content. Empty media was loaded with [³H] oleic acid, precipitated, counted, and subtracted as a background reading.

Triglyceride Turnover: Cells were loaded with 100 μ M [³H] Oleic Acid overnight. The following day, cells were washed 2x, and media was replaced with William's E Media with 0.6 mM Triacsin C (Sigma). Cells were collected at indicated times, washed, and lysed in 0.5 ml lysis buffer (1% Triton-X100 in PBS), placed in vials with scintillation fluid, and counted. Counts are normalized to protein content and graphed as a percentage of time 0.

Lipolysis: Cells were loaded with 100 μ M [³H] oleic acid overnight. The following day, cells were washed 2x and media was replaced with William's E Media with 0.6 mM Triacsin C (Sigma) and 100 μ M Etomoxir (Sigma). Media was collected at the indicated times, placed in vials with scintillation fluid, and counted. Counts are normalized to protein content.

Statistical Analysis: Data were analyzed in GraphPad Prism 6 (GraphPad Software, Inc). A two-tailed student's t test with Welch's Correction was used to compare two groups of data. Where indicated, data were analyzed using a two-way ANOVA with repeated measures or a linear regression model. $P<.05$ was considered to be significant. The Grubb's test was used to determine if there were statistical outliers and if an outlier was determined, it was removed from the statistical analysis. Variance was estimated using standard error of the mean.

RESULTS

To characterize Hig2 as a potential hepatic LD protein, we first determined if it localized to LDs in primary mouse hepatocytes. We isolated primary hepatocytes from C57Bl/6J animals and transfected them with either GFP control or GFP-tagged Hig2 constructs and incubated them with oleic acid to induce LD formation. While the GFP control construct displayed a diffuse, cytoplasmic distribution in the cells, the GFP-Hig2 construct clearly localized around the perimeter of Oil-Red-O positive LDs (**Figure 1b**). Gimm et al. used deletion analysis to determine that the 37 N-terminal amino acids of Hig2 are required for LD targeting in cancer cell lines (20), which we termed the "Putative Lipid Droplet Binding Domain" (**Figure 1a**). To confirm that this was also the targeting domain in hepatocytes, we

created a Hig2 truncation mutant with a loss of amino acids 1-28 of this putative binding domain. When the Hig2 truncation mutant was transfected into primary hepatocytes, it also localized diffusely, similar to the GFP control, demonstrating that Hig2 localizes to LDs in hepatocytes through this putative LD binding domain (**Figure 1b**). As Gimm et al. determined by sequence analysis that an amphipathic helix is located in this domain (20), it is possible that Hig2 may interact with the lipid droplet directly via surface interaction (26).

Hepatic LD protein expression is highly sensitive to nutritional status. As both Fsp27 and Cidea, two bona fide liver LD proteins, are highly upregulated upon high fat diet (HFD) feeding in mice (8), we measured Hig2 expression in two situations of hepatic steatosis. Fasting, which liberates lipids from adipose tissue via lipolysis, causes a temporary increase in liver lipids (27). Indeed, a 24-hour fast caused a 2-fold increase in Hig2 mRNA expression in C57Bl/6J mouse livers (**Figure 1c**). Obesity-induced hepatic steatosis in C57Bl/6J mice also caused a significant 2.7-fold increase in Hig2 mRNA expression in liver (**Figure 1d**), consistent with the concept that Hig2 expression is highly correlated with liver lipid levels.

LD protein overexpression can also promote lipid deposition (7). For example, experimentally enhancing Fsp27 expression promotes TG accumulation in a variety of cell types, while Fsp27 deficiency reduces LD formation (28,29). We tested whether Hig2 expression modifies TG accumulation in liver by manipulating Hig2 expression in primary mouse hepatocytes. First, we isolated primary hepatocytes from Hig2^{f/f} animals and infected them with either control adenovirus (AV) or an AV expressing HA-tagged Hig2. As expected, Hig2-HA AV- infected cells demonstrated increased Hig2-HA levels compared with controls as determined by Western blot (**Figure 2b**). We incubated the cells with 250 μ M oleic acid for 24 hours to induce LD formation, fixed, and then stained them with Oil-Red-O to image LDs. Imaging revealed that Hig2-HA AV- infected hepatocytes had significantly more LDs compared with controls (**Figure 2a**). Though there was no significant difference in TG levels in BSA vehicle-treated hepatocytes, Hig2- infected cells

demonstrated a 1.5- fold increase in TG content compared with control cells after oleate loading (**Figure 2c**). Taken together, these results demonstrate that high Hig2 expression is sufficient to promote lipid deposition in hepatocytes. These results confirmed those of a report published while our studies were in progress showing that overexpression of Hig2 in liver via adeno-associated virus (AAV) vector injection in mice resulted in increased hepatic lipid deposition *in vivo* (23).

Conversely, to determine whether Hig2 expression is necessary for lipid deposition in hepatocytes, we genetically deleted Hig2 in primary hepatocytes using a tamoxifen-inducible mouse model (Hig2iKO). We crossed Hig2^{f/f} mice to Ubc ERT2 cre positive mice (**Figure 3a**), isolated hepatocytes from these Hig2iKO animals, plated them for 3 hours, and treated them with either 2.5 μ M 4-OH-tamoxifen to induce deletion or ethanol vehicle as a control for 48 hours before analysis (**Figure 3b**). Tamoxifen treatment resulted in a 90% reduction in Hig2 mRNA and protein expression compared with ethanol vehicle-treated controls as assessed by qRT-PCR and Western blot, respectively (**Figure 3c-d**). Cells were fixed and stained with Oil-Red-O, and LDs were quantified. Strikingly, tamoxifen- treated hepatocytes had less Oil-Red-O- positive LD compared with ethanol vehicle- treated controls (**Figure 3f**). To confirm that this was not a side effect of tamoxifen treatment or the Ubc ERT2 cre transgene, we treated Hig2^{f/f} or Ubc ERT2 positive hepatocytes on a wild type background with tamoxifen and observed no alterations in lipid accumulation (data not shown). When TG was extracted and quantified, we found that ethanol vehicle- treated cells had 1.7-fold more TG after BSA treatment and 1.4-fold more TG after oleic acid loading compared with tamoxifen-treated cells (**Figure 3e**). The ethanol vehicle- treated hepatocytes demonstrated an average of 63 ± 8 LD per cell, while tamoxifen- treated hepatocytes displayed over a 67% reduction in LD content and had an average of only 18 ± 3 LD per cell (**Figure 3g**). Furthermore, the LDs in Hig2iKO hepatocytes were approximately 50% smaller than ethanol vehicle - treated controls and displayed an average size of $2.3 \pm 0.6 \mu\text{m}^2$ compared with $4.3 \pm 0.6 \mu\text{m}^2$ for ethanol vehicle- treated controls (**Figure 3h**). Interestingly, Hig2-deficient

hepatocytes had significantly more LDs than controls after loading with 500 μ M oleic acid for 24 hours (41 ± 6 compared with 75 ± 11); however, these LDs were over 50% smaller than control LDs ($4.0 \pm .8 \mu\text{m}^2$ vs. $9.3 \pm 1.8 \mu\text{m}^2$) (**Figure 3g, h**). This phenomenon is similar to the smaller LDs found in Fsp27-deficient adipocytes (29). These results demonstrate that Hig2 deficiency greatly reduced LD abundance and TG deposition in hepatocytes. Taken together, these experiments in primary hepatocytes suggest Hig2 expression is required for hepatocyte lipid deposition and LD growth *in vitro*.

Excess accumulation of liver lipids is often associated with insulin resistance in obese mice and humans (4). Therefore, we generated mice with liver-specific deletion of Hig2 to address whether Hig2 deficiency could reduce hepatic steatosis and preserve glucose tolerance in a model of diet-induced obesity. We crossed the Hig2^{fl/fl} mouse with mice expressing albumin cre to generate a mouse with liver-specific Hig2 deletion (Hig2LKO, **Figure 4a**). Hig2LKO mice demonstrated a significant, 89% reduction of Hig2 mRNA specifically in hepatocytes compared with fl/fl controls (**Figure 4b**) and a concomitant reduction in Hig2 protein levels as determined by Western blot (**Figure 4c**). Other tissues such as white adipose tissue (WAT), spleen, and kidney did not show significant reductions in Hig2 mRNA (**Figure 4d**), demonstrating that the deletion was specific for hepatocytes. We placed these animals on HFD or normal diet (ND) for 16 weeks and measured their body weight weekly. Although there was no significant difference in the body weights of the Hig2LKO animals versus the fl/fl controls (**Figure 4e**), the Hig2LKO animals demonstrated significantly improved glucose tolerance as measured by a glucose tolerance test (GTT) in both the ND group and at early time points following glucose injection in the HFD-fed group (**Figure 4f**). However, no significant difference between genotypes was observed in an insulin tolerance test (ITT, **Figure 4g**).

We measured fasting circulating insulin levels, serum TGs, serum cholesterol, serum non-esterified fatty acids (NEFA), β - hydroxybutyrate and liver cholesterol, but all were unchanged among the groups in both ND and HFD-fed conditions (**Table 1**). Because Hig2 deletion reduces TG content *in vitro*, we examined the

livers of the Hig2LKO animals fed ND or HFD for 16 weeks. The gross liver weights were not significantly different in either diet condition, but in the ND condition, the Hig2LKO animals had lighter livers than the fl/fl controls ($1.14 \pm .04$ vs. $1.23 \pm .03$ g, $p=.08$) (**Figure 4h**). Though differences in H&E stained histology sections from fl/fl and Hig2LKO animals were unremarkable (**Figure 4j**), ND-fed Hig2LKO animals had 30 % less liver TGs than fl/fl controls ($p=.08$); however, this difference was abrogated in HFD-fed animals (**Figure 4i**). To assess liver inflammation, we isolated RNA from mice fed HFD for 16 weeks and performed qRT-PCR to assess the expression of genes involved in inflammatory pathways. No changes in gene expression were observed in Hig2LKO mouse livers compared with fl/fl controls for TNF α , IL6, IL1 β , and the macrophage marker F4/80 (data not shown).

We sought to examine potential mechanisms by which Hig2 controls TG accumulation in hepatocytes. Hepatic TG accumulation is controlled by FA uptake and hepatic lipogenesis versus hepatic lipolysis (TG turnover) (30). First, we performed qRT-PCR on RNA isolated from livers isolated from fl/fl and Hig2LKO mice on ND and assessed the expression of several genes that are critically involved in the aforementioned pathways. However, no changes in gene expression were observed in Hig2LKO mouse livers compared with fl/fl controls for CD36 (FA uptake), Cpt1a, Mttfa (mitochondrial oxidation), Ppary, Fasn, Srebp1c (lipogenesis), Srebp2 (cholesterol synthesis), ApoB (lipid export), or Atgl (lipolysis) (data not shown). Hig2 deficiency could potentially reduce the expression of other LD proteins in liver to reduce the lipid content, thus we also measured the expression of Perilipin 2, Cidea, and Fsp27 by qRT-PCR and the expression of these genes was also unchanged in Hig2LKO mouse livers compared with fl/fl livers (data not shown). Because there were no transcriptional changes in the targets we examined, we hypothesized that Hig2 was promoting hepatic lipid deposition in a post-transcriptional manner. Thus, lipid flux in Hig2LKO hepatocytes was assessed to determine whether they demonstrated a difference in lipid handling. We isolated hepatocytes from ND-fed fl/fl and Hig2LKO

hepatocytes, incubated the cells with [³H] oleic acid and then measured the total amount of radiation in Hig2LKO and fl/fl controls after overnight [³H] oleic acid loading. As expected, the Hig2LKO hepatocytes displayed a 45% reduction in lipid uptake compared with controls ($p=.07$; **Figure 5a**), confirming results obtained in Figure 3 with the Hig2iKO hepatocytes.

Genetic deletion of LD proteins such as Fsp27, Perilipin1, and Cidea in mice, has demonstrated a role for LD proteins in TG turnover and β -oxidation (29,31-33). Hepatic TG turnover has been experimentally determined to be on the timescale of 10-30 hours *in vivo* (34). Thus, parameters were assessed by loading our ND-fed Hig2LKO or fl/fl hepatocytes with [³H] oleic acid overnight and then TG turnover, lipolysis, and β -oxidation were measured. Strikingly, despite the absence of changes in gene expression, Hig2LKO hepatocytes had significantly increased TG turnover compared with fl/fl controls as determined by linear regression analysis (**Figure 5c**). Hig2LKO hepatocytes also exhibited double the amount of lipolysis at two hours as compared with controls (**Figure 5d**). Similar to Fsp27-deficient animals, Hig2LKO hepatocytes also displayed 3.3-fold higher degree of FA oxidation compared with control fl/fl hepatocytes as detected by accumulation of soluble [³H] oleic acid oxidation products (**Figure 5b**). Taken together, these results suggest that Hig2 promotes lipid deposition in a healthy liver, at least in part, by localizing to LDs in hepatocytes and inhibiting TG lipolysis.

DISCUSSION

The findings presented here define Hig2 as a physiologically important LD-associated protein that functions to promote TG accumulation in liver *in vivo*. We demonstrated that GFP-tagged Hig2 localizes specifically to hepatocyte LDs in a manner that was dependent on its putative lipid binding domain (**Figure 1**). While ectopic expression of Hig2 promoted LD abundance and TG deposition (**Figure 2**), Hig2 deletion in hepatocytes *in vitro* reduced TG accumulation (**Figure 3**). Liver-specific Hig2 deletion reduced hepatic TGs in ND-fed mice and improved glucose tolerance in both ND and HFD-fed

conditions (**Figure 4**). Hepatocytes isolated from these animals show increased TG turnover and FA oxidation, suggesting that Hig2 promotes TG deposition by inhibiting lipolysis (**Figure 5**). Indeed, direct measurement of TG hydrolysis in hepatocytes deficient in Hig2 revealed increased lipolytic rates over controls, analogous to what has been reported for other LD proteins, such as Fsp27 (15).

As the data in Figure 4 indicate, Hig2 may not be nearly as crucial for hepatic lipid deposition in HFD-fed liver as it is in the ND-fed condition. We found no decrease in total liver TG in the Hig2LKO mice compared with fl/fl controls when both groups of mice were on a HFD. This contrasts with a decrease in liver TG observed in these Hig2LKO mice on ND (**Figure 4**). One likely possibility to explain this result is other proteins that are redundant in function to Hig2.

Many LD proteins in the PAT family and the CIDE family have been shown to alter lipid deposition by inhibiting lipolysis (7,8,35). Some of these are upregulated under HFD conditions and could replace Hig2 action on lipolysis. For example, it is well known that the expression of the LD protein Fsp27 is highly upregulated under the fatty liver conditions we have examined here (36). Fsp27 was shown to inhibit lipolysis in adipocytes (28), similar to the present findings on Hig2 and hepatocytes. Taken together, it seems likely that compensation by other upregulated LD proteins explains the failure of Hig2 depletion to lower liver TG under HFD conditions.

Our findings complement the recent findings of Mattijssen et al. (23) that were published while our studies were in progress. The authors demonstrated that overexpression of Hig2 in mouse livers driven by AAV results in hepatic steatosis, similar to our results using AV as expression vector for Hig2 (**Figure 2**). Consistent with their findings, we also observe increased lipid product export in hepatocytes from Hig2LKO mice compared to controls (**Figure 5**), but find that this export consists almost entirely of FA oxidation products rather than lipoproteins. It has been shown that increases in lipolysis can shunt FAs to the mitochondria, leading to increases in β -oxidation (37). We also observed that Hig2LKO hepatocytes had increased lipolysis and TG turnover, which is phenotypically similar to what is observed after loss of LD proteins and further

suggests that Hig2 acts similarly to other proteins in this class. Thus, the increased β -oxidation products we observe in Hig2-deficient hepatocytes most likely reflects the higher levels of lipolysis and free FA availability for oxidation observed in the Hig2-deficient hepatocytes.

The increased lipolysis in Hig2LKO hepatocytes does not appear to be the result of altered expression of Atgl (data not shown), the rate-limiting TG lipase in adipose tissue. However, Atgl activity is known to be inhibited by G0/G1 Switch Protein 2 (G0S2) (38,39). Hig2 could function similarly to negatively regulate lipolysis by interacting with and inhibiting Atgl or other lipases in liver. The lipolysis pathway in liver is not well studied, but liver-specific Atgl depletion in mice increases liver TG, while overexpression reduces liver TGs and increases β -oxidation independent of changes in hepatic gene expression or serum TGs, much like we observe in the Hig2LKO mouse (40-42). Sequence alignments of Hig2 and G0S2 show 13.5% sequence identity, mostly located in the area where G0S2 is known to bind and inhibit Atgl (unpublished observation). Another target of Hig2 regulation could be Adiponutrin, a lipase from the patatin-like phospholipase domain containing (PNPLA) family that contains the most sequence similarity to Atgl (37). If Hig2 physically interacts with a lipase, it could either inhibit its activity or restrict its access to LDs. The exact mechanism by which Hig2 inhibits lipolysis will be assessed in future studies.

A remarkable finding in this study was the significant improvement in glucose tolerance observed in liver-specific Hig2 deficiency, even

under HFD conditions in which liver TG was unchanged (**Figure 4**). Although in obese animals and humans liver TG accumulation generally correlates with insulin resistance, many experimental models show dissociation of liver lipid accumulation from glucose tolerance, and the precise mechanistic connections between liver fat and metabolism and insulin sensitivity are far from clear (5). The mechanism by which Hig2 improves glucose tolerance in HFD animals, primarily in early time points following glucose injection is also unclear at this point. Hig2LKO animals trend toward enhanced insulin sensitivity in the ITT (Fig. 4), although the differences did not reach statistical significance, and the basis of improved glucose clearance in these animals is under further investigation.

Although this work on Hig2 has been performed in murine cells and tissues, mutations in other LD proteins such as Fsp27 and Perilipin 1 are associated with human disease (16,43). It will therefore be of interest to investigate whether Hig2 plays an important role in human biology. Tissue expression analysis of Hig2 shows that it is ubiquitously expressed (unpublished observation). This expression pattern parallels that of Perilipin 2, which can be found coating LDs in most tissues. This raises the question of whether Hig2 is required for lipid deposition in other tissues, particularly metabolically active tissues such as adipose tissue and muscle or for macrophage foam cell formation. The full range of Hig2 functions in diverse cell types in human biology is a key question for future research.

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FOOTNOTES:

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¹ To whom correspondence should be addressed: Michael P. Czech, Ph.D. Program in Molecular Medicine, University of Massachusetts Medical School, 373 Plantation Street, Worcester, MA, USA, Tel: (508) 856-2254; Fax: (508) 856-1617 ; Email: Michael.Czech@umassmed.edu

² The abbreviations used are: TG: triglyceride, FA: fatty acid, NAFLD: non-alcoholic fatty liver disease, LD: lipid droplet, CIDE: cell death - inducing DNA fragmentation factor 45-like effector, Hig2: hypoxia-inducible gene 2, RCC: renal clear cell carcinoma, Hif1 α : hypoxia-inducible factor 1 alpha, PPAR α : peroxisome proliferator-activated receptor alpha, IACUC: Institutional Animal Care and Use Committee, HFD: high fat diet, AV: adenovirus, AAV: adeno-associated virus, Hig2iKO: Hig2 inducible knockout, Hig2LKO: Hig2 liver knockout, WAT: white adipose tissue, ND: normal diet, GTT: glucose tolerance test, ITT: insulin tolerance test, NEFA: non-esterified fatty acids, G0S2: G0/G1 Switch Protein 2, PNPLA: patatin-like phospholipase domain containing

FIGURE LEGENDS**Figure 1. Hig2 is localized to lipid droplets and is modified by nutritional status.**

A. Amino acid sequence of murine Hig2 with putative lipid droplet binding domain residues 1-37 indicated. **B.** Hepatocytes were transfected with GFP-tagged constructs (green), loaded with 500 μ M oleic acid for 4 hours, fixed, and stained with Oil-red-O (red) and DAPI (blue). Truncated Hig2-GFP is missing residues 1-28. **C,D** Whole livers were isolated from C57Bl/6J animals, RNA was extracted, and qRT-PCR was performed for Hig2 and normalized to 36B4. **C.** Animals were fasted for 24 hours or fed. Data are represented as the mean \pm S.E.M. (*p<0.05, N=6). **D.** Animals were fed ND or HFD for 12 weeks. (*p<0.05, N=5-6). Data are represented as the mean \pm S.E.M.

Figure 2. Ectopic expression of Hig2 promotes hepatocyte lipid deposition.

Hepatocytes isolated from Hig2^{fl/fl} animals and infected with HA (control) or Hig2-HA (Hig2) adenovirus. **A, C.** Hepatocytes were loaded with 250 μ M oleic acid for 24 hours. **A.** Cells were fixed and stained with Oil-red-O (red) and DAPI (blue). **B.** Representative immunoblots of Hig2 (light and dark exposure), HA tag, and β -Actin. **C.** Triglyceride content from control and Hig2-HA infected cells. (*p<0.05, N=6). Data are represented as the mean \pm S.E.M

Figure 3. Inducible Hig2 deficiency reduces lipid droplet triglyceride in hepatocytes.

A. Schematic of Hig2 deletion with tamoxifen-inducible UbcERT2-cre. **B.** Schematic of experimental design. Hepatocytes were isolated from Hig2iKO mice, plated for 3 hours, and treated with ethanol vehicle or 2.5 μ M tamoxifen in ethanol for 48 hours. **C.** qRT-PCR was performed for Hig2 and normalized to 36B4. (**p<0.01, N=8). **D.** Representative immunoblots of Hig2 and β -Actin. **E.** Triglyceride content from cells treated with 500 μ M oleic acid or BSA vehicle for 4 hours. **F.** Hepatocytes were treated with BSA vehicle or 500 μ M oleic acid for 24 hours, fixed, and stained with Oil-red-O (red) and DAPI (blue). **G,H.** Hepatocytes were treated with 500 μ M oleic acid or BSA vehicle for 24 hours **G.** Number of lipid droplets per cell. **H.** Total lipid droplet area per cell. (*p<0.05, **p<0.01, N=5-6). Data are represented as the mean \pm S.E.M.

Figure 4. Liver-specific Hig2 deficient mice display decreased liver triglyceride under normal diet conditions and improved glucose tolerance.

A. Schematic of Albumin-cre-mediated Hig2 deletion. **B-C.** Hepatocytes were isolated from fl/fl and Hig2LKO mice. **B.** qRT-PCR was performed for Hig2 and normalized to 36B4. (#p<0.005, N=5). Data are represented as the mean \pm S.E.M. **C.** Representative immunoblots of Hig2 and β -Actin. **D.** White adipose tissue (WAT), kidney, and spleen were isolated from fl/fl and Hig2LKO mice. qRT-PCR was performed for Hig2 and normalized to 36B4. (N=8-9). Data are represented as the mean \pm S.E.M. **E-I.** Fl/fl or Hig2LKO animals were fed ND or HFD for 16 weeks. **E.** Body weight curves. (N=10-13). **F.** Glucose tolerance test. (+p=.08, *p<0.05, **p<0.01; \$ p<0.05, 2-way ANOVA, N=7-11). **G.** Insulin tolerance test. (N=9-17). Data are represented as the mean \pm S.E.M. **H.** Liver weights. (+ p=0.08, N=8-11). Data are represented as individual values \pm S.E.M. **I.** Lipids were extracted from livers and triglyceride content was assessed. (+ p=0.08, N=8-11). Data are represented as individual values \pm S.E.M. **J.** Livers were sectioned and stained with H&E.

Figure 5. Hig2 deficiency increases hepatocyte lipolysis, β -oxidation and triglyceride turnover.

A-D. Hepatocytes were isolated from fl/fl or Hig2LKO animals, plated, and loaded with 100 μ M [³H] oleic acid overnight. **A.** Oleate uptake. (+ p=0.07, N=8). **B.** Total β -oxidation. (*p<0.05, N=6). **C.** Triglyceride turnover. (*p=0.05 via linear regression analysis of the slope, N=8-10). **D.** Lipolysis. (*p<0.05, N=3) Data are represented as the mean \pm S.E.M.

Table 1. Liver cholesterol and serum metabolites were assessed from fl/fl or Hig2LKO animals fed ND or HFD for 16 weeks. Data are the mean \pm S.E.M (N=5-13). BD, below detection; NEFA, non-esterified fatty acids.

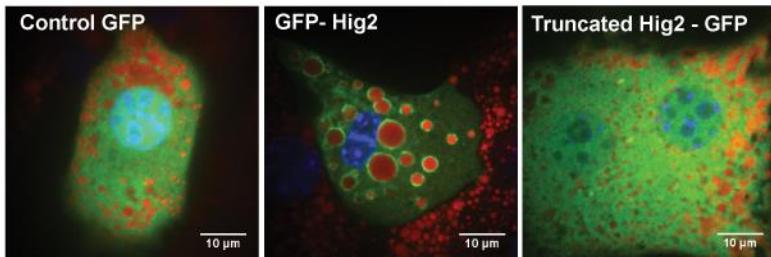
Table 1

Parameters	Normal Diet		16wks HFD	
	HIG2 fl/fl	HIG2LKO	HIG2 fl/fl	HIG2LKO
Insulin (ng/ml)	BD	BD	2.831 \pm 0.477	2.640 \pm 0.268
Serum triglycerides (mg/dL)	64.283 \pm 4.302	51.456 \pm 5.113	107.384 \pm 6.634	102.954 \pm 10.707
Serum cholesterol (mg/dL)	64.401 \pm 5.782	70.218 \pm 5.929	127.834 \pm 6.993	137.622 \pm 6.255
Liver cholesterol (μ g/mg)	1.745 \pm 0.106	1.717 \pm 0.071	1.804 \pm 0.226	2.181 \pm 0.082
NEFA (mmol/liter)	0.416 \pm 0.028	0.348 \pm 0.038	0.536 \pm 0.068	0.476 \pm 0.058
β -Hydroxybutyrate (mmol/liter)	0.469 \pm 0.042	0.569 \pm 0.078	0.373 \pm 0.038	0.274 \pm 0.017

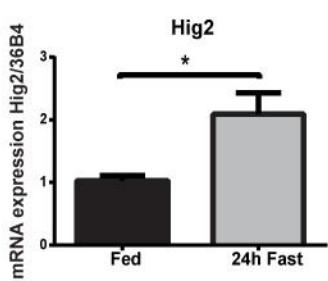
A. Putative Lipid Droplet Binding Domain

MKFMLNLYVLGIMLTLSIFVRVMESLGGLLESPLPG
SSWITRGQLANTQPPKGLPDHPSRGVQ

B.



C.



Hig2

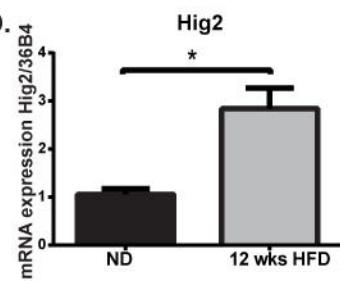


Figure 1

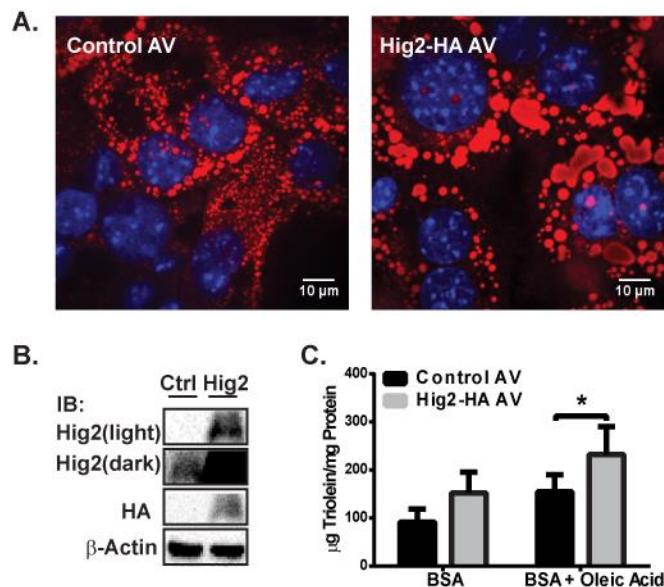


Figure 2

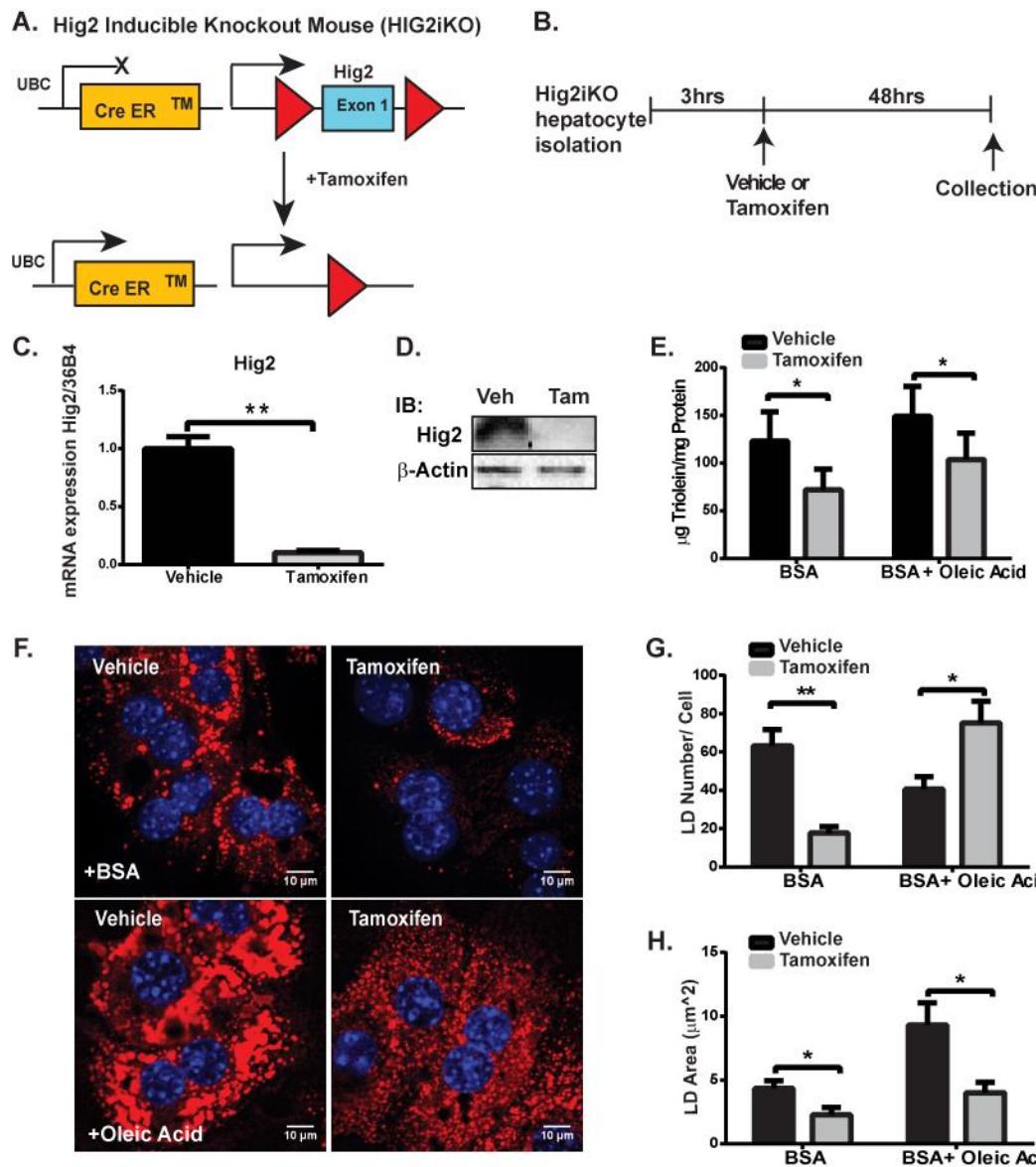


Figure 3

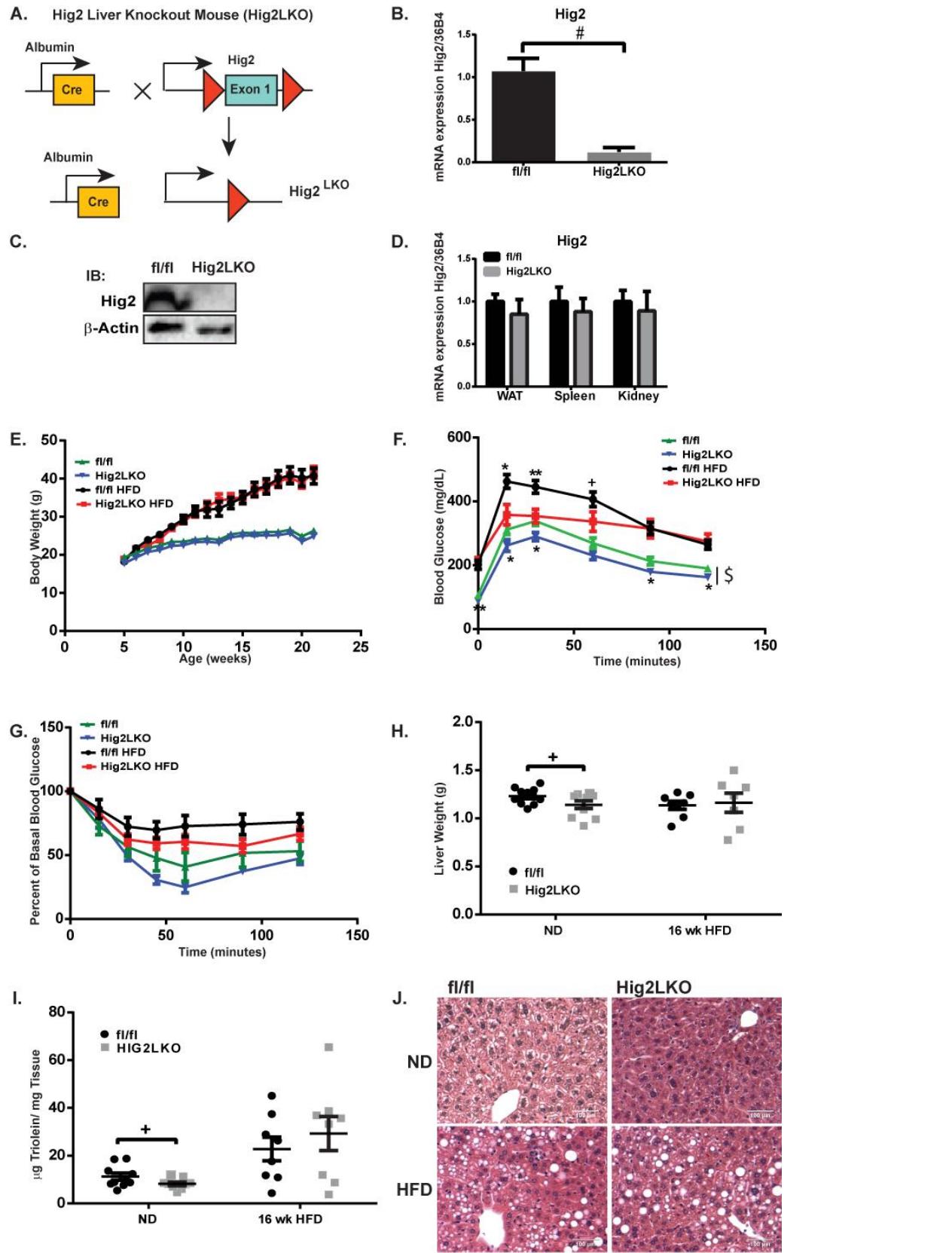


Figure 4

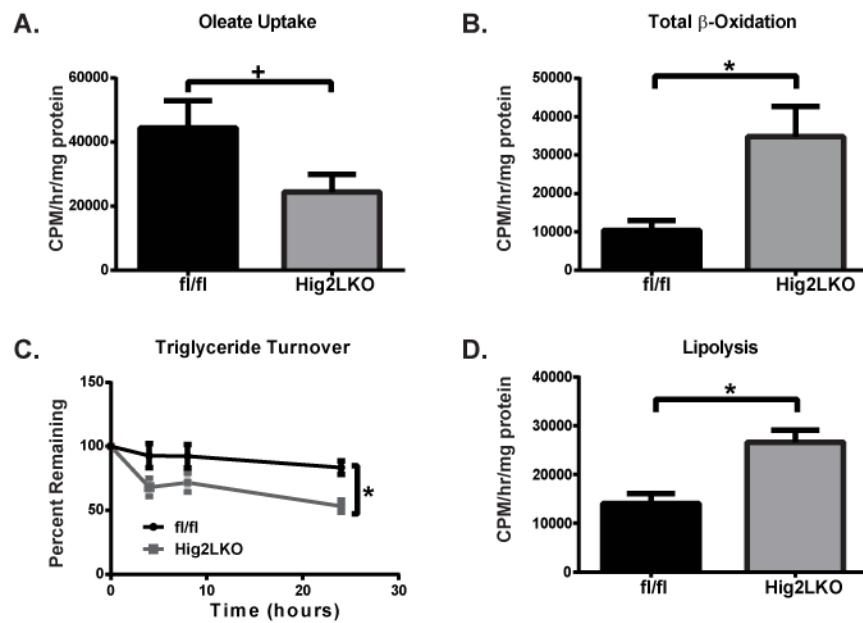


Figure 5