

Role of *trans*-10, *cis*-12 Conjugated Linoleic Acid and Unconventional Prefoldin

RPB5 Interactor 1 Protein in Non-Alcoholic Fatty Liver Disease

by

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Abstract

Non-alcoholic fatty liver disease (NAFLD) is the hepatic manifestation of metabolic syndrome. Due to its high prevalence, and its link to hepatocellular carcinoma (HCC), studying the pathogenesis of NAFLD is crucial. Unconventional prefoldin RPB5 interactor 1 (URI1) belongs to the prefoldin family of proteins that have been shown to coordinate nutrient availability by transcriptional regulation of genes involved in glucose and lipid metabolism. URI1 has been shown to be elevated in mouse liver after induction of NAFLD by a high fat diet and contributes to the development of HCC.

Trans-10, *cis*-12 conjugated linoleic acid (t10,c12 CLA) is marketed as a weight loss supplement and feeding t10,c12 CLA to mice results in hepatic steatosis (the earliest stage of NAFLD characterized by hepatic lipid accumulation). In this thesis, it is hypothesized that t10,c12 CLA feeding of lean and obese *db/db* mice facilitates hepatic steatosis through upregulation of URI1 protein expression. The validity of this hypothesis was assessed using *in vivo* (mouse) and *in vitro* (H4IIE cell line) experimental models, which included heterozygous and homozygous *Uri1* knock out (KO) H4IIE cell lines, which were generated using the CRISPR/Cas9 system.

T10,c12 CLA feeding promoted hepatic steatosis in both lean and *db/db* mice, compared to mice fed the control diet, by upregulating hepatic *de novo* lipogenesis and fatty acid uptake which, potentially, may occur by t10,c12 CLA-mediated activation of the mammalian target of rapamycin pathway. Obesity- and t10,c12 CLA-induced hepatic steatosis also resulted in

increased hepatic URI1 levels. In parallel, URI1 protein levels and neutral lipid accumulation *in vitro* were both elevated the most after 24 hours of oleic acid (OA) treatment. Lastly, in comparison to wild type H4IIE cells, *Uri1* KO H4IIE cells exhibited fewer lipid droplets in response to OA treatment, suggesting a role for URI1 in hepatic lipid accumulation.

In conclusion, t10,c12 CLA may promote hepatic steatosis by upregulating hepatic URI1 expression since t10,c12 CLA feeding resulted in increased hepatic URI1 protein levels and URI1 facilitates hepatic lipid accumulation. This study identifies URI1 as a potential novel promoter of hepatic steatosis which may also contribute to the progression of NAFLD to HCC.

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List of Abbreviations

AA	Amino acid
ACC	Acetyl CoA carboxylase
ADP	Adenosine diphosphate
AMP	Adenosine monophosphate
AMPK	AMPK activated kinase
ANOVA	Analysis of variance
AP1	Activating protein 1
ATP	Adenosine triphosphate
AU	Arbitrary units
AUROC	Area under the receiver-operating characteristic curve
BAD	Bcl-associated agonist of cell death
BAT	Brown adipose tissue
BCA	Bicinchoninic acid assay
BFP	Blue fluorescence protein
BMI	Body mass index
bp	Base pair
BSA	Bovine serum albumin
BW	Body weight
c9,t11 CLA	<i>cis</i> -9, <i>trans</i> -11 conjugated linoleic acid
CACS	Coronary artery calcium score
CAMKKβ	Calmodulin-dependent protein kinase kinase β
C-terminus	Carboxyl-terminus
ChREBP	Carbohydrate-responsive element binding protein
CI	Cell index
CI/hour	Cell index per hour
CIMT	Carotid intima-media thickness
CK-18	Cytokeratin-18
CLA	Conjugated linoleic acid
CLS	Cytoplasmic localization signal
CoA	Coenzyme A
CRISPR/Cas	Clustered regularly interspaced short palindromic repeats/CRISPR associated proteins
CST	Cell Signaling Technology
CTL	Control
CVD	Cardiovascular disease
DAG	Diacylglycerol
DAMP	Damage associated molecular patterns
dNTP	Deoxynucleoside triphosphate
DSB	Double strand DNA break
EDTA	Ethylenediaminetetraacetic acid

EMT	Epithelial mesenchymal transition
ER	Endoplasmic reticulum
ETC	Electron transport chain
FA	Fatty acid
FABP1	Fatty acid binding protein-1
FACS	Fluorescence activated cell sorting
FAS	Fatty acid synthase
FAT/CD36	Fatty acid translocase/cluster of differentiation 36
FBS	Fetal bovine serum
FFA	Free fatty acids
FLI	Fatty Liver Index
g	Gram
gDNA	Genomic DNA
GFP	Green fluorescence protein
H4IIE^{BFP+} cells	H4IIE cells expressing blue fluorescence protein
H4IIE^{BFP+/GFP+} cells	H4IIE cells expressing both blue and green fluorescence protein
HBS	HEPES-buffered saline
HCC	Hepatocellular carcinoma
HDL	High-density lipoprotein
HFD	High-fat diet
IGF-1	Insulin-like growth factor 1
IGFR1	Insulin-like growth factor-1 receptor
IRS-1	Insulin receptor substrate 1
JNK	c-jun N terminal kinase
kDa	Kilodalton
KO	Knock out
LA	Linoleic acid
LD/nucleus	Lipid droplets per nucleus
LKB1	Liver kinase B1
LB	Luria broth
LD/nucleus	Lipid droplets per nucleus
mA	Milliamp
MAPK	Mitogen activated protein kinases
MCP-1	Monocyte chemoattractant protein-1
MetS	Metabolic syndrome
mg	Milligram
mg/dL	Milligram per decilitre
mg TG/ mg protein	Milligram of triacylglyceride per milligram of protein
mL	Mililitre
mM	Millimole per litre
mm	Millimeter
MRI	Magnetic resonance imaging

MRI-PDFF	Magnetic resonance imaging-determined proton density fat fraction
mRNA	Messenger RNA
N-terminus	Amino-terminus
mTOR	Mammalian target of rapamycin
NAD⁺	Nicotinamide adenine dinucleotide
NADP	Nicotinamide adenine dinucleotide phosphate
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NCBI	National Centre for Biotechnology Information
ng	Nanogram
NHEJ	Non-homologous end joining
NLS	Nuclear localization signal
nm	Nanometer
NMD	Nonsense mediated mRNA decay
O-GlcNAcylation	O-linked N-acetylglucosaminylation
OA	Oleic acid
OGT	O-linked N-acetylglucosamine transferase
PA	Palmitic acid
PAM	Protospacer adjacent motif
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDRG1	P53 and DNA damage regulated gene 1
PFA	Paraformaldehyde
PFDN	Prefoldin
PI	Propidium iodide
PP1γ	Protein phosphatase 1 γ
PS	Ponceau Stain
PTC	Premature termination codon
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PVDF	Polyvinylidene difluoride
R	Correlation coefficient
R²	Goodness of fit
RISC	RNA-induced silencing complex
RNA	Ribonucleic acid
ROC	Rate of change
ROS	Reactive oxygen species
RTCA	Real time cell analysis
S	Serine
SNP	Single nucleotide polymorphism
S6K1	p70 S6 Kinase
S6RP	S6 Ribosomal protein
SDS	Sodium dodecyl sulfate

SDS PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
SERCA	Sarco/endoplasmic reticulum
sgRNA	Synthetic guide RNA
siRNA	Short interfering RNA
SREBP-1c	Sterol regulatory-element binding protein-1c
SRR	Summary relative risk
T	Threonine
t10,c12 CLA	<i>trans</i> -10, <i>cis</i> -12 conjugated linoleic acid
TBE	Tris-borate EDTA buffer
TFIIIF	Transcription Factor II F
TLR4	Toll-like receptor 4
TALEN	Transcription activator-like effector nuclease
TBST	Tris-buffered saline, 0.1% Tween 20
TCAG	The Centre of Applied Genomics
TG	Triacylglyceride
TNF-α	Tumour necrosis factor- α
T2DM	Type 2 diabetes mellitus
UPR	Unfolded protein response
URI1	Unconventional prefoldin RPB5 interactor 1
UXT	Ubiquitously expressed prefoldin-like chaperone
V	Volt
VLDL	Very-low-density lipoprotein
WAT	White adipose tissue
WDR92	WD repeat containing protein 92
WT	Wild type
Y	Tyrosine
ZFN	Zinc finger nuclease
α-MEM	Minimal essential media- α
$\mu\text{g}/\mu\text{L}$	Microgram/microlitre
μL	Microlitre
μM	Micromole per Litre
μm	Micrometer

Introduction

Liver

Role in Homeostasis

As the largest internal organ of the human body, the liver contributes to many aspects of normal health. These aspects include carbohydrate, protein, lipid and bile acid metabolism and detoxification of xenobiotics (chemical substances that are not typically found within the organism) (1).

The liver is essential for keeping blood glucose concentrations in a normal range. Insulin is key for the body to communicate its physiological state (fed or fasted) to the liver and to facilitate the appropriate response. The liver helps to maintain normal blood glucose levels by removing glucose from the circulation and storing it as glycogen when levels are too high. The liver also releases glucose into circulation by glycogen breakdown or glucose synthesis from non-carbohydrate precursors (gluconeogenesis) when levels are low (2).

The liver acts as a focal point for lipid metabolism (3). The liver receives free fatty acids (FFA) from the diet via chylomicron remnants, from adipose tissue during the post-absorptive/fasting state, and from *de novo* FFA synthesis (*de novo* lipogenesis) when there is an excess of carbohydrates (4). From here, hepatocytes (the functional cell type of the liver) convert FFAs into triacylglycerides (TG) and secrete them as very low-density lipoprotein (VLDL) particles. Alternatively, when this process is saturated, TGs are stored within cytoplasmic lipid droplets. These lipid droplets act as an accessible energy source for hepatocytes (3).

The liver is essential for the detoxification of ammonia produced by protein catabolism. The body cannot store protein and must deaminate all amino groups of the amino acids (AA)

which make up the protein. While carbohydrate can be burned or stored, the ammonia produced from deamination must be detoxified and excreted (5). Ammonia is transported to the liver from peripheral tissues using glutamine or alanine as nontoxic transporters. Hepatocytes mediate deamination reactions which release ammonia. From here, ammonia is taken up by the hepatocyte mitochondria and the ammonia is ultimately converted into urea. Urea is released into circulation and then excreted (6).

The body is exposed to various pharmaceutical agents and chemicals. The liver is important for the metabolism and clearance of these xenobiotics, which occurs by converting xenobiotics into more water-soluble metabolites that are more readily excreted (7).

Metabolic Dysfunction in the Liver

The liver is known for being a tenacious organ, exhibiting exemplary regenerative capabilities after toxic injuries, chronic inflammation, and surgical resection (8). The liver's resilience to injury is essential since it is the first internal organ which comes in contact with food from the diet, potential pathogens, and dangerous chemicals entering the body through the digestive system.

Despite this, the liver is not immune to gradual accumulation of damage which may be brought on by excessive calorie intake and a sedentary lifestyle. In the background of excessive calorie intake and a sedentary lifestyle, TGs may accumulate within the liver due to a persistently high influx of FFAs and insufficient energy expenditure (9). All FFAs entering or synthesized in the liver are converted into TGs to prevent cell damage from certain harmful lipid species, like diacylglycerols (DAG) or palmitic acid (PA) (4). DAGs and PA have been shown

to mediate negative consequences for hepatocyte cell health, including increased insulin resistance and apoptosis, respectively (10,11).

As TGs accumulate, so do these harmful lipid species causing chronic inflammation, insulin resistance, and hepatocyte cell death (12). However, these consequences often go unnoticed and this disease state can deteriorate without being detected until there is a severe outcome like liver failure or hepatocellular carcinoma (HCC) (13). Therefore, this pathological disease state is identified as a range of disease severities, ranging from mild hepatic steatosis to severe cirrhosis. Non-alcoholic fatty liver disease (NAFLD) is the term which encompasses all of these diseases. NAFLD is the accumulation of TGs and inflammatory damage within the liver in the absence of significant alcohol intake and/or viral infection, and is closely tied to the metabolic syndrome (MetS) (9).

Non-Alcoholic Fatty Liver Disease

Non-Alcoholic Fatty Liver Disease Spectrum

NAFLD may manifest as a mild disease or progress to where there are severe outcomes like liver failure or cirrhosis (13). Hepatic steatosis is defined when the liver contains at least 5% fat by weight (14) and is often asymptomatic and diagnosed incidentally (15). Non-alcoholic steatohepatitis (NASH) is the more aggressive form of the disease, where steatosis is accompanied by inflammatory changes and hepatocyte ballooning (16). High amounts of harmful lipid species and pro-inflammatory conditions lead to excessive hepatocyte cell death (17). While the liver has extensive capacities for wound healing, the rate of hepatocyte cell death may overwhelm these regenerative efforts. As a result, dead hepatocytes accumulate

and alternative wound healing responses, mediated by immune and hepatic stellate cells, are initiated. Wound healing by these cells results in abnormal matrix deposition and scarring which is termed fibrosis (18). If conditions persist, fibrosis can develop into cirrhosis where the scarring is so extensive that there is hepatocellular dysfunction and increased resistance to blood flow resulting in hepatic insufficiency and portal hypertension, respectively (19). Lastly, NAFLD can directly contribute to the development of HCC, a major subtype of liver cancer, making HCC one of the most severe outcomes of NAFLD (20).

Prevalence

NAFLD is the most common chronic liver disease in the world (21). The global prevalence of NAFLD is estimated to be 25.4% (22). NAFLD incidence has increased from 15% in 2005 to 25% in 2015 (23). Regarding Canada specifically, in a cohort of 450 Canadians, the prevalence of NAFLD was 24.7% when diagnosed by computed tomography (24).

The main risk factor for NAFLD is obesity, and due to the growing rates of obesity in Canada, rates of NAFLD are expected to increase as well (25). A modelling study by Swain et al. (26) estimated that yearly cases of NAFLD, NASH and HCC in Canada would increase by 20% (7.8 million to 9.3 million), 35% (1.9 million to 2.6 million) and 80% (660 to 1200), respectively, between 2019 and 2030 (26).

A study by Younossi et al. (27) assessed the economic cost of NAFLD in the United States. The annual predicted cases of hepatic steatosis and NASH were 12 million and 600 thousand, respectively, and the resultant annual economic burden of NAFLD in the United

States would be \$103 billion. This burden will likely only increase after each year since yearly rates of obesity are increasing, and NAFLD is expected to follow this trend (27).

Diagnosis

Most cases of NAFLD are asymptomatic. When symptoms are present, they consist of malaise, fatigue, and right upper quadrant discomfort (28). Investigation into whether an individual has NAFLD often begins after identification of MetS, raised blood liver enzyme levels, or Type 2 Diabetes Mellitus (T2DM) (29). Diagnostic tests for NAFLD should be considered for individuals with MetS and/or T2DM due to the close relationship between these 3 pathologies (29). Blood liver enzyme levels (i.e., alanine transaminase, gamma glutamyl transaminase, or aspartate transaminase) are often elevated in individuals with NAFLD, however, normal liver enzyme levels can also be present in individuals with NAFLD (30).

Individually, these indicators are poor predictors of the presence of NAFLD. However, several parameters can be combined to make tests which more reliably predict NAFLD. The Fatty Liver Index (FLI) assesses body mass index (BMI), waist circumference, gamma-glutamyl transferase, and serum TG levels to provide a score out of 100. A FLI score under 30 rules out fatty liver, while a score over 60 accurately detects fatty liver (31). This test was validated in an Italian cohort and in an elderly Dutch cohort, where the area under the receiver-operating characteristic curve (AUROC; a parameter for determining the performance of diagnostic test where 1 indicates perfect accuracy and 0.5 indicates random chance) was 0.84 and 0.813, respectively (31,32). There are more tests available, and particular tests may be better suited for certain populations. For example, a Korean developed index system test that uses four

separate parameters (ratio of circulating alanine aminotransferase/aspartate aminotransferase levels, γ -glutamyl transpeptidase blood levels, TG blood levels, and BMI), may predict NAFLD better in their respective population compared to the FLI test since it was developed using clinical information from a Korean population (33).

When NAFLD is suspected, further diagnostic tests are warranted. The gold standard for diagnosis is a liver biopsy since this can inform the level of steatosis, inflammation, and fibrosis. Liver biopsies involve attaining a liver sample using a troughed needle from a sedated patient. Liver samples are then fixed, and a pathologist carries out histological assessment of the tissue sample (34). Hepatic steatosis is then evaluated by determining the percentage of hepatocytes containing visible cytoplasmic lipid droplets. Samples containing at least 5% of hepatocytes with visible lipid droplets are diagnosed with hepatic steatosis. Histological analysis of liver samples can also diagnose NASH by identifying inflammatory indices like ballooned hepatocytes, Mallory-Denk bodies (protein aggregates of cytokeratin which accumulate in the cytoplasm of hepatocytes, are commonly found in ballooned hepatocytes and are indicative of NASH), necro-inflammatory foci, and fibrosis (16).

Even though liver biopsy is the gold standard of NAFLD diagnosis, this method is invasive and expensive. It is also limited by sample variability and intra- and inter-observer variability (35). Alternative non-invasive diagnostic tests are often preferred for these reasons. Ultrasound is the most common imaging technique used to assess liver steatosis, because of its low cost, safety, reproducibility, and wide availability (36). A meta-analysis using 49 studies and a total of 4720 participants showed that the sensitivity for ultrasound to detect moderate-severe fatty liver, compared to liver biopsy, was 85% (37). However, ultrasound is less accurate at detecting

mild steatosis. Sensitivity of detecting hepatic steatosis by ultrasound was shown to be reduced when there was $\leq 12.5\%$ liver TG content (38).

Whether hepatic steatosis has progressed to NASH cannot be elucidated using ultrasound and liver biopsy is often avoided due to its invasiveness. However, different circulating biomarkers, such as cytokeratin-18 (CK-18) serum fragments and fibroblast growth factor 21, may help determine the presence of NASH. Increased hepatocyte apoptosis occurs in NASH livers compared to livers with isolated steatosis. Apoptosis requires the activation of effector caspases (proteolytic enzymes) which cleave CK-18, a major intermediate filament protein in hepatocytes (35). A meta-analysis using 9 studies and 852 patients showed that elevated CK-18 fragment blood levels had a pooled AUROC of 0.82 for detecting NASH (39). In contrast, Cusi et al. (40) observed limitations in using CK-18 as a blood biomarker for NASH. In the multi-ethnic study using 424 participants, circulating CK-18 levels increased significantly with increased steatosis, inflammation and NASH, but the AUROC for detecting NASH was only 0.65. There is some promise of combining CK-18 measurements with other circulatory biomarkers of NASH like adipocyte fatty acid binding protein, fibroblast growth factor 21, and soluble Fas levels (35).

Measuring the extent of liver fibrosis is important since this is the best predictor of severe NAFLD outcomes like cirrhosis, need for liver transplant, and liver failure (41). Therefore, a non-invasive method for measuring the degree of fibrosis in a NAFLD patient is essential as well. Transient elastography (also called FibroScan®) measures the stiffness of the liver by placing a probe on the skin, which emits a sheer wave across the liver parenchyma. The velocity of the sheer wave in the liver parenchyma can be calculated using the Doppler technique. The

velocity of the shear wave is directly proportional to the stiffness of the liver, a surrogate marker of fibrosis (42). A meta-analysis has shown the AUROC of transient elastography for detecting significant fibrosis and cirrhosis is 0.82 and 0.94, respectively, in NAFLD patients (42). Transient elastography is useful for ruling out significant fibrosis in NAFLD patients and for sparing them from invasive or expensive tests like magnetic resonance imaging (MRI) or liver biopsies.

MRI can determine the percentage of fat which constitutes the liver more accurately than liver biopsy (43). The MRI-determined proton density fat fraction (PDFF) can quantify the proportion of fat in the liver (44). A meta-analysis by Gu et al. (45) using 6 studies with a total of 635 participants, showed that MRI-PDFF has excellent diagnostic accuracy. When assessing steatosis using biopsy, severity is graded between 0, 1, 2, and 3. The AUROC of MRI-PDFF for identifying biopsy proven steatosis grades between 0 vs. 1-3, 0-1 vs. 2-3, and 0-2 vs. 3 were 0.98, 0.91, and 0.90, respectively (45).

Clinical Significance

NASH is often the cause for individuals requiring a liver transplant in the United States. The proportion of patients waiting for liver transplants due to NASH in the United States has increased 170% between 2004 and 2013. NASH has shown the greatest increase as a cause of liver transplant waitlist registration compared to other chronic liver diseases. It is the second leading aetiology of liver disease among adults waiting for liver transplants since 2013 and is projected to becoming the leading cause of liver transplants in the United States this decade (46). Furthermore, NASH patients with fibrosis have increased rates of death due to

complications from cirrhosis, HCC or liver transplant compared to patients with hepatic steatosis (41).

At the first glance, hepatic steatosis appears to be a benign pathological outcome, and interventions should aim for preventing the deterioration of the disease state into NASH and fibrosis. However, several studies have revealed individuals with NAFLD are predisposed to suffer from cardiovascular disease (CVD). The most prevalent cause of death in individuals with NAFLD is CVD (13). A meta-analysis using 16 observational studies with 34,043 participants comparing adults with and without NAFLD, showed that individuals with NAFLD are 1.6 times more likely to experience fatal or non-fatal CVD outcomes. Furthermore, if the individual was classified as having severe NASH (identified by elevated gamma-glutamyl transferase, a high NAFLD fibrosis score, or histology analysis) their risk of experiencing fatal or non-fatal CVD outcomes is 2.58 times higher than those without NAFLD (47).

Hepatic steatosis contributes to the development of CVD. Computed tomography can assess the calcification of the coronary artery and provide a coronary artery calcium score (CACS). Also, ultrasonography of the carotid artery can detect plaque and measure carotid intima-media thickness (CIMT). These biomarkers serve as indicators of risk for CVD events (48). A retrospective cohort study including 4731 participants showed that those with hepatic steatosis (diagnosed by ultrasound) had an annual rate of CACS progression of 22% compared to participants without hepatic steatosis who had an annual rate of CACS progression of 17% (49). Furthermore, it was shown in a population of biopsy proven NAFLD patients that CIMT is greater in NAFLD patients (1.14 mm) compared to a healthy control population (0.82 mm) (50).

In addition to this, there is evidence that NAFLD can lead to the development of T2DM. NAFLD is prevalent in individuals with T2DM. Dain et al. (51) performed a meta-analysis on studies investigating the prevalence of NAFLD in patients with T2DM using 24 studies involving 35,599 T2DM patients. All but one study used ultrasound to diagnose NAFLD. The pooled prevalence of NAFLD in T2DM patients was 59.67% (51). As previously discussed, ultrasound is a poor diagnostic test of mild NAFLD, which makes it likely that these studies underreported the true prevalence of NAFLD. Furthermore, serum levels of gamma glutamyl transferase and alanine aminotransferase, surrogate markers for NAFLD, have been shown to predict the incidence of T2DM (52). This may indicate that NAFLD and T2DM develop through similar mechanisms. Lastly, interindividual differences in insulin resistance between healthy men were associated with differences in hepatic fat content (53). These findings contribute to the evidence that hepatic fat content and the level of liver injury are directly associated with insulin resistance.

There is also substantial mechanistic evidence that NAFLD contributes to the development of T2DM. When mice are fed a high-fat diet (HFD), tissue-specific insulin resistance occurs in the liver and adipose tissue after 1 week, while it takes 3 weeks for the muscle to become insulin resistant (54). Isolated liver insulin resistance could initiate the development of T2DM, because there is reduced insulin-mediated suppression of hepatic gluconeogenesis. Absence of insulin-mediated hepatic gluconeogenesis suppression would contribute to hyperglycemia and progress whole-body insulin resistance to T2DM. It has been demonstrated that humans with high hepatic fat content ($\geq 6\%$) have a 30% higher rate of hepatic gluconeogenesis compared to those with low hepatic fat content ($\leq 6\%$) (55). There is

also evidence that hepatic fat content is the main determinant of insulin resistance in different tissues. In obese men and women, it was shown that hepatic fat content, and not visceral adipose tissue volume, was able to predict insulin resistance in the liver, skeletal muscle, and adipose tissue (56). The consequence of insulin resistance in the liver is a cascade of events which leads to dysfunctional whole-body glucose homeostasis. Importantly, hepatic fat content appears to be a main determinant for the extent of hepatic insulin resistance. In addition to this, beyond the 5% hepatic fat content threshold for having NAFLD, there are several negative health outcomes to NAFLD development that are not isolated to the liver. These negative health outcomes are outlined in a study by Brile et al. (57) using a cohort of 352 participants. This study showed that individuals with $\geq 6\%$ hepatic fat content had a reduced insulin-mediated suppression of hepatic gluconeogenesis, increased skeletal muscle insulin resistance, reduced hepatic insulin extraction, increased plasma insulin, increased plasma TGs, reduced high-density lipoprotein (HDL) cholesterol, and increased plasma alanine aminotransferase and aspartate transaminase levels (57).

Therefore, despite the facile interpretation that isolated hepatic steatosis is a benign disease, there are various negative health outcomes that are caused by the development of this disease. Interventions should aim to reduce hepatic fat content below 5% to avoid the increased risk of CVD and the development of T2DM. How increased fat content in the liver leads to hepatic insulin resistance will be explored in the NAFLD Pathogenesis section.

Metabolic Syndrome and Non-Alcoholic Fatty Liver Disease

NAFLD is closely tied to MetS since NAFLD is considered to be the hepatic manifestation of MetS (15). MetS has been defined in the Third Report of the National Cholesterol Education Program Adult Treatment Panel as having at least 3 of the following metabolic abnormalities: increased waist circumference (>102 cm for men and >88 cm for women), high TG levels (≥ 1.7 mM), low levels of HDL cholesterol (<1.03 mM for men and <1.3 mM for women), high blood pressure ($\geq 130/\geq 85$ mm Hg), or high levels of fasting glucose (≥ 6.1 mM) (58). It was shown in a population from the United States that 43% of individuals with MetS had NAFLD (59).

Furthermore, the prevalence of NAFLD is even higher in individuals with more than 3 metabolic abnormalities (59). As the global rate of obesity has increased, so has the global rate of NAFLD. The global mean BMI has increased between 1974 to 2014 from 21.7 kg/m^2 to 24.4 kg/m^2 in men and from 22.1 kg/m^2 to 24.4 kg/m^2 in women (25). Since obesity is a major risk factor for NAFLD, the global obesity trends have likely contributed to the increased global incidence of NAFLD (60).

Non-Alcoholic Fatty Liver Disease Pathogenesis

Lipid Droplets

NAFLD involves the accumulation of TGs within the liver which occurs through receiving fat from the diet via chylomicron remnants, recycling of fatty acids (FA) released from the adipose tissue during fasting, and *de novo* lipogenesis within the liver (4). Conventionally, these TGs are not stored for long in the liver as they are packaged into VLDLs for secretion and delivery to extrahepatic tissues, or the TGs are used for energy production in the liver (3).

Alternatively, if TG stores saturate the liver's capacity for VLDL secretion and exceed the liver's energy requirements, then TGs are used to form cytoplasmic lipid droplets for their safe storage (4). Lipid droplets are dynamic organelles consisting of a neutral lipid core, a phospholipid monolayer which covers the exterior, and lipid droplet specific regulatory proteins decorating the surface (61). The incorporation of FFA into TGs and their storage in lipid droplets is an adaptation used by the liver to avoid lipotoxicity, since certain lipid intermediates are inherently toxic to hepatocytes. Furthermore, once FFA influx overwhelms the physiological capacity of the liver to handle FFAs, hepatocyte apoptosis will occur (62).

Lipid Metabolism Proteins

Various proteins are responsible for proper lipid metabolism in the liver. Furthermore, the expression of several of these proteins is dysregulated during the development of NAFLD (63). The most relevant pathways by which the liver accumulates TGs for NAFLD are by hepatic FA uptake and *de novo* lipogenesis.

Fatty acid translocase/cluster of differentiation 36 (FAT/CD36) facilitates the uptake of FFAs in several cell types, including hepatocytes (64). Hepatic FAT/CD36 expression and protein levels have been shown to be elevated in patients with NAFLD compared to healthy controls (65). Importantly, hepatic FAT/CD36 protein levels are associated with deleterious outcomes of NAFLD, like hyperinsulinemia, insulin resistance, and steatosis grade (65). This indicates that FAT/CD36 expression is necessary for mediating the deleterious outcomes which occur due to NAFLD. Hepatic FAT/CD36 expression was also shown to correlate with apoptosis markers such as TUNEL-positive cells in obese patients with varying degrees of NASH (66). This demonstrates

that elevated FAT/CD36 expression is associated with NASH severity and may indicate that FAT/CD36 contributes to the progression of hepatic steatosis to NASH. As evidence for this, FAT/CD36 genetic deletion has been shown to protect mice from HFD-induced hepatic steatosis (67,68). Also, hyperinsulinemia may play a role in increased FAT/CD36 expression during NAFLD. For instance, CBA/J mice consuming a high sucrose diet developed hyperinsulinemia and hepatic steatosis which coincided with increased hepatic expression of FAT/CD36 whereas C57BL/6 mice consuming a high sucrose diet did not develop hyperinsulinemia or hepatic steatosis (69). Furthermore, exposing isolated hepatocytes from obese Zucker rats (a well-established animal model of hepatic steatosis and MetS) to high concentrations of insulin results in increased FAT/CD36 content at the plasma membrane and increased oleic acid (OA; a monounsaturated FA) uptake compared to control hepatocytes from lean Zucker rats (70). This is evidence that hyperinsulinemia, which is common during NAFLD, promotes FAT/CD36 expression and contributes to NAFLD progression.

Fatty acid binding protein-1 (FABP1) is a cytosolic protein that is highly enriched in hepatocytes. It functions similarly to albumin in the serum since it facilitates the intracellular transport of FFAs (71). It also has an indispensable role in hepatic FA uptake. Liver levels of FABP1 are increased in individuals with NAFLD compared to control subjects (72). Furthermore, mice with a FABP1 genetic deletion show reduced hepatic TG accumulation (73) and reduced hepatic lipid deposition after an oral bolus of OA (74) compared to WT controls.

Hepatic *de novo* lipogenesis is an important component for the development of NAFLD. In individuals with high hepatic fat content (>18.4%), hepatic *de novo* lipogenesis contributes to their hepatic fat content 3.5 times more than in individuals with low hepatic fat content (<3.1%)

(75). Furthermore, rates of hepatic *de novo* lipogenesis have been shown to correlate with whole-body insulin resistance (76).

Among the proteins responsible for mediating hepatic *de novo* lipogenesis, fatty acid synthase (FAS) is crucial for this process, and has been observed to be elevated in the livers of NAFLD patients (77,78). FAS is a multifunctional, homodimeric protein which catalyzes the last step in FA biosynthesis. FAS catalyzes the synthesis of PA (a saturated long chain FA), using acetyl-coenzyme A (CoA) as a primer, malonyl CoA as a carbon donor, and reduced nicotinamide dinucleotide phosphate (NADPH) as a reducing equivalent (79). Sterol regulatory-element binding protein-1c (SREBP-1c) and carbohydrate-responsive element-binding protein (ChREBP) are transcription factors which control the expression of lipogenic genes, including FAS (80,81). Levels of SREBP-1c and ChREBP are elevated in response to hyperinsulinemia and hyperglycemia, respectively (82). Both conditions are common during NAFLD. Also, mammalian target of rapamycin (mTOR) activation has been shown to lead to increased SREBP-1c protein levels, which leads to increased FAS levels (83). The mTOR pathway will be discussed in a later section.

Lipotoxicity

Lipotoxicity refers to the detrimental effects of accumulated harmful lipid species, like DAG and PA, on cellular homeostasis and tissue function (12). To avoid lipotoxicity, these lipid species are shunted into TG synthesis pathways and stored away within lipid droplets. The detrimental consequences of the accumulation of harmful lipid species in hepatocytes include

inflammation (84), oxidative stress (85), insulin resistance (10), endoplasmic reticulum (ER) stress (86), and hepatocyte cell death (87).

It has been shown that NAFLD patients have increased levels of lipid peroxidation, a consequence of elevated oxidative stress (88). Oxidative stress is caused by increased levels of reactive oxygen species (ROS), which are generated from “electron leakage” within the electron transport chain (ETC) in the mitochondria. This condition is worse in the context of NAFLD since there are higher concentrations of FFAs, which can fuel the ETC and generate higher levels of ROS (89). Sunny et al. (55) showed that subjects with high hepatic fat content ($\geq 6\%$) had increased mitochondrial oxidative flux through the tricarboxylic acid cycle (~ 2 -fold induction) compared to subjects with low hepatic fat content ($\geq 6\%$) (55). This is evidence that in individuals with hepatic steatosis, there is increased mitochondrial oxidative activity which would lead to increased ROS production. ROS and its lipid peroxidation products cause damage to proteins involved in the ETC and result in loss of mitochondrial function. Loss of mitochondrial function impairs β -oxidation of FFAs and oxidative phosphorylation and would allow FFAs levels to increase. Elevated FFA levels cause even more damage to mitochondrial function and perpetuate this vicious cycle, where FFA accumulation promotes conditions for further FFA accumulation and deterioration of the pathological state. (90). This is exhibited by NAFLD patients having hepatic mitochondria with structural abnormalities and impaired oxidative phosphorylation (91,92). Depletion of cellular anti-oxidants and reduction of oxidative phosphorylation capacity results in shifting the hepatocyte redox status towards a pro-oxidative state and adenosine triphosphate (ATP) depletion, respectively. If these conditions persist, hepatocyte cell death occurs, and the disease state deteriorates (93).

The ER is a cellular organelle where post-translational modifications and protein folding of secretory and membrane proteins occur (94). It is also the main site of VLDL assembly, and lipid droplet formation (61). Lastly, the ER is important for maintaining Ca^{2+} homeostasis (95). Obesity has been shown to disrupt ER homeostasis and induce ER stress (96). ER stress is the result of disruptions in the physiologic function of the ER by the accumulation of unfolded proteins or disturbances to ER Ca^{2+} homeostasis (95). ER stress activates the unfolded protein response (UPR) which aims to relieve ER stress, and also results in the upregulation of cellular processes that facilitate hepatic fat accumulation, inflammation, and apoptosis (97). Since the ER membrane is the main site for lipid metabolism (61), when hepatocytes are charged to process large amounts of lipids (conditions found in NAFLD), it results in the disruption of the ER membrane composition and stiffening of the membrane (97). It was shown that the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) in the ER membrane is sensitive to changes in the membrane composition and its Ca^{2+} transport activity is suppressed as a result of this. This disrupts ER homeostasis and triggers activation of the UPR (86). The UPR results in the upregulation of proteins which further drive lipogenesis and cause hepatic lipid accumulation and inflammation (98,99).

As previously discussed, insulin resistance and NAFLD are tightly associated (51). In brief, insulin resistance is the inability of tissues to respond to insulin. Insulin resistance has been demonstrated to be associated with increased liver FFA uptake (100) and hepatic *de novo* lipogenesis (76,77). This shows that insulin resistance is implicated with processes that drive hepatic lipid accumulation in NAFLD. An initial consequence of liver insulin resistance is the inability to suppress hepatic gluconeogenesis through insulin signaling which contributes to

hyperglycemia and subsequent hyperinsulinemia (53). Paradoxically, the insulin signaling cascade which upregulates TG synthesis remains intact, despite liver insulin resistance (101). High insulin and glucose levels lead to increased expression of SREBP-1c and ChREBP, respectively (82). Furthermore, hepatic DAG (an intermediate metabolite in TG synthesis) levels in the cytoplasm have been shown to be associated with hepatic insulin resistance in obese individuals (10). This association could be explained by DAG's ability to activate protein kinase C ϵ which phosphorylates insulin receptor substrate 1 (IRS-1) on a serine (S) residue which deters insulin signaling (102).

The importance of inflammation in the development of NAFLD was highlighted when diet-induced hepatic steatosis in mice was prevented through the deletion of liver resident immune cells known as Kupfer cells (84). Also, the inflammatory status of individuals is reportedly associated with NAFLD severity (103–105). NAFLD creates an environment which favours Kupffer cell activation. Hepatocytes undergoing metabolic stress or apoptosis (due to lipotoxicity) release intracellular molecules called damage associated molecular patterns (DAMP). DAMPs then activate Kupffer cells which initiate a local inflammatory response that causes injury amplification (106). Activated Kupffer cells release pro-inflammatory cytokines that induce mitochondrial dysfunction, ER stress and apoptosis in hepatocytes (107). Apoptotic hepatocytes can also secrete various chemokines like monocyte chemotactic protein-1 (MCP-1). MCP-1 secretion results in the recruitment of more immune cells and continues the pro-inflammatory positive feedback loop between lipotoxic hepatocytes and activated immune cells (17). Importantly, several pro-inflammatory cytokines have been shown to induce insulin resistance which would further deteriorate the pathological state (108).

NAFLD develops through these multiple mechanisms or “hits”. Initially, NAFLD was believed to be carried out through the “the two-hit hypothesis”. This is where the hepatic TG accumulation is the first hit and then sequentially oxidative stress and inflammation (the second hit) takes place and causes hepatocyte damage (15). This is outdated and has been replaced by the “multiple hit” model. This is where multiple hits contribute to NAFLD severity and feed into each other (109). Many of these cellular processes share similar pathways. For example, c-jun N-terminal kinase (JNK) is activated by the inflammatory cytokine, tumour necrosis factor α (TNF- α) (110), which is associated with NAFLD (111). Additionally, oxidative stress inhibits a phosphatase which downregulates JNK activation (112). JNK activation also contributes to the UPR (97) and induces insulin resistance through phosphorylation of IRS-1 at S371 (113). JNK promotes hepatocyte cell death by stimulating the activation of caspases and the formation of Activating Protein 1 (AP-1), a transcription factor which upregulates the expression of pro-apoptotic genes (114). JNK has been shown to be important for the development of obesity and insulin resistance in animal models of MetS, given that genetic deletion or pharmacological inhibition of JNK in mice is protective against HFD-induced NASH (115,116). In summary, JNK activation connects all of these “hits” and drives the upregulation of all of these cellular processes. Thus, the “multiple hit” model explains how these separate cellular processes which augment NAFLD severity do not proceed in isolation and occur simultaneously.

Mammalian Target of Rapamycin Signaling

The mTOR signaling cascade coordinates cell growth in accordance with nutrient status and influences the balance between anabolic and catabolic processes (117). High levels of insulin, nutrient availability, energy status, and growth factors shift the balance towards anabolic processes partially through the phosphorylation of mTOR and downstream kinases, including p70 S6 Kinase (S6K1) and S6 Ribosomal protein (S6RP) (118). Conditions which favour mTOR activation are prevalent in the livers of individuals with NAFLD (15).

Hepatic mTOR signaling is often found to be elevated in animal models of obesity and in hepatocyte cell culture after FA treatment (119,120). The hepatic fat content of obese *db/db* mice was reduced through hepatic injection of an adenoviral vector encoding small interfering (si)RNA that targets and depletes S6K1 mRNA, thus showing mTOR contributes to hepatic fat accumulation (121). Furthermore, PA treatment results in ER stress, insulin resistance, and apoptosis which coincides with S6K1 phosphorylation. The importance of S6K1 activation for PA-induced lipotoxicity was exhibited when the combination treatment of PA and rapamycin (a pharmacological inhibitor of mTOR signaling) resulted in blunted PA-induced lipotoxicity. Furthermore, immortalized neonatal hepatocyte cell lines from livers of S6K1 deficient mice also showed blunted PA-induced lipotoxicity (122). Mechanistic studies have revealed that mTOR signaling is required for the expression of SREBP-1c (83). The aforementioned studies provide evidence that mTOR has an important role for mediating hepatic fat accumulation during NAFLD.

AMP-Activated Protein Kinase

AMP-activated protein kinase (AMPK) determines cellular energy status by sensing ATP and adenosine diphosphate (ADP) levels and promotes catabolic processes and inhibits anabolic processes by phosphorylating target proteins (123). AMPK directly represses mTOR activation to promote catabolic processes (117). The anabolic processes AMPK inhibits include *de novo* lipogenesis, which is achieved by phosphorylating acetyl CoA carboxylase (ACC) (124). ACC phosphorylation prevents ACC-mediated carboxylation of acetyl CoA and the formation of malonyl CoA, a vital carbon donor for FA biosynthesis (125). Furthermore, reductions of malonyl CoA levels result in the alleviation of malonyl CoA-mediated inhibition of FFA transport into the mitochondria, and, ultimately, increased β -oxidation (126,127).

High levels of ADP/AMP:ATP promote the phosphorylation and activation of AMPK. Periods of fasting and exercise will increase ADP/AMP:ATP levels and promote AMPK activation. In contrast, when there is excessive caloric intake, ATP is readily available, AMPK phosphorylation is allosterically inhibited, and anabolic process are promoted (123).

AMPK has also been shown to be important for the upregulation of autophagy by decreasing mTOR activation. Autophagy is an intracellular cellular recycling and quality control mechanism where dysfunctional/aged organelles, proteins or lipid droplets are degraded and used to provide an alternative source of energy during starvation (128). Several bioactives which alleviate NAFLD in animal models have been shown to do so by upregulating autophagy through AMPK activation (129–131). AMPK activation is considered a viable treatment strategy for NAFLD disease regression through the use of pharmaceuticals, diet, or exercise (132).

Progression of Non-Alcoholic Fatty Liver Disease to Hepatocellular Carcinoma

In 2018 liver cancer was the 6th most commonly diagnosed form of cancer and the 4th leading cause of cancer death worldwide (133). Furthermore, rates of liver cancer incidence and mortality are higher in men, such that it is the 5th and 2nd most common form of cancer incidence and death in men, respectively (133). While liver cancer has been more common in developing countries, it has been consistently increasing in developed countries (134). The most prominent subtype of primary liver cancer is HCC, comprising 70-85% of cases (135). HCC incidence has been increasing in the United States, with 1.5 cases per 100,000 in 1973 versus 6.2 cases per 100,000 in 2011 (136).

The rising incidence of HCC can be attributed to rising rates of obesity and T2DM, which are both major risk factors for NAFLD. A meta-analysis using 11 cohort studies, showed that the summary relative risk (SRR) for liver cancer is 1.17 for overweight individuals and 1.89 for obese individuals compared to normal weight individuals (137). The association between HCC and T2DM is even clearer. Another meta-analysis using 25 studies showed the SRR for HCC incidence and mortality in individuals with T2DM, compared to individuals without T2DM, is 2.01 and 1.56, respectively (138).

HCC often emerges from end-stage liver disease and cirrhosis, and underlying aetiologies including viral infection (Hepatitis B and C Virus), toxicity (aflatoxin or alcohol), and MetS (NASH) (134). Hepatitis B and C Virus-associated cirrhosis is the most common cause of HCC, particularly in developing countries (139). This is also the case in developed countries, but this trend is shifting due to improvements in antiviral treatments and the growing prevalence of NAFLD (134). This was the case in a study investigating the aetiologies of HCC in 13,648

Medicare (federal insurance for people over 65 in the United States) participants from 2005 to 2014, where NAFLD was the most prominent underlying aetiology for developing HCC (NAFLD – 20.7%, HCV – 19.4%, alcoholic liver disease – 4.9%, and HBV – 4.2%) (140). A similar finding was observed in a smaller German cohort of 162 HCC patients (141).

NAFLD creates an environment which favours the development of HCC through several mechanisms. *In vitro*, incubating HepG2 cells with OA results in the reduced expression of a tumour suppressor phosphatase, Phosphatase and Tensin Homolog deleted on chromosome 10 (PTEN), which leads to increased expression of genes associated with epithelial mesenchymal transition (EMT), angiogenesis, inflammation, and cell cycle regulation (142). These processes are important for the development of HCC (20,143). Furthermore, it was shown in human HCC that the levels of FFA uptake via FAT/CD36 were directly proportional to EMT scores, demonstrating that FFA uptake is important for EMT and the development of HCC (144). Also, pro-inflammatory cytokines (TNF- α and interleukin-8), which have been implicated in the pathogenesis of NAFLD, have been shown to promote HCC development (145–147). Lastly, hyperinsulinemia, which is prevalent in individuals with NAFLD, may also promote tumorigenesis. It was shown that IRS-1 expression was elevated in human HCC tumours compared to adjacent non-tumorigenic tissue within the same liver (148). Furthermore, liver-specific IRS-1 genetic deletion in mice is protective against diethyl nitrosamine-induced HCC (149). Therefore, IRS-1 expression is implicated in HCC progression and elevated IRS-1 expression may allow HCC cells to take better advantage of hyperinsulinemic conditions compared to non-tumorigenic cells. Furthermore, insulin resistance has been shown to be directly associated with hepatic levels of insulin-like growth factor 1 (IGF-1) (150) and IGF-1

stimulates proliferation and survival of many types of cancer cells, including HCC cells (151,152). Additionally, IGF-1 expression is increased in HCC tumours from patients with T2DM compared to non-tumour adjacent tissue and the use of insulin-like growth factor-1 receptor (IGFR1) monoclonal antibodies to block IGF-1 and -2 signalling was effective for reducing proliferation and promoting apoptosis in HCC cells *in vitro* (152,153). This is evidence that IGF-1 contributes towards hepatocarcinogenesis since impedance of its signaling pathway prevents HCC development. The aforementioned evidence suggests that NAFLD promotes the development of HCC.

Unconventional Prefoldin RPB5 Interactor 1

Unconventional Prefoldin RPB5 Interactor 1 (URI1) belongs to the prefoldin class of proteins. Prefoldins are chaperone proteins which contribute to the assembly of cytoplasmic protein complexes and prevent protein aggregation by mediating proteolytic degradation. In combination with other proteins, prefoldin subunits form the prefoldin-like complex. Human prefoldin-like complex subunits include prefoldin (PFDN) 2, PFDN6, URI1, ubiquitously expressed PFDN-like chaperone (UXT), P53 and DNA damage regulated gene 1 (PDRG1), and WD repeat containing protein 92 (WDR92). The prefoldin-like complex participates in the assembly of proteins such as RNA Polymerase II and phosphatidylinositol-3 kinase-related protein kinases like mTOR (154). URI1 is integral for prefoldin-like complex-mediated assembly of RNA polymerase II due to its binding site to the RNA polymerase subunit, RPB5 (155).

Prefoldin-like complexes may also interact functionally with transcription factors in a sub-unit specific manner (154). URI1 inhibits the transcription factors it interacts with, including

androgen receptor (156) and Hepatitis B Virus X protein (157).

URI1 is referred to as 'unconventional' due to the presence of additional, highly conserved protein domains. These include a binding site for the RNA polymerase subunit, RPB5, a domain for binding to protein phosphatase 1 γ (PP1 γ), and a URI1 box (the function of the URI1 box has not been identified). Furthermore, it has nuclear and cytoplasmic localization signals (CLS and NLS, respectively), which influence its subcellular location (158). A protein domain map of URI1 is shown on the following page (Figure 1).

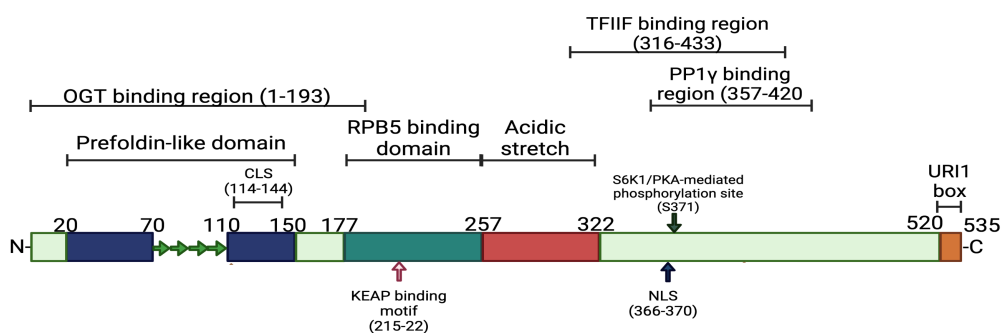


Figure 1: Depiction of URI1 protein domains. URI1 is an atypically large prefoldin protein, due to its additional evolutionarily conserved functional domains, including an RPB5 binding domain, an acidic stretch, and the URI1 box (158). The prefoldin-like domain interacts with other prefoldin proteins to form heterohexameric prefoldin complexes (164). URI1 belongs to the α class of prefoldin subunits, which consist of α -helical coil coiled structures and connected by 2 β -hairpins (green arrows) (158). URI1 contains CLS and NLS sequences (165). RBP5 binding domain has been shown necessary for RPB5 binding and regulation (164). Directly after the RBP5 binding domain is an acidic stretch and its exact function is unknown (155). A phosphorylation site is found after the acidic stretch (S371). The kinases S6K1 and PKA have been shown to induce URI1 S371 phosphorylation (160,166). Phosphorylated URI1 results in URI1-PP1 γ complex disassociation and OGT activity inhibition (160,166). Lastly, an evolutionarily conserved URI1 box is located at the C-terminus (158). Its function is unknown (158). OGT was able to bind to a truncated URI1 segment which corresponded to URI1 AA residues 1-193 (166). Therefore, OGT interacts with URI1 within this region. The same is also true for transcription factor II F (TFIIF) and PP1 γ and their respective binding regions (160,167). URI1 contains a binding motif for kelch-like ECH-associated protein 1 (KEAP1; a negative regulator of nuclear factor erythroid 2-related factor 2 (NRF2)) and facilitates increased expression of NRF2-associated antioxidant genes (168). Created with BioRender.com.

URI1 has been shown to have an integral role in coordinating cell growth and survival in accordance with nutrient availability. Rapamycin treatment in control HeLa cells resulted in the altered expression of 28 genes associated with cell growth, maintenance, and communication. This was altered after short interfering (si)RNA-mediated URI1 ablation which indicates the importance of URI1 in proper rapamycin or nutrient deficiency transcriptional responses in human cells (159). URI1 has been shown to function within a negative feedback mechanism which prevents excessive S6K1 signaling when nutrients and/or growth factors are present and deter apoptosis pathways. An unphosphorylated form of URI1 complexes with PP1 γ in the absence of growth factors. Upon the addition of growth factors, S6K1 phosphorylates URI1 which liberates PP1 γ from the URI1-PP1 γ complex. Upon its release, PP1 γ dephosphorylates S6K1, rendering it inactive. S6K1 dephosphorylation favours apoptosis since the phosphorylation of the pro-apoptotic protein, Bcl-associated agonist of cell death (BAD), is prevented, thus allowing it to facilitate the release of cytochrome C, causing apoptosis (160).

The oncogenic properties of URI1 have been widely explored. High levels of URI1 were found in human ovarian cancer specimens. It was then revealed that elevated URI1 protein levels sustain ovarian cancer cell survival through inhibition of PP1 γ activity and preservation of S6K1-mediated survival signaling (161). In prostate cancer cells, URI1, in collaboration with UXT, represses androgen receptor-mediated transcription. This resulted in reduced ability for prostate cancer cell anchorage-independent cell growth and thus less tumorigenicity (156).

URI1 expression in the liver favours the development of HCC (162). Liver specific overexpression of URI1 in mice results in spontaneous liver tumours. This is due to URI1-mediated repression of aryl hydrocarbon and estrogen receptors transcriptional activities.

These transcription factors facilitate the expression of enzymes required for L-tryptophan/kynurenine/nicotinamide adenine dinucleotide (NAD⁺) catabolism. Perturbation of this pathway leads to low NAD⁺ levels, defective NAD⁺-dependent DNA repair mechanisms, and tumour development (162). Furthermore, phosphorylated URI1 (S371) has been shown to inhibit O-linked N-acetylglucosamine transferase (OGT) O-linked N-acetylglucosaminylation (O-GlcNAcylation) activity (163). URI1 is found to be phosphorylated in glucose deprived conditions by PKA. This results in a reduction of the O-GlcNAcylation of c-MYC (a potent oncogene) thus reducing c-MYC stability, promoting its degradation, and preventing hepatocarcinogenesis (163). In human HCC samples, O-GlcNAcylation levels were found to be elevated compared to normal tissues, and URI1 levels were also elevated but S317 phosphorylation was not detected. Also, mice expressing a non-phosphorylatable mutant form of URI1 had abundant protein levels of c-MYC compared to controls. This suggests that unphosphorylated URI1 may induce c-MYC O-GlcNAcylation and promote the development of HCC (163). Gomes et al. (169) were the first to report that hepatic URI1 protein levels increased in response to HFD in mice. Furthermore, heterozygous deletion of hepatic URI1 protected mice from HFD-induced hepatic steatosis (169). Due to URI1's close association with HCC, URI1 being shown to be important for the development of hepatic steatosis, changes to URI1 protein levels as a result of dietary changes, and the increased risk of HCC that is associated with MetS, URI1 may contribute to the development of NAFLD and its progression to HCC.

trans-10, cis-12 Conjugated Linoleic Acid

Conjugated linoleic acid (CLA) consists of a family of positional and geometric isomers of linoleic acid (LA) with a conjugated double bond system. They are found in ruminant food products such as dairy products and ground beef (170). The *cis-9, trans-11* CLA (c9,t11 CLA) isomer accounts for 85% of the CLA isomers in the diet (171). Even though the *trans-10, cis-12* CLA (t10,c12 CLA) isomer is not present in physiologically relevant levels in food, when CLA is produced synthetically, it makes up about half of all the CLA isomers present (172). Therefore, a relevant amount of t10,c12 CLA is present in dietary supplements. LA, c9,t11 CLA, and t10,c12 CLA, are shown below in Figure 2.

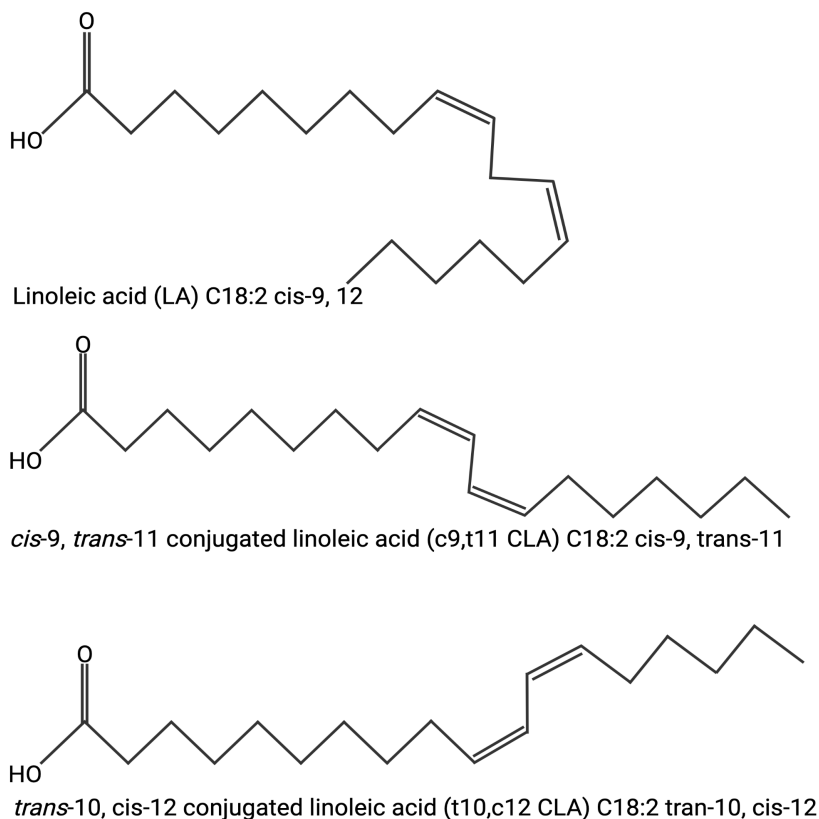


Figure 2: LA, c9,t11 CLA, and t10,c12 CLA. Created with BioRender.com.

Based on studies in murine models, numerous potential positive health benefits have been attributed to the intake of CLA isomers including prevention of cancer, prevention of atherosclerosis, improved immune responses, and weight loss. It has been shown that the health effects of CLA are isomer specific, with most of the focus being on the more abundant c9,t11 and t10,c12 CLA isomers (171).

It has been established that the t10,c12 CLA isomer is responsible for the anti-obesity effect of CLA supplements in mice (173). Feeding either wild type (WT) C57BL/6 mice or leptin receptor deficient *db/db* mice with t10,c12 CLA caused a reduction in whole-body adiposity of 54% and 75%, respectively (173). However, consumption of t10,c12 CLA by lean C57BL/6 mice was accompanied with increased liver weight, increased hepatic TG concentration (unpublished data) and hyperglycemia (173). In obese *db/db* mice, t10,c12 CLA consumption resulted in hyperglycemia (173), increased liver weight, and a trend ($P=0.0794$) for an increase in hepatic TG accumulation (unpublished data). Although t10,c12 CLA consumption did not result in a significant increase of hepatic TG concentration in *db/db* mice, the significant increase of liver weight suggests t10,c12 CLA consumption resulted in elevated liver injury (e.g., macrophage infiltration) (174). Importantly, the effects of t10,c12 CLA on adiposity and hepatic fat content are species specific. In a different animal model of hepatic steatosis, *fa/fa* Zucker rats, dietary t10,c12 CLA was shown to reduced hepatic lipid concentrations and improve features of MetS (175).

The increased hepatic TG accumulation observed in mice could be due to t10,c12 CLA's effect on the mTOR pathway in hepatocytes. Go et al. (176) showed that incubating HepG2 cells with 100 μ M t10,c12 CLA upregulates hepatic *de novo* lipogenesis through the mTOR pathway

(176). Also, a 16-hour co-treatment of t10,c12 CLA (80 μ M) with PA (250 or 500 μ M) resulted in increased PA-induced ER stress (177). This shows that t10,c12 CLA treatment may increase the sensitivity of HepG2 cells to PA-induced ER stress. Augmentation of PA-induced ER stress by t10,c12 CLA treatment may be mediated by t10,c12 CLA inhibition of stearoyl CoA desaturase (SCD). SCD catalyzes the conversion of saturated FAs to monounsaturated FAs. The inhibition of SCD activity leads to increased saturated FA composition of total FAs which would result in elevated PA-induced lipotoxicity and ER stress (177). ER stress had previously been discussed as being capable of inducing a cellular stress response which upregulates lipogenic gene expression (99). This highlights another potential way t10,c12 CLA may encourage hepatic fat accumulation and liver injury in lean and *db/db* mice, respectively.

In humans, the intake of 4.5 grams of a 1:1 CLA isomer mixture (c9,t11 and t10,c12 CLA) everyday for over a year resulted in a statistically significant reduction in body fat mass (178). However, many studies have failed to show a significant effect of CLA on body fat mass in humans (179–181). This may possibly be due to differences in study design (182). On another note, the supplementation of t10,c12 CLA in humans has resulted in negative health outcomes like insulin resistance (183), lipid peroxidation, and inflammation (184). Increases in insulin resistance and inflammation in adipose tissue has also been observed in mice being fed a diet with t10,c12 CLA (185–187).

Weight loss has been shown as an effective method for resolving NAFLD, where weight loss of at least 7% results in meaningful disease regression (60). However, t10,c12 CLA-induced weight loss in mice results in a shift of TG deposition from adipose tissue to the liver (173). This shows that the manner by which weight loss is achieved is important for achieving disease

regression. More research is warranted to investigate how the liver changes to become the preferential location for lipid deposition during t10,c12 CLA-induced NAFLD in mice.

Experimental Models

Animal Models of Non-Alcoholic Fatty Liver Disease

It is estimated that about 34% and 11% of the American and Canadian adult populations, respectively, have MetS (188,189). In order to investigate interventions that help relieve its burden on the healthcare system, well established experimental models are essential. Mice (*Mus musculus*) are often used as animal models, which constitute about 60% of all preclinical research (190). This is likely due to their small size, short reproductive cycle, similar physiology to humans, feasibility of genetic manipulation, and various standardized methods and devices for phenotyping mice which make them the economical and practical choice (190).

For investigating MetS in mice, MetS can be induced through dietary intervention, genetic manipulation, or chemical induction (191). The mouse strain that is often used is the C57BL/6, which are prone to developing MetS characteristics including obesity, glucose intolerance, and insulin resistance. Other strains such as SWR/J and A/J mice are less susceptible to developing MetS (190).

Leptin is a 16 kilodalton (kDa) hormone that is principally produced by adipose tissue in proportion to the size and amount of adipose tissue. Circulating leptin is taken up by the brain, where it imparts signaling that regulates food intake, appetitive behavior, and energy

expenditure. The importance of leptin signaling for maintaining energy homeostasis is revealed by genetic models, such as *ob/ob* and *db/db* mice, which have impaired leptin signaling (192).

Db/db mice are an animal model of MetS since they exhibit various MetS characteristics. They have a single autosomal recessive G-to-T point mutation in the leptin receptor gene, which leads to abnormal mRNA splicing of the leptin receptor and the production of a non-functional leptin receptor (191). A major advantage of using genetic animal models of MetS is that they rapidly and reliably develop MetS without time consuming dietary interventions or invasive procedures which may influence mouse characteristics (193).

Leptin signaling abnormalities lead to uncontrolled appetite, hyperphagia, and reduced energy expenditure, which reflects how humans develop MetS (191). *Db/db* mice have severe early onset obesity, which is apparent at 4 weeks of age. They may attain body weights up to 3 times greater than WT controls. *Db/db* mice develop hyperglycemia by 2 months of age, and their blood glucose levels can be 4-fold greater compared to WT mice on the same genetic background. Plasma insulin is elevated after 10-14 days and peaks at about 3 months. Furthermore, insulin secreting pancreatic β -cells become dysfunctional and their secretory capacity begins declining after 6 months. Therefore, *db/db* mice exhibit several features of MetS (193).

Since NAFLD is considered the manifestation of MetS in the liver, this animal model has been used to study NAFLD. *Db/db* C57BL/6 mice develop hepatic steatosis through excess caloric intake. However, hepatic steatosis will often not progress into NASH and requires additional intervention to induce the inflammatory profile that is typical in NASH. Interventions which induce NASH include a methionine/choline deficient diet (methionine and choline are

necessary for β -oxidation, VLDL production, and protection from oxidative stress which leads to the hepatic lipid accumulation and NASH), HFD, or endotoxin infusions (infusion of toxins produced by bacteria which activate a strong inflammatory response by Kupffer cells and results in liver injury and NASH) (194). Therefore, *db/db* mice are a good model for hepatic steatosis, but not necessarily for NASH (195).

In Vitro Cell Culture Models of Non-Alcoholic Fatty Liver Disease

In vitro models used to study NAFLD include the use of primary cell cultures, and immortalized cell lines. These models are beneficial since they allow for analysis of molecular mechanisms of liver damage and TG accumulation. Nevertheless, *in vitro* models are simplistic and limited since they cannot fully reflect the complexities associated with NAFLD pathogenesis in a whole organism (196). Among these immortalized cell lines is the rat hepatoma cell line, H4IIE. H4IIE cells are a conventional cell line for investigating liver disease since they grow and respond to stimuli in a physiologically relevant manner (197).

In mammals, hepatocytes receive FFA in the form of non-esterified FFAs which are released by lipolysis from adipose tissue (4). Hepatocytes also receive FFAs from chylomicron remnants. Chylomicron remnants carry TG (sourced from dietary fats) to the liver. Chylomicron remnants are internalized into hepatocytes by low density lipoprotein receptors and are degraded by lysosomes. As a result, TGs carried by the chylomicron remnant are hydrolyzed to constituent FFAs which are available for intermediary metabolism (198). Lastly, the liver can synthesize FAs using acetyl-CoA as precursor. In *in vitro* models, FFA treatment of cell cultures involves complexing FFAs with bovine serum albumin (BSA) to address the low solubility of FFA

in media and assist its uptake into cells (199). The manner in which hepatocytes are treated with FFA *in vitro* is reminiscent of how the hepatocytes receive FFA from adipose tissue since FFAs released from adipose tissue are bound to albumin to be transported to the liver (3). This source of FFAs for the development of NAFLD is relevant because it was reported that in a cohort of patients with NAFLD, 59% of the TG content in their livers was derived from FFAs from adipose tissue (200).

The accumulation of lipid droplets within hepatocytes is the hallmark of NAFLD and is initially observed before more severe forms of NAFLD develop. Exposing hepatocyte cell culture to high concentrations of FFA will also induce intracytoplasmic lipid droplet formation (196). Due to their relevance to NAFLD, reliable methods for visualizing lipid droplets are imperative. The commercial fluorescent dye, BODIPY 493/503, reliably stains and identifies lipid droplets with bright green fluorescence (201).

The two most prevalent FA species in the liver are PA (16:0) and OA (18:1,n-9) (202). Studies have identified that the treatment of PA and OA in hepatocytic cell lines leads to different effects. OA is more steatogenic but less apoptotic than PA and vice versa (203). OA is well tolerated by hepatocytes in comparison to other FAs. This allows researchers to investigate the consequences of lipid accumulation that are not related to hepatocyte apoptosis (204).

CRISPR/Cas9 Gene Editing

CRISPR/Cas (clustered regularly interspaced short palindromic repeats/CRISPR associated proteins) is a microbial adaptive immunity system which eliminates foreign genetic elements using RNA-guided nucleases (205). This adaptive immunity system is utilized in

genome editing by delivering to cells the Cas endonuclease and their RNA guides that are designed to target a specific genetic sequence using a ~20-nucleotide long guide sequence. The CRISPR/Cas9 gene editing system consists of a designed synthetic guide RNA (sgRNA) (made from fusing CRISPR RNA and transactivating CRISPR RNA) and the Cas9 endonuclease (206).

Once present in the cell, the RNA guided Cas9 endonuclease identifies a 3-base pair (bp) sequence called the protospacer adjacent motif (PAM). Cas9 then binds to the PAM and proceeds to facilitate bp complimentary binding between the flanking DNA sequence and the sgRNA (207). The capacity to change the guide sequence in sgRNA allows Cas9 to be directed to any gene target of interest (205). Once positioned, Cas9 facilitates a double strand DNA break (DSB) 3 bp upstream from the 3' end of the PAM sequence (207).

This genetic editing system exploits the host cell's DNA repair mechanism to mediate genetic alterations exactly where the DSB had occurred. Non-homologous end-joining (NHEJ) is the DNA damage repair pathway that is often utilized after DSBs and is required for facilitating genetic knockouts. This pathway is error-prone and often results in insertion/deletion alterations (indels) (205). These indels may lead to frameshift mutations, and the addition of a premature termination codon (PTC) in the transcribed RNA. The PTC is detected by the mRNA quality control mechanism, nonsense mediated mRNA decay (NMD), and degrades it (208). Functionally, the gene which harbours the CRISPR/Cas9 induced-PTC is knocked out since its RNA is degraded by NMD. A limitation CRISPR/Cas9 gene editing possesses is the potential of off-target effects (209). There is potential that sgRNA strands may facilitate non-specific binding in genomic DNA (gDNA) and DSBs at unintended locations of gDNA. The consequences of off-target effects are unpredictable and may cause cells to adopt an undesirable phenotype (i.e.,

cancer cells) (209). Therefore, proper screening of sgRNA for preventing off-target effects is crucial.

Other methods for genome editing include the use of zinc finger nuclease (ZFN) and transcription activator-like effector nuclease (TALEN) enzymes. ZFN and TALEN utilize protein-DNA binding to recognize and target specific genetic sequences (210,211). These methods have certain disadvantages. For example, since multiple zinc fingers are required to bind to their gene target, individual zinc fingers may interact with each other and impair target specificity (212). This complicates the process of ZFN targeting, and makes it difficult for non-specialists to design ZFNs which target their gene of interest (213). DNA recognition in TALEN-mediated gene editing is mediated by transcription activator-like (TAL) effectors which are comprised of AA tandem repeats that are 33-35 AAs long. Each repeat varies by only 2 AAs and allows the repeat to target a specific nucleotide (210). Therefore, this requires engineering large repetitive sequences which encode targeted TAL effectors. In contrast, CRISPR/Cas9 editing only requires a short RNA molecule that can easily be manipulated (214). For these reasons, CRISPR/Cas9 gene editing is becoming the preferred method of DNA manipulation, due to its reliability and feasibility compared other gene editing methods.

siRNA serves as another method of inhibiting gene expression through RNA interference. siRNA are synthetic double stranded RNA that are targeted towards specific mRNAs. siRNA can be exogenously introduced into cells to become processed into a single stranded antisense RNA strand which facilitates RNA-induced silencing complex (RISC)-mediated targeted mRNA degradation. RISC-mediated mRNA degradation achieves reduced expression of a gene of interest (215). The manner by which siRNA facilitates gene suppression

is fundamentally different to how CRISPR/Cas9 achieves this, since siRNA represses gene expression at the level of post-transcriptional mechanisms while CRISPR/Cas9 represses gene expression at the level of the genome (216). CRISPR/Cas9-induced genetic alterations are permanent and heritable while siRNA genetic alterations are transient and potentially incomplete (217). Furthermore, if cells are charged with processing excessive amounts of exogenous siRNA, it may saturate the endogenous siRNA system and impair cell viability (218). Lastly, compared to CRISPR/Cas9-induced gene edit off-target effects, siRNA genetic manipulation has been shown to be more susceptible to off-target effects (219).

Study Rationale

Gaps in Research

The increasing rate of obesity is mirrored by the increasing incidence of NAFLD (23,25). Individuals with NAFLD are at increased risk of suffering from CVD and developing T2DM (47,220). Furthermore, a significant proportion of liver transplants are required for NASH patients (46). Evidently, this disease adds a considerable burden to the health care system due to its strong prevalence and deleterious outcomes (27).

Weight loss is an effective method for improving hepatic steatosis, NASH resolution and fibrosis regression (60). However, an animal experiment in our lab where significant fat mass loss was achieved through a dietary intervention with t10,c12 CLA, a dietary component known for inducing weight loss, resulted in increased hepatic fat accumulation in lean C57BL/6 mice (173). Also, rather than improving steatosis in genetically obese *db/db* mice the extent of steatosis was aggravated by t10,c12 CLA diet (unpublished data). Investigating the metabolic

changes in the liver associated with this phenomenon is important for understanding why the liver may become steatotic during t10,c12 CLA-induced fat mass loss. Understanding how the liver may become steatotic in the context of weight loss may help us understand why individuals with healthy BMIs are susceptible to developing NAFLD (221). This research highlights the importance of considering side effects in the liver when developing weight loss therapies.

Interestingly, NAFLD and HCC share similar risk factors (137,138). The most common aetiology of HCC is viral infection, but in developed countries this trend has been shifting due to improvements in antiviral therapies and the high prevalence of NAFLD (134). Recent prospective studies have shown that in cohorts of HCC patients, NASH was the most common aetiology for developing HCC (140,141). Additionally, in cases where the underlying aetiology of HCC could not be determined (cryptogenic HCC), evidence has been mounting that NAFLD could be this missing link (222). The reason for this idea is that there is a high prevalence of T2DM and obesity in cryptogenic HCC cohorts, with many of them having histological and clinical features of NAFLD (135).

Previous research in mice has shown that hepatic levels of the protein, URI1, which has been shown to promote hepatocarcinogenesis, are increased with a HFD diet, a dietary regimen which also induces hepatic steatosis (169). Furthermore URI1 protein levels are elevated in human HCC samples compared to healthy controls (162,163,169). Given the importance of URI1 in the context of HCC and NAFLD, determining how the expression of this protein is regulated by diet is crucial. Studies investigating how obesity and T2DM are linked to HCC are limited and URI1 is a potential and important link between the two.

T10,c12 CLA has previously been revealed to promote hepatic lipid accumulation in the context of t10,c12 CLA induced-fat mass loss in mice (223). However, the role of URI1 in this process has never been discussed. Furthermore, the proposed analyses will include both lean C57BL/6 and genetically obese *db/db* mice. This will enable investigation into whether changes in hepatic lipid concentrations are due to obesity or due to t10,c12 CLA, specifically.

Almost exclusively, the oncogenic properties of URI1 have been widely discussed and investigated. However, discoveries by Gomes et al. (169) have shown a potential link between URI1, hepatocarcinogenesis, and MetS (169). It is crucial that the link between URI1 and MetS is further explored due to the high prevalence of MetS and NAFLD, the hepatic manifestation of MetS (13,25), the projected increased incidence of these pathologies over time (26), and the increased risk of HCC associated with MetS (137,138). This would be the first study that explores the roles of dietary factors for influencing hepatic URI1 expression.

No research has explored the importance of URI1 in neutral lipid accumulation in an *in vitro* model of hepatic steatosis. Several studies have used this model to show the relevance of certain proteins or cellular process for *in vitro* neutral lipid accumulation (99,224,225). This study will involve the first generation of a URI1 KO rat hepatoma cell line whereas previous studies have achieved *in vitro* URI1 depletion through the use of siRNA (160,161,163).

Hypothesis

It is hypothesized that t10,c12 CLA promotes hepatic lipid accumulation through upregulation of URI1 expression and that genetic ablation of URI1 in H4IIE cells results in reduced FA-induced neutral lipid accumulation.

Experimental Approach

To explore the relationship between URI1, t10,c12 CLA and NAFLD, three different experimental models were employed. An animal model was used to assess the relevance of URI1 in the development of t10,c12 CLA induced-fatty liver disease. *In vitro* experiments in WT H4IIE cells were used to investigate the consequences of neutral lipid accumulation on URI1 protein levels and identify specific pathways by which t10,c12 CLA promotes hepatic lipid accumulation. Lastly, CRISPR/Cas9 gene editing was used to knock-out the *Uri1* gene in H4IIE cells to determine whether the relationship between URI1 and neutral lipid accumulation is causal or incidental.

Objectives

1. To evaluate the relationship between hepatic lipid accumulation and URI1 protein levels in the livers of lean C57BL/6 mice and genetically obese *db/db* mice fed a control (CTL) diet or a diet with t10,c12 CLA. This was achieved by:
 - a. Measuring hepatic TG concentration with a colorimetric assay,
 - b. Measuring hepatic URI1 protein levels by Western blotting,
 - c. Comparing hepatic TG concentrations with hepatic URI1 levels to determine the relationship between hepatic lipid accumulation and hepatic URI1 protein levels, and

- d. Measuring the levels of proteins which are relevant for hepatic lipid accumulation to identify potential pathways by which URI1 and t10,c12 CLA may influence this process.
2. To determine if URI1 protein levels change in response to FA-induced neutral lipid accumulation and to identify pathways by which t10,c12 CLA may influence hepatic lipid accumulation. This was done by:
 - a. Treating H4IIE cells with FAs and assessing neutral lipid accumulation over time by using BODIPY staining,
 - b. Determining if URI1 protein levels change in response to FA treatments by using Western blotting, and
 - c. Identifying potential cellular processes by which t10,c12 CLA may influence hepatic lipid accumulation by treatment of H4IIE cells with t10,c12 CLA and Western blotting of proteins involved in hepatic lipid accumulation.
3. To determine whether genetic ablation of *Uri1* in H4IIE cells affects FA-induced neutral lipid accumulation by:
 - a. Using the CRISPR/Cas9 gene editing system to knock out (KO) the *Uri1* gene in H4IIE cells, and
 - b. Treating WT H4IIE cells and *Uri1* KO H4IIE cells with OA to induce neutral lipid accumulation and visualizing the neutral lipid accumulation by using BODIPY staining.

Methods

Cell Culture

H4IIE rat hepatoma cells (American Type Culture Collection, CRL 1548) were grown in minimal essential media α (α -MEM) (Gibco™) supplemented with 10% fetal bovine serum (FBS) (Hyclone). Cells were grown on either 150 mm plates (Thermo Scientific) or 100 mm plates (Sarstedt) in a humidified 37°C incubator with 5% CO₂. Upon reaching at least 70% confluency (3-4 days after seeding), the medium was aspirated from the plate and the adherent cells were washed with either sterile HEPES-Buffered Saline pH 7.4 (HBS) (5 mM EDTA pH 8.0, 150 mM NaCl, 20 mM HEPES) or phosphate buffered saline (PBS) (137 mM NaCl, 2.7 mM KCl, 10.1 mM Na₂PO₄, 1.8 mM KH₂PO₄). To detach the cells from the plate, 0.25% trypsin with ethylenediaminetetraacetic acid (EDTA) (Gibco) in HBS was added (3 mL for 150 mm plates, 2 mL for 100 mm plates) and the plates were incubated at 37°C for 5 minutes. Cell detachment was confirmed via microscopy. To neutralize the trypsin, an equal amount of α -MEM with 10% FBS was added to the plate. The mixture of trypsin, media, and cells was collected and transferred to a 15 mL conical tube (FroggaBio or Sarstedt). Cells were pelleted by centrifugation at 140 g for 5 minutes in a 5804 R centrifuge (Eppendorf) using the A-4-44 rotor (Eppendorf) or in a Legend XFR centrifuge using the TX-750 rotor (Thermo Scientific). The media was aspirated, and the cell pellet was resuspended in 6 mL of HBS/PBS. For passaging cells, new medium was placed in the plate and 0.5 mL of the cell suspension was added back to 150 mm or 100 mm plates.

H4IIE cells were also grown on coverslips for BODIPY staining experiments. Square coverslips (18 mm) (Fisherbrand™) were placed within wells of a 6-well plate (Eppendorf). Two

mL of 70% ethanol was then added to the wells to sterilize the coverslips. Coverslips were left in 70% ethanol for at least 20 minutes at room temperature in the biosafety cabinet. After, the ethanol was aspirated and coverslips were washed twice with HBS (2 mL for each wash). Two mL of α -MEM supplemented with 10% FBS with 2×10^5 H4IIE cells was then added to the wells of the plate.

Cell Counting

Prior to different experiments, the cell density of the cell suspension was calculated. At the Taylor/Zahradka lab, cell density was determined using a haemocytometer (Hausser Scientific). Cell suspensions (10 μ L) were added into the 2 chambers in the haemocytometer. Cells were counted in two different sets of 16 squares. This was repeated for the other chamber. The average of the number of cells in each set of 16 squares was calculated and then multiplied by 10,000. The final value represents the number of cells per mL of cell suspension.

At the McManus lab, 40 μ L of the cell suspension was mixed with 40 μ L of 0.2% Trypan Blue Dye (Gibco). Then 10 μ L of the mixture was pipetted onto a cell counter slide (Cedex Smart Slide, Roche) in duplicate. The smart slide was inserted into the automatic cell counting machine, a Cedex XS Cell Counter (Roche). Using this machine and the Cedex XS software (Roche) cell densities of viable and non-viable cells were calculated. The two viable cell concentration measurements were averaged and used as the final cell density of the cell suspension.

Cell Treatments for Western Blotting

Cells were grown in α -MEM supplemented with 10% FBS in 24 well cell culture plates (Thermo Scientific) and wells were seeded with 5×10^4 H4IIE cells and grown until confluent. Upon reaching confluency, H4IIE cells were placed into serum-free medium for 72 hours to allow the cells to become quiescent (226). FAs were conjugated with BSA (Roche) in a 5% FA-free BSA in PBS solution and used to treat cells. Cells were incubated with FAs in serum free media for a specific amount of time and then lysed with 2 $\times$ sample buffer (2% (v/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, and 125 mM Tris-HCl pH 6.8). Lysates were collected and then briefly sonicated with the Sonic Dismembrator Model 100 (Fisher Scientific) using power level 1. Protein concentrations were determined using the Pierce™ Bicinchoninic Acid Assay (BCA) protein assay (Thermo Scientific) to allow for equal protein loading onto SDS polyacrylamide gels. The procedure for the Pierce™ BCA is described in the following section.

Protein Concentration Assay

Protein concentrations were measured with the Pierce™ BCA (Thermofisher Scientific). A standard curve with known concentrations which ranged from 0.2 mg/mL to 2 mg/mL was constructed by diluting a BSA stock of 2 mg/mL. Ten μ L of each sample and standard were pipetted in triplicate into the wells of a clear 96 well plate (Thermo Scientific). Reagent A and Reagent B from the kit were mixed 50:1, then 200 μ L was added to each well with samples or standards. The 96 well plate was left to incubate at 37°C for 30 minutes, and then was inserted

into the FLUOstar Omega (BMG LABTECH) microplate reader which measured the absorbance at 562 nm.

Cell Treatments for BODIPY Staining

Cells were seeded onto coverslips in 6 well plates (Eppendorf) and were grown and serum starved under the same conditions as described in the Cell Treatments for Western Blotting section. OA was used to induce lipid droplet formation in H4IIE cells. Despite t10,c12 CLA being the focus of the study, OA is the standard FA used for *in vitro* neutral lipid accumulation. OA is well tolerated by hepatocytes and allows researchers to investigate the consequences of lipid accumulation that are not related to hepatocyte apoptosis. Future experiments should include t10,c12 CLA-induced neutral lipid accumulation, but it is important to assess the benchmark FA used for *in vitro* neutral lipid accumulation. A stock solution of 0.1 M OA was made by diluting 1.77 M OA dissolved in ethanol (Cayman Chemical) in PBS with 5% FA-free BSA (Roche). Two μL of the stock solution was added to wells with 2 mL of serum free media for a final concentration of 100 μM of OA for each well. Quiescent cells were left incubating with OA for 0, 1, 4, 8, and 24 hours (depending on the framework of the experiment), after which the media was aspirated, and the cells were fixed with 100 μL of 4% paraformaldehyde (PFA) (4% (w/v) PFA in PBS) for 25 minutes. Then, cells were washed 4 times for 5 minutes with 200 μL of new PBS each time. After, a stock solution of BODIPY (Invitrogen) was made by dissolving BODIPY powder in ethanol to give a 2 mg/mL concentration. This stock solution was diluted 1:100 in PBS. Washed cells were incubated in 100 μL of BODIPY for 10 minutes at 37°C in a dark chamber to protect from the light. From here on, the protocol was

performed in the dark. Cells were then washed twice with 200 μ L of PBS. Next, a 1 mg/mL stock solution of Hoechst 33342 (Sigma-Aldrich) was diluted 1:100 in PBS. Washed cells were incubated with 100 μ L of Hoechst 33342 for 10 minutes at 37°C. Then, cells were washed with 200 μ L of PBS twice. A drop of Mounting Media (Eprelia™ Immu-mount™) was applied onto the microscope slides, and then coverslips were mounted onto the microscope slides (Fisherbrand™ Superfrost™). Stained cells were imaged using a Zeiss Axiovert 3.0 microscope and the images were processed using Axiovision Rel. 4.7 Software.

Image Analysis

For each coverslip, 5 images were taken. The numerical values produced from 5 images from the same coverslip were averaged to produce one data point. Exposure time and gain were optimized for each experiment and kept constant for every image of an experiment. ImageJ was used to analyze each image (227). BODIPY images were not altered. For Hoechst 33342 images, brightness & contrast were altered to optimize the visibility of the nuclei. Both images were merged and saved. After, the channels were split, and the images were individually analyzed. For the BODIPY images, a threshold was applied to the image to allow pixels which exhibit a minimum intensity to be included in the analysis. The same level of threshold was used for all BODIPY images in a single experiment. For Hoechst 33342, a threshold was also applied but the level would change per image to ensure all nuclei were included. The Watershed function was then applied to separate conjoined nuclei for analysis. The images were scaled and the 'analyze particles' function was used. Particle count, average size and area were recorded for the BODIPY and Hoechst 33342 images.

Animal and Diets

Tissue samples used in the research described in this thesis were acquired from a previous animal dietary intervention trial conducted in the lab by Azadeh Yeganeh (228). The animal ethics protocol was approved by the University of Manitoba Animal Care Committee. The animals used in the dietary intervention trial were lean C57BL/6 mice to model a healthy population, and *db/db* mice developed from the C57BL/6 genetic background to model an unhealthy population with MetS. Male *db/db* (n=18) and lean C57BL/6 (n=28) mice from Jackson Laboratory (JAX Mice and Services) were 6-weeks old when acquired and once acclimatized (1-2 weeks) were randomly assigned to different groups. A total of 16 mice (8 *db/db* mice and 8 C57BL/6 mice) were placed into the CTL diet group while the other 30 mice (10 *db/db* and 20 C57BL/6) were placed into the t10, c12 CLA diet (0.4% w/w) group. The diet formulation is listed below in Table 1. The mice were on diet for 4 weeks, after which they were weighed and euthanized by decapitation. Liver tissue was dissected, weighed, flash frozen in liquid nitrogen and stored at -80°C.

Table 1: Diet Formulation

<i>Ingredients (g/kg diet)</i>	<i>Control Diet</i>	<i>t10,c12 CLA Diet</i>
Cornstarch ¹	363	363
Maltodextrin ¹	132	132
Sucrose ¹	100	100
Egg White ¹	212.5	212.5
Cellulose ¹	50	50
AIN-93G-Mineral Mix ¹	35	35
AIN-93VM-Vitamin Mix ¹	10	10
Choline ¹	2.5	2.5
Biotin Mix ¹ (200 mg biotin/kg cornstarch)	10	10
Tert-butylhydroquinone ²	0.014	0.014
Soy Oil ¹	85	80.57
t10,c12 CLA (90% pure) ³	0	4.44

¹Dyets Inc.

²Sigma Aldrich

³Larodan

Triacylglyceride Concentration Assay

The LabAssay™ Triglyceride colorimetric assay (FUJIFILM Wako Pure Chemical Corporation) was used to determine liver TG concentrations. Fifty mg of liver was added to 200 µL of PBS. The tissue-PBS mixture was homogenized by sonicating the mixture with the Sonic Dismembrator Model 100 (Fisher Scientific) at level 1 power setting for 10 seconds, then centrifuged in a 5804 R centrifuge (Eppendorf) using the FA-45-30-11 rotor (Eppendorf) at 17,950 g for 10 minutes. The supernatant and the upper fatty layer (if visible) were collected, and the pellet was discarded. The supernatant and the upper fatty layer were used for the subsequent analysis.

All standards and samples were assayed in triplicate in a clear 96 well plate (Thermo fisher). A standard curve ranging from 0.25 mg/dL to 8.88 mg/dL was prepared at the time of the assay using the TG standard provided in the kit. The colour reagent was prepared by reconstituting 1 bottle of chromogen substrate with 1 bottle of buffer.

For each sample, 1 μ L was pipetted into each well. Samples from lean C57BL/6 mice on the CTL diet were not diluted whereas all samples from *db/db* mice and t10,c12 CLA-fed lean mice were diluted 1:4 with PBS so that their absorbance fell within the range of the standard curve. After addition of 150 μ L of the colour reagent to each well, the 96 well plate was gently shaken and allowed to incubate at 37°C for 5 minutes. The 96 well plate was then put into the FLUOstar Omega (BMG LABTECH) microplate reader and the absorbance was measured at both 600 (main) and 700 (reference) nm.

TG concentration was standardized to the relative protein concentration of the respective sample. The BCA assay (described in a previous section) was employed to measure the protein concentrations in these samples. Samples were diluted 1:49 with PBS so that their protein concentration measurements would fall within the range of the standard curve.

Protein Extraction from Liver Tissue

Protein was extracted from 40-50 mg of liver tissue by flash freezing the tissue with liquid nitrogen. Soon after, the frozen tissue was ground using a mortar and pestle and 1.2 mL of 3 $\times$ Sample Buffer (187.5 mM Tris-HCl pH 6.8, 3% SDS and 30% glycerol) and 1.2 μ L of both 1000 $\times$ protease and 1000 $\times$ phosphatase inhibitors were added. The mixture was left at room temperature for at least 15 minutes to allow the tissue to lyse. The lysates were collected in 15

mL conical tubes, centrifuged in a 5804 R centrifuge (Eppendorf) using the FA-45-30-11 rotor (Eppendorf) at 17,950 g for 20 minutes and the supernatant was transferred to a new 1.5 mL tube (Fisherbrand™). The pellet was discarded. The supernatant was sonicated with the Sonic Dismembrator Model 100 (Fisher Scientific) at power level 1 for 10 seconds. The BCA assay (described in a previous section) was used to measure protein concentration. Tissue samples were diluted 1:9 with 3× sample buffer so that their protein concentration measurements would fall within the range of the standard curve.

Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis

SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) was employed to separate proteins in protein lysates depending on their size and allow antibodies to detect the presence of the proteins of interest. The percentage of acrylamide used in the SDS polyacrylamide gels was dependent on the size of the protein of interest. For example, if the protein of interest was less than 20 kDa, then the protein lysate would be run in a 15% acrylamide gel. The appropriate volumes of reagents were mixed, poured into the appropriate gel casting apparatus, and allowed to polymerize for 40 minutes. After, the stacking gel was made by mixing the required reagents, pouring it on top of the separating gel, and promptly inserted a comb to form the wells. The mixture was allowed to polymerize for 20 minutes. SDS polyacrylamide gel recipes are provided below in Tables 2-5.

The majority of the SDS-PAGE was done with the Triple Wide Mini Vertical Gel System (Expedeon) for Western blots when there were more than 15 samples. When fewer samples were to be compared, SDS-PAGE was done with the Mini-Protein® II Cell (Bio-Rad) gel

electrophoresis system. Fifteen μg of protein from each sample was topped up to the same volume with 3 $\times$ sample buffer for liver lysates or 2 $\times$ sample buffer for cell culture lysates. Protein samples were denatured and stained for visualization during SDS-PAGE by adding 1 μL of a 1:1 mixture of 10% bromophenol blue and β -mercaptoethanol to each sample. For further protein denaturation, samples were heated for 5 minutes in a water bath which was heated to 100°C, using a microwave. Each sample was loaded into a separate well of the SDS polyacrylamide gel. To determine the molecular weight of these proteins, the BLUelf Pre-stained Protein Ladder (Froggabio) was loaded into a separate well alongside the protein lysates.

SDS PAGE was carried out inside a tank filled with 1 $\times$ running buffer (25 mM Tris Base, 192 mM Glycine, and 0.1% SDS). The SDS polyacrylamide gel was run at a constant current, 20 mA per mini gel or 70 mA for the triple wide gel, for 60-90 minutes. The separated proteins were transferred electrophoretically to a sheet of polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich), with 100V for 60 minutes using 1 $\times$ transfer buffer (161 mM glycine, and 31.2 mM Tris Base, and 20% methanol). The Triple-Wide Mini Blotting System (Expedeon) was used for the triple wide gel wet transfers and the Mini Trans-Blot[®] cell (Bio-Rad) was used for wet transfers with mini gels.

Ponceau Stain (PS) was used to evaluate the quality of the transfer and to create a loading control for Western blots. PS binds reversibly with protein and serves to visualize the proteins present on PDVF membranes and to measure relative amounts of total protein. After transfer, the PVDF membranes were dipped in PS (0.1%) (VWR), rinsed with Milli-Q water, and

placed between two sheets of transparent plastic. The PS-stained membrane was then imaged using the ChemiDoc Imaging System (Bio-Rad).

After, the membranes were washed with 1× Tris-buffered saline, 0.1% Tween 20 (TBST) (50 mM Tris-HCl pH 7.4, 150 mM NaCl, and 0.05% Tween 20) for 5 minutes twice on a rocking platform to remove the PS. Then, the PVDF membranes were incubated in TBST containing 3% (w/v) BSA (Roche) for at least an hour at room temperature to block non-specific binding sites.

Antibodies, targeting proteins of interest, were diluted 1:1000 in 3% BSA-TBST, and are listed in Table 6. Membranes were incubated with diluted primary antibodies overnight at 4°C on a rocking platform. After, the primary antibody was removed and the membranes were washed 5 times with TBST for 5 minutes using fresh TBST each time. Next, the membrane was incubated with horseradish peroxidase (HRP) conjugated secondary antibody for 1 hour at room temperature on a rocking platform. Secondary antibodies were diluted 1:10000 in 1% BSA-TBST. After this, the same washing procedure used for the primary antibody was carried out.

Next, the membrane was briefly incubated in Immobilon® Crescendo Western HRP Crescendo Substrate (Millipore) and imaged using the ChemiDoc Imaging System (Bio-Rad). The intensity of the bands produced from the image was measured by densitometry using Image Lab software (Bio-Rad). A prominent band on the PS image of the membrane was selected as the loading control and the intensity of that band was also measured with Image Lab.

Table 2: Separating Gel Recipes for Triple Wide Gels

% Acrylamide	20% acrylamide (mL)	1.5 M Tris pH 8.8 (mL)	Milli-Q Water (mL)	10% SDS (μL)	10% APS (μL)	TEMED (μL)
5%	5	4.5	10.3	200	200	16
7.5%	7.5	4.5	7.8	200	200	16
10%	10	4.5	5.3	200	200	16
12.5%	12.5	4.5	2.8	200	200	16
15%	15	4.5	0.3	200	200	16

Table 3: Stacking Gel Recipe for Triple Wide Gels

% Acrylamide	20% acrylamide (mL)	1.5 M Tris pH 8.8 (mL)	Milli-Q water (mL)	10% SDS (μL)	10% APS (μL)	TEMED (μL)
5%	2	2	3.76	80	80	20

Table 4: Separating Gel Recipes for One 1.5 mm Mini-Gel

% Acrylamide	20% acrylamide (mL)	1.5 M Tris pH 8.8 (mL)	Milli-Q (mL)	10% SDS (μL)	10% APS (μL)	TEMED (μL)
5%	5	2.25	5.15	200	200	16
7.5%	7.5	2.25	3.9	200	200	16
10%	10	2.25	2.65	200	200	16
12.5%	12.5	2.25	1.4	200	200	16
15%	15	2.25	0.15	200	200	16

Table 5: Stacking Gel Recipe 1.5 mm Mini Gel

% Acrylamide	20% acrylamide (mL)	1.5 M Tris pH 8.8 (mL)	Milli-Q water (mL)	10% SDS (μL)	10% APS (μL)	TEMED (μL)
5%	1	1	1.88	40	40	10

Table 6: Antibodies Used in Western Blotting Analysis

Antibody	Secondary	Predicted Size (kDa)	Catalog #	Company	Function
p-AMPK (T172)	Rabbit	62	2535	Cell Signaling	Activated kinase
FABP1	Rabbit	14	227066	Abcam	FFA uptake
URI1	Rabbit	79	5844	Cell Signaling	Coordination of gene expression with nutrient availability
URI1	Rabbit	60	PA5-103250	ThermoFisher Scientific	Coordination of gene expression with nutrient availability
p-S6RP (S235/236)	Rabbit	32	2211	Cell Signaling	Cell growth and proliferation promotion.
S6RP	Rabbit	32	2217	Cell Signaling	Cell growth and proliferation promotion.
FAS	Mouse	265	610962	BD Biosciences	FA synthesis
p-JNK (T183/Y185)	Rabbit	46, 54	9251	Cell Signaling	Stress signaling
FAT/CD36	Rabbit	110 (varies due to post-translational modifications)	NB400-144	Novus Biologicals	FFA Uptake

URI1 Overexpression Lysate for URI1 Antibody Characterization

Confirmation that URI1 is detected by URI1-specific antibodies was performed by Western blotting with a URI1 overexpression lysate. The molecular weight of the bands obtained with the lysate were compared to the molecular weight of the bands obtained using mouse liver and H4IIE cell lysates. The URI1 overexpression lysate (Origene, catalogue # LC418431) was prepared by transfecting HEK293 cells with a human URI1 expression vector.

Transcription of the recombinant Uri1 gene was driven by a cytomegalovirus (CMV) promoter packaged within the expression vector. The mRNA produced from the expression vector should be translated into a protein equivalent to native human URI1 (with the exception of a carboxy (C)-terminus myc-DDK (FLAG) tag) since the cellular machinery within HEK293 cells is expected to direct appropriate protein folding and post-translational modifications. The URI1 overexpression lysate was reconstituted using 2× sample buffer to a final concentration of 1 µg/µL.

Amino Acid Sequence Alignment and Analysis of URI1 Isoforms

The amino acid (AA) sequence of the URI1 isoforms was obtained from the National Centre for Biotechnology Information (NCBI) (229). For comparative analysis, these sequences were aligned using the online Clustal Omega Multiple Sequence Alignment tool (230). Molecular weights were calculated using the Protein Molecular Weight calculator from The Sequence Manipulation Suite (231).

CRISPR/Cas9

The CRISPR/Cas9 system was used to create H4IIE cells with a genetic knockout (KO) in the Uri1 gene. First, 2 plasmids with sgRNAs, which targeted two different exons in the Uri1 gene (exons 4 and 10), and blue fluorescence protein (BFP) expression cassettes were packaged into lentiviral particles. Then, transduction was used to deliver the sgRNA with BFP to H4IIE cells. After, fluorescence activated cell sorting (FACS) was used to isolate cells that successfully

expressed BFP which is indicative of successful sgRNA transduction. After, nucleofection was used to deliver the Cas9 endonuclease with green fluorescence protein (GFP) to sgRNA transduced H4IIE cells. FACS was used again to isolate cells expressing both GFP and BFP which identifies cells that simultaneously express the Cas9 endonuclease and sgRNA. Of these cells, clonal populations were isolated and were then screened for successful CRISPR/Cas9 gene edits in the *Uri1* gene, and the presence of a homozygous or heterozygous *Uri1* gene KO by Western blotting and sequencing. The sgRNA plasmid expansion, transduction, and nucleofection was carried out at the McManus lab in the CancerCare Research Institute in Winnipeg.

Synthetic Guide RNA Plasmid Expansion

Two sgRNA strands targeting 2 different exons in the *Uri1* gene were used to facilitate generation of H4IIE *Uri1* KO cells. SgRNA strands were designed by Dr. Lucile Jeusset. The sgRNA strands were designed using the Millipore CRISPR Design Tool (https://www.milliporesigmabioinfo.com/bioinfo_tools/faces/secured/crispr/crispr.xhtml) and purchased from Sigma. The sgRNA sequences are provided in Table 8. The sgRNA plasmid DNA concentration was measured using a Nano-Drop spectrophotometer (Thermofisher) which measures the amount of light waves absorbed by the DNA containing buffer which correlates to DNA concentration. *Escherichia coli* (*E. coli*) transformation was used to expand the sgRNA plasmids. Five ng of sgRNA plasmid DNA (Sigma) was added to 50 μ L of Stellar Competent *E. coli* cells (Clontech). Tubes containing the *E. coli* and DNA mixture were placed on ice for 30 minutes. After, the bacteria were heat shocked by placing the cells at 42°C for 45 seconds. Tubes were returned to ice for 1-2 minutes. The volume was then topped up to 500 μ L with

Super Optimal broth with Catabolite Repression medium (Takara Bio). This mixture was then incubated at 37°C with moderate shaking for an hour. Luria Broth (LB) agar plates with 60 µg/mL carbenicillin were prepared and the bacteria mixture was spread over the LB agar gel. The LB agar plates were left to incubate overnight at 37°C to allow the growth of transformed *E. coli* colonies. The day after, one colony of *E. coli* was selected to inoculate 2 mL of liquid LB with 60 µg/mL carbenicillin. Liquid cultures were left to incubate overnight at 37°C with moderate shaking. The next day, the liquid was centrifuged at 6800 g for 10 minutes in the CS-6R centrifuge using the G.H 3.8 rotor (Beckman Coulter). The LB was removed and the QIAprep Spin MiniPrep Kit (Qiagen) was used to isolate plasmid DNA by following the manufacturer's instructions. DNA concentration and purity were measured using the Nano-Drop Spectrophotometer. DNA purity was acceptable as long as the 260/280 ratio was ~1.8.

Table 7: sgRNA Sequences and Gene Targets

sgRNA	Sequence ¹	sgRNA Target
sgUri1-1	CGTGGGTTTAGTTGAGCAT	Uri1 gene, exon 4
sgUri1-2	CGGCTACGAGACTTCAGGA	Uri1 gene, exon 10

¹The sequences shown depict the variable 19 nucleotide sequence at the 3' end of sgRNA used to facilitate complimentary hybridization with gDNA within the Uri1 gene. The opposite 5' end of sgRNA facilitates Cas9-sgRNA complex formation and the DSB at the targeted location.

Transduction

Transduction is the process of using a viral vector to introduce foreign DNA into a cell. All work with lentiviral vectors was done in the Class 2 Regulated products (CL2-R) room at the CancerCare Manitoba Research Institute in Winnipeg. The Lenti-X 293T cell line (Takara Bio), which is a subclone of the human embryonic kidney cell line, HEK 293, is used since it supports high levels of virus titer. Lenti-X 293T cells were grown on Corning® Biocoat™ Collagen coated

100 mm TC culture dishes in Dulbecco's Modified Eagle Medium (Hyclone) supplemented with tetracycline free FBS (Clontech). sgRNA expression plasmids were packaged within Lentiviral vectors by adding 7 ng of sgRNA expression plasmids to the Lenti-X Packaging Single Shots (Takara Bio) and topped up to 600 μ L with nuclease free water. sgRNA expression plasmids were allowed to incubate inside the Lenti-X packaging single shots for 10 minutes to allow for lentiviral packaging. The contents were then added dropwise to 80-90% confluent Lenti-X 293T cells and allowed to incubate with the complexed lentiviral vector for 4 hours. After the 4 hours, 6 mL of complete media was added and cells were left incubating in a 5% CO₂ incubator at 37°C for another 48 hours. To determine if there was sufficient virus production, 20 μ L of the media from the transduced Lenti-X 293T cells was applied to a Lenti-X GoStix strip (Clontech). The presence of a band on the strip indicated that there was enough virus present for transduction (which occurred ~48 hours after incubating Lenti-X 293T cells with Lentiviral vectors). The supernatant was collected, passed through a 0.45 μ m filter, combined 3:1 with Lenti X Concentrator (Clontech) and incubated overnight at 4°C. After, the supernatant was centrifuged at 1,500 g for 45 minutes at 4°C. The ensuing lentiviral pellet was reconstituted in 1 mL of PBS and stored in -80°C

H4IIE cells were seeded into the wells of a 24 well plate and allowed to attach for 24 hours. After, the media was aspirated. Viral stock was diluted 1:2 with serum-free media and 200 μ L of the diluted viral stock was added to the cells with 1 μ L of polybrene (Sigma). Cells were incubated with viral vectors in serum free media for 4 hours. After, complete media was added for a total 500 μ L per well and incubation was continued for about 24 hours. After, this media was safely removed and disposed. Any plastics and reagents used for lentiviral

manipulations were disposed in the appropriate waste containers which were filled with bleach. Wells were washed 3 times with PBS to remove all traces of virus and fresh new complete media was added. Cells were allowed to expand and were continually moved into larger well plates until there were enough cells for FACS.

CRISPR/Cas9 Plasmid Expansion

CRISPR/Cas9 with GFP plasmids and their respective DNA concentrations were provided by the McManus lab. *E. coli* transformation (as described above for sgRNA plasmid expansion) was used to expand the CRISPR Cas9 plasmid, with each tube containing 5 ng of DNA and 50 μ L of DH5 α competent cells (Invitrogen). Transformed bacteria were plated on LB Agar with 100 μ g/mL kanamycin (Fisher Scientific) and left to incubate overnight at 37°C. A single colony was expanded by inoculating 0.75 mL of liquid LB with 100 μ g/mL kanamycin and after incubating at 37°C for 4-5 hours with moderate shaking, the liquid culture was transferred to a sterile 500 mL Erlenmeyer flask with 200 mL of liquid LB containing 100 μ g/mL kanamycin, which was incubated overnight at 37°C with moderate shaking.

After, the culture was transferred to a sterile 500 mL centrifuge bottle (Nalgene). The culture was centrifuged at 6000 g for 15 minutes using the Sorvall RC 6 Plus centrifuge with the SLA-1500 rotor (Thermo Scientific) to pellet the bacteria. The supernatant was removed, and the pellet was resuspended in 10 mL of the P1 resuspension buffer (50 mM Tris base, 0.01 M EDTA disodium salt dihydrate and 100 μ g/ml RNase A). Then, 10 mL of the P2 Lysis buffer (50 mM NaOH and 0.25% SDS) was added and mixed thoroughly. The mixture was left to incubate at room temperature for 5-10 minutes. Then, 10 mL of prechilled P3 neutralization buffer (3 M

potassium acetate, adjusted to pH 5.5 with glacial acetic acid) was added and was left to incubate on ice for 20 minutes. The mixture was then centrifuged at 20,000 g for 30 minutes at 4°C using the 5430r centrifuge with the F-35-6-30 rotor (Eppendorf). A Qiagen Genomic-Tip 500/G Tip column was equilibrated using 10 mL of the QBT equilibration buffer (835 mM NaCl, 50 mM MOPS, 15% isopropanol, 0.15% Triton X-100, and pH adjusted to 7.0 using 4 M NaOH). The buffer was allowed to flow through the column using gravity. The supernatant was then added to the column and allowed to pass through the column. The column was then washed twice by applying 30 mL of the QC wash buffer (1 M NaCl, 50 mM MOPS, 15% isopropanol, adjusted to pH 7 using 4 M NaOH). The DNA was then eluted out of the column using 15 mL of the QF elution buffer (1.25 M NaCl, 50 mM Tris-HCl, 15% isopropanol, adjusted pH to 8.5 with 1 M HCl) and collected. DNA was then precipitated out of the elution buffer using 0.7 volumes of isopropanol. The mixture was then centrifuged at 15,000g for 30 minutes at 4°C using the 5430r centrifuge with the F-35-6-30 rotor (Eppendorf). The supernatant was removed and 5 mL of 70% ethanol was added to it. The tube was inverted 5 times to wash the pellet and then centrifuged for 10 minutes at 15,000 g with same centrifuge and rotor. The supernatant was decanted, and the pellet was left out to dry until the pellet became clear (15-20 minutes). The pellet was redissolved in Tris-HCl EDTA pH 8.0 buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, and adjusted to pH 8.0 with 1 M HCl). The DNA concentration was measured using a spectrophotometer (Thermofisher) and DNA purity was acceptable as long as the 260/280 ratio was ~1.8. Compositions of all buffers used are provided below in Table 8.

Table 8: Buffers used for CRISPR Cas9 Expansion

Buffer	Composition
P1 Resuspension Buffer	50 mM Tris base, 0.01 M EDTA disodium salt dihydrate and 100 µg/ml RNase
P2 Lysis Buffer	50 mM NaOH and 0.25% SDS
P3 Neutralization Buffer	3 M potassium acetate, adjusted to pH 5.5 with glacial acetic acid
QBT Equilibration Buffer	750 mM NaCl, 50 mM MOPS, 15% isopropanol, 0.15% Triton X-100, and pH adjusted to 7.0 using 4 M Na OH
QC Wash Buffer	1 M NaCl, 50 mM MOPS, 15% isopropanol, adjusted to pH 7 using 4 M NaOH
QF Elution Buffer	1.25 M NaCl, 50 mM Tris-HCl, 15% isopropanol, adjusted pH to 8.5 with 1 M HCl
Tris-HCl EDTA pH 8.0	10 mM Tris-HCl pH 8.0, 1 mM EDTA, and adjusted to pH 8.0 with 1 M HCl

Nucleofection

The next step is nucleofection to deliver the Cas9 endonuclease with GFP plasmid into H4IIE cells, which have undergone transduction and express the designed *Uri1* targeting sgRNA strands. This will allow expression of Cas9 in sgRNA expressing H4IIE cells and the mediation of CRISPR/Cas9 gene editing. Nucleofection is a transfection method which uses a combination of electrical parameters and cell type specific reagents to permeabilize cells and allow the introduction of foreign DNA.

sgRNA expressing H4IIE cells which had undergone transduction (700,000) were seeded one day prior on a 150 mm plate (Sarstedt). H4IIE cells were trypsinized and collected into a 15 mL conical tube (Sarstedt). The cell concentration of the cell suspension was calculated using the Cedex XS Cell Counter. Then, a portion of the cell suspension containing 3×10^6 cells were transferred to another conical 15 mL tube. Cells were then pelleted by centrifugation in the Legend XFR centrifuge with the TX-750 rotor (Thermo Scientific) at 140 g for 5 minutes. The

supernatant was then removed from the pellet. Using the Lonza Cell line Nucleofector™ Kit V, the nucleofection solution was made by mixing Cell Line Nucleofector® Solution V with Supplement 1 2:9. The cell pellet was resuspended in 100 µL of the nucleofection solution and 3 µg of Cas9-GFP DNA was added. The mixture was then added to the aluminum nucleofector cuvette. The cuvette was inserted into the cuvette holder in the Nucleofector™ 2B Device (Lonza) and the T-020 program was initiated. The T-020 program applies an electrical field, with a voltage and a time length of pulse specific to the T-020 program, to facilitate DNA uptake by the H4IIE cells. After the program concluded, the cells were promptly removed from the cuvette and added into a well of a 6 well plate with fresh media. Cells were allowed to attach for an hour and a half, and then the media was replaced to remove cells that didn't survive the process. GFP expression was determined to be best within 16 hours of nucleofection. FACS was used to isolate cells expressing BFP and GFP to obtain cells which were both successfully transduced and nucleofected with sgRNA and Cas9 plasmids.

Fluorescence Activated Cell Sorting

FACS was used twice to isolate H4IIE cells expressing BFP and GFP. FACS was first used after transduction to isolate cells expressing BFP. This was conducted by Monroe Chan in the Regenerative Medicine Flow Cytometry facility at the Rady Faculty of Health Science Campus of the University of Manitoba. Another round of FACS was used to isolate cells which successfully co-expressed GFP and BFP. This round of FACS was done by Christine Zhang in the Flow Cytometry Core Facility at the Rady Faculty of Health Science Campus of the University of Manitoba because the flow cytometry apparatus in the Regenerative Medicine Flow Cytometry

Facility was not operational. H4IIE cells that were not transduced or nucleofected were used to determine the gating parameters for detecting BFP and GFP. Also, to identify non-viable cells, propidium iodide (PI) (Sigma-Aldrich) was added to the sorting buffer (PI positive cells represent non-viable cells and vice versa). Cells which underwent transduction or nucleofection were collected into a 15 mL conical tube and pelleted. After, the cell pellet was resuspended in sorting buffer (1% FBS, 2 mM EDTA, 1×PI, and topped up to 4 mL with PBS). The samples were transported to their respective sorting facility on ice and all PI negative cells with appropriate GFP and/or BFP expression levels were collected in collecting media (1× penicillin-streptomycin, 20% FBS, and topped up to 2 mL with α -MEM). Returned cells were seeded into a single well of a 6 well plate with complete media. After allowing sufficient growth, several aliquots of cells were frozen.

Limiting Dilution

Limiting dilution was used to isolate clonal populations of H4IIE cells which successfully co-expressed BFP and GFP (H4IIE^{BFP+/GFP+}). These clones were later screened to assess whether a CRISPR/Cas9-induced edit occurred in the Uri1 gene. CRISPR/Cas9 DNA edits may not have been achieved, despite BFP/GFP co-expression, for many reasons, including inability of sgRNA to hybridize with complimentary gDNA or insufficient Cas9 expression. Furthermore, there is vast variation in the type of mutations which may have occurred between cells. Therefore, individual cells are isolated by limiting dilution to identify cells with a successful Uri1 gene edit and to create a clonal population of cells with a specific genetic mutation of interest. A cell suspension of H4IIE^{BFP+/GFP+} cells was diluted such that it had a cell density of 5 cells/mL in

complete media. Then, 100 μ L of the cell suspension was added to every well of two 96 well plates so that there would be on average 0.5 cells per well. This was done to minimize the chance that a well contained more than one cell. The goal of this was to obtain exactly one cell per well to create a clonal population of cells. These wells were monitored daily for growth. If more than one colony of cells was observed, these populations were not included in the downstream screening process. Once a clonal population reached confluency, the cells were moved to 6 well plates. Once they reached confluency in the 6 well plates, cells were moved to 12 well plates, which were then extracted upon reaching confluency to screen for URI1 protein by Western blotting. Any clones which exhibited diminished or absent URI1 protein levels were reserved for additional screening by DNA sequencing.

Genomic DNA Extraction

gDNA was extracted from clones exhibiting diminished URI1 expression to determine whether the CRISPR/Cas9 endonuclease had facilitated an edit in the Uri1 gene. For this, a phenol chloroform extraction method was employed. A minimum of 1×10^7 cells were pelleted in a 15 mL conical tube, and resuspended in one mL of cell lysis buffer (100 mM NaCl, 10 mM Tris-HCl pH 8.0, 25 mM EDTA, 0.5% SDS, and 0.1 mg/mL proteinase K). Cell lysates were incubated overnight in a 54°C water bath. An equal volume of a phenol:chloroform:isoamyl alcohol solution (25:24:1) (Sigma-Aldrich) was added to the cell lysate, which was then vortexed for 1 minute. This mixture was centrifuged in a 5804 R centrifuge (Eppendorf) using the A-4-44 rotor at 2,000 g for 5 minutes. The upper aqueous phase was separated from the organic phase by careful pipetting and transferred to a new 15 mL conical tube. RNase A was

then added to the aqueous phase to give a final concentration of 20 µg/mL. The tube was then incubated in 37°C for 20 minutes. Then, 100% ethanol (2.5× the volume of aqueous phase) and 3 M sodium acetate pH 5.2 (0.1× the volume of the aqueous phase) were added to the aqueous phase. The tube was then inverted several times to precipitate the DNA. Then, the tube was centrifuged at 2,000 g for 10 minutes to pellet the DNA. The supernatant was then decanted and 1 mL of 70% ethanol was added to the pellet. The tube was inverted several times to wash the DNA pellet and then centrifuged again at 1,700 g for 5 minutes. Next, the ethanol was decanted, and the pellet was allowed to dry for 15-20 minutes or until the pellet became translucent. The pellet was resuspended in 200 µL of Tris-HCl EDTA pH 8.0 buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA, and adjusted to pH 8.0 with 1 M HCl) and the DNA concentration measured using a NanoDrop Spectrophotometer (Thermofisher).

Polymerase Chain Reaction

With the DNA from the clones which exhibited diminished Uri1 expression, Polymerase Chain Reaction (PCR) was used to amplify the DNA from the exons which were targeted by the CRISPR/Cas9 endonuclease (exons 4 and 10). Primers were designed to complement and bind to DNA approximately 300 bp upstream and downstream from the edit regions of both CRISPR Cas9 target sites. The nucleotide sequences of these primers are presented in Table 9. Primers were ordered from Integrated DNA Technologies as a DNA pellet, which was reconstituted in nuclease free water (Promega) to a concentration of 100 µM.

On ice, gDNA was combined with appropriate amounts of 5× Phusion HF buffer (ThermoFisher), 2.5 mM deoxynucleoside triphosphate (dNTP) mixture (Takara Bio), Phusion™ High-Fidelity DNA polymerase (ThermoFisher), MgCl₂ (ThermoFisher), and primers and topped up to 20 µL with nuclease-free water. Pipetting volumes for PCR experiments are provided in Table 10.

Upon addition of all the reagents in 8-strip PCR tubes, the MyCycler™ Thermal Cycler (Bio-Rad) was used to apply the cyclic thermal changes to facilitate the PCR. Cyclic thermal changes involved:

- a single initial denaturation phase (98°C, 30 seconds, ×1 cycle) to break hydrogen bonds between complementary bp present on opposite DNA strands to dissociate double stranded DNA into single strands and to expose DNA binding sites for the designed PCR primers
- a denaturation phase (98°C, 10 seconds, ×30 cycles) to dissociate newly synthesized double stranded DNA into single stranded DNA at the start of every new cycle
- an annealing phase (64.7°C for exon 4 amplification and 64.6°C for exon 10 amplification, 30 seconds, ×30 cycles) where primers hybridize with their complementary DNA targets and where optimal temperature is selected for binding DNA primers with their complement gDNA (as calculated by the T_m Calculator from ThermoFisher)
- an extension phase (72°C, 18 seconds, ×30 cycles) where the temperature is optimal for thermostable DNA polymerase mediated DNA strand replication of new DNA strands

- a single final extension phase (72°C, 5 minutes, ×1 cycle) at the end of the cycles to allow DNA polymerase mediated DNA synthesis to finish any incomplete newly synthesized double stranded DNA

After DNA amplification, PCR products were visualized by electrophoresis in a 1.5% agarose gel. Enough agarose (Invitrogen) to make a 1.5% gel was added to 60 mL of 1× Tris-Borate-EDTA buffer (TBE) (89 mM Tris base, 89 mM boric acid, and 2 mM EDTA) and was heated, using a microwave, to dissolve the agarose. Once the solution cooled to a lukewarm temperature, SYBR™ Safe DNA Gel Stain (Invitrogen) was added to allow for DNA visualization. Molten agarose was poured onto a horizontal gel apparatus with a comb inserted at the top to form the wells in the gel. Molten agarose was allowed to cool and solidify into a gel. The agarose gel was transferred into a Mini Sub Cell GT Cell (Bio-Rad) horizontal electrophoresis tank filled with 1× TBE buffer. Five µL of 100 bp DNA Ladder (New England Biolabs) was added to one of the wells to allow for estimation of the PCR product size. Five µL of PCR product from each respective clone was mixed 6:1 with Gel Loading Dye, Purple (6×) (New England Biolabs) and loaded onto the agarose gel. Agarose gel electrophoresis was performed at 80V until the dye front travelled 80% of the distance to the end of the gel. The gel was then imaged using the Chemi-Doc Imaging System.

Table 9: Primers Used for Polymerase Chain Reaction and DNA Sequencing

Primer	Sequence	Gene Target	Amplicon Length (bp)
Forward	CCAGTGCATAGGTAAGAACTCAGTA	<i>Uri1</i> , Exon 4	666
Reverse	CCTACAAGGGGCATCTTTCCA		
Forward	TTGGCAAATCAAGCTGCTCC	<i>Uri1</i> , Exon 10	599
Reverse	CCTGAGGGCAAAGGCTTCTT		

Table 10: Pipetting Volumes for High Fidelity Polymerase Chain Reaction

Reagent	Volume
5× Phusion HF Buffer	4 µL
2.5 mM dNTP mixture	1.6 µL
100 µM Forward Primer	0.1 µL
100 µM Reverse Primer	0.1 µL
100 ng/µL Template gDNA	1 µL
50 µM MgCl ₂	0.4 µL
Phusion Polymerase	0.2 µL
Nuclease free H ₂ O	12.6 µL

Polymerase Chain Reaction Product Purification and Sequencing

Once it was confirmed that the PCR product had the predicted size, PCR products were purified from primers, nucleotides, enzymes, salts, and other impurities using the QIAquick PCR Purification Kit (QIAGEN). The PCR product purification was carried out by following the manufacturer's protocol.

Purified PCR products were shipped to The Centre of Applied Genomics (TCAG) DNA Sequencing/Synthesis Facility in Toronto for Sanger sequencing. Samples were prepared by mixing 30 ng of DNA from each clone with 50 ng of primer in a total of 7.7 µL of nuclease-free water in 8-strip PCR tubes. DNA was sequenced in the forward direction using the same primer used in the initial PCR. Upon receiving the DNA samples at TCAG facilities, DNA sequencing results were returned as chromatograms. Chromatogram files which showed double peaks starting close to the CRISPR/Cas9 target region were uploaded to CRISP-ID. CRISP-ID is an online program that predicts the size and location of CRISPR/Cas9-induced indels from Sanger sequencing (232). Sanger sequencing in a diploid cell with a CRISPR/Cas9-induced indel results in 2 different alleles and a mixture of 2 Sanger sequencing outputs in a single chromatogram.

CRISP-ID analyzes the mixture of alleles and resolves the differences with the use of a reference sequence.

If the chromatogram did not show double peaks, then the sequencing results were aligned with the reference DNA sequence using the online Clustal Omega Multiple Sequence Alignment tool (230). This was done to check if there were any homozygous CRISPR/Cas9 induced-indels, since these would not be apparent in the chromatogram.

Upon identification of indels, the impact of the indels on the AA sequence was predicted using the ExPASy translate tool (233). This is an online application which allows for the translation of a nucleotide sequence to a protein sequence. Both the reference coding DNA of the *Uri1* gene and the resulting coding DNA of the *Uri1* gene in clones with a CRISPR/Cas9 edit were translated into an AA sequence using this tool. The AA sequences of CRISPR/Cas9 edited *Uri1* alleles from *Uri1* KO H4IIE clones and the AA sequence from the reference sequence of *Uri1* were aligned with each other using the Clustal Omega Multiple Sequence Alignment tool. This was done to predict whether an out-of-frame insertion or deletion occurred, leading to NMD, and ultimately gene silencing.

Real Time Cell Analyses

Real time cell analysis (RTCA) is a real-time cell counting assay that measures the presence of adherent cells through the interruption of conductance between the media and the surface to which the cells adhere. The xCELLigence RTCA system (Acea Biosciences) measures electrical impedance by using E-plates with wells that contain two biosensors on the bottom of

the wells. When the wells are filled with a conductive solution (like cell culture media) and an electric potential is applied to the biosensors, electrons exit out of the negative terminal, travel through the solution, and reach the positive terminal. However, when adherent cells populate the bottom of the wells, the conductance of the media is reduced. The extent that conductance is impeded is directly related to the number of adherent cells present and the area they occupy. Therefore, impedance of electron flow caused by adherence is used by real-time cell analysis to measure cell proliferation and is reported as a unitless parameter called cell index (CI), where $CI = (\text{impedance at time point } n - \text{impedance in the absence of cells}) / \text{nominal impedance value}$. Upon seeding the E-Plate, the E-plate is docked onto the RTCA SP station which is placed inside a 37°C incubator with 5% CO₂. The RTCA DP Station is connected to the RTCA control unit with the preinstalled RTCA software. This way, the CI can be monitored in real time, and starts once cells are seeded.

All wells of the E-plate 16 were filled with 100 µL of complete media. Then the E-plate was docked onto the RTCA DP station. Upon docking, the background measurement was performed. After, 100 µL of H4IIE cell suspensions with a cell density of approximately 10 000 cells/mL (from WT cells and the various clones to be monitored) were seeded in the E-plate 16 in quadruplicate to achieve 1000 cells and a total of 200 µL of media per well. The plate was docked onto the RTCA DP Station and CI measurements were taken every 15 minutes until the cells became confluent (~7 days).

For RTCA data analysis, the mean of the CI values was calculated for each 10-hour increment. Also, the mean maximal CI and the number of hours to reach maximal CI were determined. Lastly, the rate of change (ROC) when cells reached 50% confluence was

measured, to assess the speed of growth of H4IIE clones of interest. This was done by using curve fitting to construct a curve which fit the RTCA data set. MATLAB was used to generate 10th order polynomials that produce a curve that resembles the data set, such that the goodness of fit, (R^2 ; a value which quantifies how close the model and the data points are, where 1 indicates a perfect fit), was ≥ 0.999 (234). The derivative of the curve fitting formula was then used to calculate the ROC when cells were 50% confluent (1/2 maximal CI).

Statistical Analyses

For experiments involving animal tissue, two-way Analysis of Variance (ANOVA) was employed to determine whether main effects (diet and genotype) or interaction effects (diet $\times$ genotype) were statistically significant. Correlations were analyzed using Spearman's rank correlation coefficient since data were not normally distributed or homogeneous as determined by the Shapiro-Wilk test and Levene's test for homogeneity of variance, respectively. For *in vitro* experiments with WT H4IIE cells, data were analyzed by one way ANOVA followed by Duncan's multiple range test to identify specific differences among means. Experiments with *Uri1* KO and WT H4IIE cells used two-way ANOVA to determine whether main effects (treatment and genotype) or the interaction effect (treatment $\times$ genotype) were statistically significant.

The Shapiro-Wilk test for normality and Levene's test for homogeneity of variance were used to determine if the raw data and log transformed data were normal and homogenous, respectively. Log transformed data were used if the raw data were not normal and homogenous. If both data sets could not satisfy this requirement, non-parametric testing was

done using the Kruskal-Wallis test, followed by post hoc testing with least significant differences and Tukey correction for multiple comparisons. Data are reported as means $\pm$ standard error of the mean (SEM) and the statistical significance was set at $P < 0.05$. All statistical tests were done using SAS software (235).

Results Section A: URI1 Protein and Antibody Characterization

The purpose of this section is to establish the characteristics of the URI1 antibodies being employed for Western blot analysis in this research study. As part of this process, it was observed that there are discrepancies between the calculated molecular weight of URI1 and its molecular mass as determined by SDS-PAGE. The experiments described in this section were done to explore these discrepancies and to confirm the identity of the bands detected by immunoblotting as URI1.

Detection of URI1 in a HEK293 URI1 Over-Expression Lysate Using the CST URI1 Antibody

All the commercially available antibodies were made by immunizing animals with synthetic peptides corresponding to human URI1. Thus, these antibodies are likely best suited for detecting human URI1.

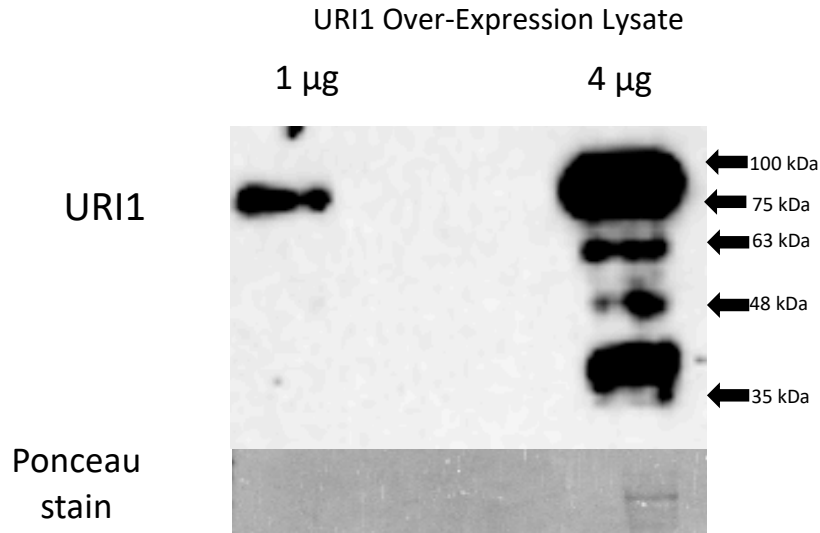
HEK293 cells are derived from human embryonic kidney. The URI1 overexpression lysate was made by transfecting HEK293 cells with a human URI1 expression vector. Transcription of the recombinant URI1 gene is driven by a CMV promoter. The mRNA produced from the expression vector should be translated into a protein equivalent to native human URI1 (with the exception of an added C-terminus myc-DDK (FLAG) tag) since the cellular machinery within HEK293 cells is expected to direct appropriate protein folding and post-translational

modifications. It is important to note that HEK293 cells independently express URI1. The providers of the URI1 over-expression lysate predict the molecular weight of URI1 to be 60 kDa.

The overexpression lysate was reconstituted in 2× Sample Buffer and 1 µg and 4 µg of the overexpression lysate were loaded into separate wells during SDS-PAGE. Western blotting was carried out using a URI1 antibody from Cell Signaling Technology (CST). The antibody provider predicts the molecular weight of URI1 to be 79 kDa.

For the lane where 1 µg of lysate was loaded, one band was observed at 79 kDa (Figure 3A), matching the CST datasheet. Interestingly, when 4 µg of URI1 was loaded, there were an additional 3 bands situated near the 63, 48, and 35 kDa molecular weight markers. There was a significantly stronger signal from the 79 kDa URI1 band compared to the other 3 bands (Figure 3B). It is important to note that URI1 is expressed in most human cell lines, including HEK 293 cells. The strongest band may have been the result of the transcription of the URI1 expression vector and the remaining bands could have been due to basal expression of URI1 in HEK 293 cells.

A



B

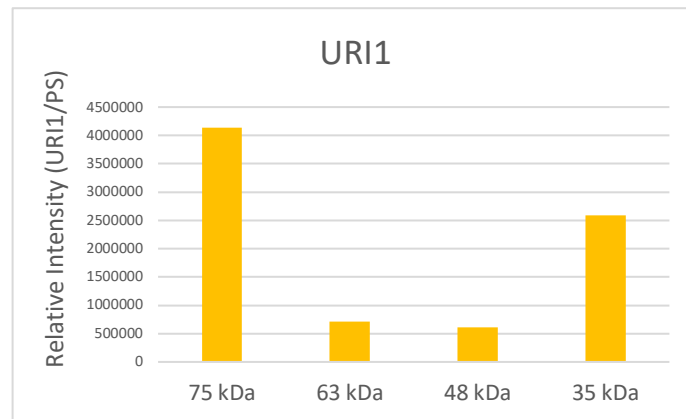


Figure 3: URI1 Western blot using URI1 over-expression lysate and the CST URI1 antibody (A). The lanes on the left and right side of the Western blot were loaded with 1 and 4 µg of URI1 over-expression lysate, respectively. The location of molecular weight markers are indicated on the right. Relative protein levels of URI1 bands within the 4 µg lane (B). Bands are designated according to the closest molecular weight marker. Densitometry was used to quantify the intensity of the URI1 bands. No attempt to account for the effect of saturation was made during quantification.

Detection of URI1 in Mouse Lysate using the Cell Signaling Technology Antibody

Mouse liver lysates were prepared as previously described (SDS-PAGE Section in the Method Chapter) for Western blotting. The CST URI1 antibody detected a single band which was nearest to the 48 kDa molecular weight marker (Figure 4A), despite it being predicted to be 79 kDa by the antibody provider. The ~48 kDa band identified by the CST URI1 antibody mirrors the band observed in the URI1 over-expression lysate Western blot that was closest to 48 kDa molecular weight marker (Figure 3A).

To confirm that this band is URI1 and not the result of non-specific binding, an alternate antibody from ThermoFisher, which recognizes a separate URI1 epitope compared to the CST URI1 antibody, was used for Western blotting. This Western blot shows 3 bands close to the 63, 48, and 35 kDa molecular weight markers (Figure 4B). These 3 bands also mirror the 3 less intense bands observed in the URI1 over-expression lysate Western blot (Figure 3A). Importantly, the band closest to the 48 kDa molecular weight marker is found in the same location as the band detected by the CST URI1 antibody. Therefore, since the likelihood that both of these antibodies non-specifically recognize proteins that are a similar size is extremely low, and a band detected by the CST antibody in the URI1 overexpression lysate has a similar molecular weight, the ~48 kDa band detected by both the CST and Thermofisher URI1 antibodies is likely URI1.

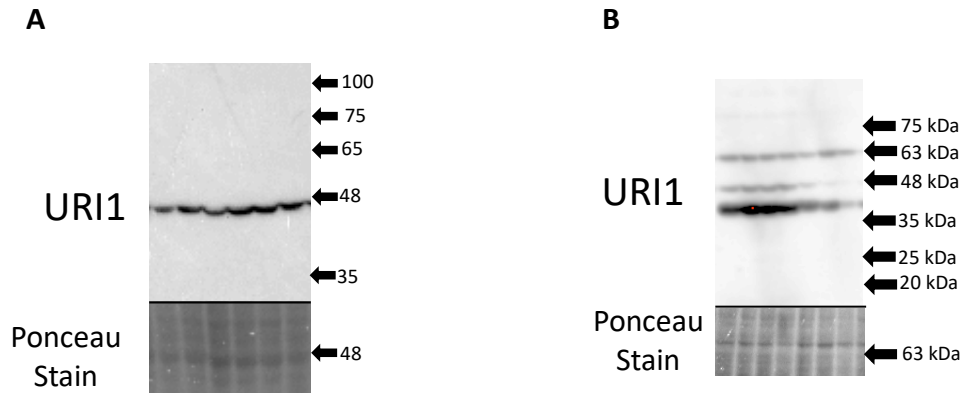


Figure 4: Mouse liver lysates immunoblotted with CST and Thermofisher URI1 antibodies. Western blots of a mouse live lysate using (A) the CST URI1 antibody and (B) the Thermofisher URI1 antibody. Black arrows on the Western blot and PS indicate the locations of the molecular weight markers.

Detection of URI1 in H4IIE Rat Hepatoma Cells

URI1 was detected in H4IIE cell samples using the CST URI1 antibody. Western blots with quiescent H4IIE cell samples showed one band near the 35 kDa molecular weight marker (Figure 5A). Interestingly, URI1 Western blots using quiescent H4IIE cells which were treated with OA for at least 4 hours, showed the same 35 kDa band and an additional ~79 kDa band (Figure 5B). The additional ~79 kDa band corresponds to the molecular weight predicted by CST.

The ~79 kDa URI1 band had been shown to be responsive to growing conditions. Since this band is sensitive to changes in the environment, it is likely mediating a biological effect to compensate for these changes. Thus, the ~79 kDa band of URI1 was the focus for Western blot analyses in H4IIE cells. Conditions which allow for detection of the ~79 kDa band may indicate a novel mechanism of regulating URI1 expression.

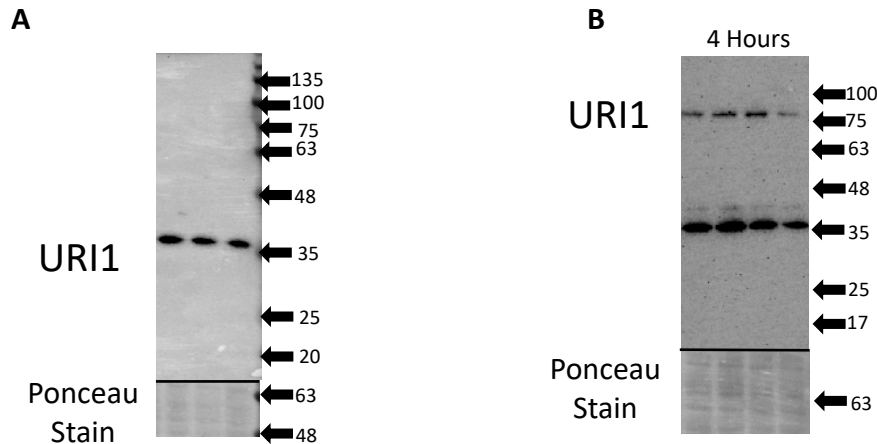


Figure 5: URI1 Western blots of H4IIE cell lysates using the CST URI1 antibody. URI1 Western blots using quiescent H4IIE cells (A), and quiescent H4IIE cells after OA treatment for 4-hours (B). The Western blot was modified to only include the lanes that show that the ~79 kDa band is detected by the CST URI1 antibody in quiescent H4IIE cells after 4-hours of OA treatment. Lanes were extracted from the Western blot presented in Figure 17 on page 108 which is shown in its entirety. There were no other modifications to this image. Black arrows on the Western blot and PS indicate the locations of the molecular weight markers.

Review of URI1 Isoforms Across Different Species Using the NCBI Database

The diversity of URI1 bands observed across different Western blots using different URI1 antibodies and types of lysates warrants inquiry into the different URI1 isoforms. The NCBI database was used to help elucidate the discrepancies between the different URI1 bands with differing molecular weights observed across the different Western blots. A summary of the different listed URI1 isoforms in humans, mouse, and rat are presented in Table 11. Furthermore, the Basic Local Alignment Search Tool (BLAST) was used to align different URI1 isoforms to identify the differences between URI1 isoforms.

Table 11: Summary of the Human, Mouse, and Rat URI1 Isoforms Listed in the NCBI Database, and their Relation to URI1 Western Blots.

Species	URI1 Isoform ^a	Molecular Weight (kDa)	URI1 Western blot Figure which exhibits a band corresponding to the respective URI1 isoform (kDa)	Nearest molecular weight marker to the URI1 isoform band (kDa)
Human	Isoform X1	58	Figure 3A	63
	Isoform c	53	N/A ^b	N/A
	Isoform X4	51	Figure 3A	48
	Isoform X3	55	N/A	N/A
	Isoform a	59	Figure 3A	63
	Isoform X2	55	N/A	N/A
	Isoform X5	51	Figure 3A	48
	Unidentified URI1 Band	~40	Figure 3A	35
Mouse	Isoform X1	60	Figure 4B	63
	Isoform X2	54	N/A	N/A
	Isoform X3	50	Figures 4A and 4B	48
	Unidentified URI1 Band	~40	Figure 4B	35
Rat	Isoform X2	42	Figures 5A, 5B and 5C	35
	Isoform X1	51	N/A	N/A
	URI1	59	N/A	N/A

^aNomenclature of the individual URI1 isoforms is based on the NCBI database which uses X and a number unique for that isoform (i.e., X1, X2, etc.) or a lower-case letter.

^bN/A indicates that a band with the molecular weight of the respective URI1 isoform was not detected amongst the Western blots presented in this thesis.

Human URI1

The NCBI database depicts 7 different isoforms for human URI1. In the NCBI database, protein isoforms are designated by X and a number unique for that isoform (i.e., X1, X2, etc.). Different isoforms may also be categorized using lowercase letter (i.e., a, b, c, and etc.) The molecular weights of human URI1 isoforms X1, c, X4, X3, a, X2, and X5 are 58, 53, 51, 55, 59, 55, and 51 kDa, respectively (summarized in Table 11). Using BLAST to align the amino acid (AA) sequences of these URI1 isoforms showed they vary in their C- and amino (N)-termini (Figure 6).

The peptide antigen sequence used to produce the CST URI1 antibody is proprietary, but the synthetic peptide used corresponds to residues surrounding AA 523 of human URI1 isoform a. Therefore, theoretically, URI1 isoforms a, X3, X1, and X4 would be recognized by this antibody, but not URI1 isoforms X2, c, and X5 (since these isoforms do not contain the epitope used by the CST URI1 antibody). The CST URI1 antibody detected 4 bands which were nearest to the 75, 63, 48, and 35 kDa molecular weight markers (Figure 3A). Since URI1 isoforms a and X1 are 59 and 58 kDa, respectively, these URI1 isoforms may be represented by the band nearest to the 63 kDa molecular weight marker. Since URI1 isoform X5 is 51 kDa, this URI1 isoform may be represented by the URI1 band closest to the 48 kDa molecular weight marker.

There were additional bands near the 75 and 35 kDa molecular weight markers, but the NCBI database does not list URI1 isoforms with similar calculated molecular weights. The URI1 band close to the 75 kDa molecular weight marker is likely the result of post-translational modifications and bands with a lower molecular weight could be URI1 lacking these modifications.

The band closest to the 35 kDa molecular weight marker could be the result of alternative splicing, cleavage, or the use of alternate start codons. URI1 AA sequences show various Methionine (M) residues dispersed throughout the protein. Methionine indicates the presence of potential mRNA translation start codons. Therefore, the presence of the lower molecular weight URI1 band may be the result of mRNA translation being initiated closer to the end of the protein. If protein translation started at Methionine 151 of URI1 isoform a, it would produce a protein that is 42 kDa which corresponds approximately to the lower molecular weight band observed in the URI1 overexpression lysate Western blot (Figure 3A). Methionine 151 in URI1 isoform a is identified by green font in Figure 6. Therefore, the presence of the ~35 kDa URI1 band is possibly the result of the use of an alternative start codon that has not yet been identified.

Human URI1 Isoform AA sequence Alignment

a	MEAPTVE TPPDPSPPSAPAPALVPLRAPDVARLREEQEKVVTNCQERIQHWKKVDNDYNA	60
X3	-----MHWKKVDNDYNA	12
X1	-----MTTWSSSLQGSHVSKRALAYALVVTNCQERIQHWKKVDNDYNA	42
X4	-----	0
X2	MEAPTVE TPPDPSPPSAPAPALVPLRAPDVARLREEQEKVVTNCQERIQHWKKVDNDYNA	60
c	-----MTTWSSSLQGSHVSKRALAYALVVTNCQERIQHWKKVDNDYNA	42
X5	-----MWRGC-ARSRKRKKVDNDYNA	20
a	LRERLSTLPDKLSYNIMVPFGPFAFM PGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHR	120
X3	LRERLSTLPDKLSYNIMVPFGPFAFM PGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHR	72
X1	LRERLSTLPDKLSYNIMVPFGPFAFM PGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHR	102
X4	-----MVPFGPFAFM PGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHR	44
X2	LRERLSTLPDKLSYNIMVPFGPFAFM PGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHR	120
c	LRERLSTLPDKLSYNIMVPFGPFAFM PGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHR	102
X5	LRERLSTLPDKLSYNIMVPFGPFAFM PGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHR	80

a	KEHVRKTIDDLKKVMKNFESRVEFTEDLQKMSDAAGDIVDIREEIKCDFEFKAKHRIA HK	180
X3	KEHVRKTIDDLKKVMKNFESRVEFTEDLQKMSDAAGDIVDIREEIKCDFEFKAKHRIA HK	132
X1	KEHVRKTIDDLKKVMKNFESRVEFTEDLQKMSDAAGDIVDIREEIKCDFEFKAKHRIA HK	162
X4	KEHVRKTIDDLKKVMKNFESRVEFTEDLQKMSDAAGDIVDIREEIKCDFEFKAKHRIA HK	104
X2	KEHVRKTIDDLKKVMKNFESRVEFTEDLQKMSDAAGDIVDIREEIKCDFEFKAKHRIA HK	180
c	KEHVRKTIDDLKKVMKNFESRVEFTEDLQKMSDAAGDIVDIREEIKCDFEFKAKHRIA HK	162
X5	KEHVRKTIDDLKKVMKNFESRVEFTEDLQKMSDAAGDIVDIREEIKCDFEFKAKHRIA HK	140

a	PHSKPKTSDIFEADIANDVKS KDLLADKELWARLEELERQEELLGELDSKPDTVIANG ED	240
X3	PHSKPKTSDIFEADIANDVKS KDLLADKELWARLEELERQEELLGELDSKPDTVIANG ED	192
X1	PHSKPKTSDIFEADIANDVKS KDLLADKELWARLEELERQEELLGELDSKPDTVIANG ED	222
X4	PHSKPKTSDIFEADIANDVKS KDLLADKELWARLEELERQEELLGELDSKPDTVIANG ED	164
X2	PHSKPKTSDIFEADIANDVKS KDLLADKELWARLEELERQEELLGELDSKPDTVIANG ED	240
c	PHSKPKTSDIFEADIANDVKS KDLLADKELWARLEELERQEELLGELDSKPDTVIANG ED	222
X5	PHSKPKTSDIFEADIANDVKS KDLLADKELWARLEELERQEELLGELDSKPDTVIANG ED	200

a	TTSSEEEKEDRNTNVNAMHQV TDSHTPCHKDVASSEPFSGQVNSQLNCSVNGSSSYHS DD	300
X3	TTSSEEEKEDRNTNVNAMHQV TDSHTPCHKDVASSEPFSGQVNSQLNCSVNGSSSYHS DD	252
X1	TTSSEEEKEDRNTNVNAMHQV TDSHTPCHKDVASSEPFSGQVNSQLNCSVNGSSSYHS DD	282
X4	TTSSEEEKEDRNTNVNAMHQV TDSHTPCHKDVASSEPFSGQVNSQLNCSVNGSSSYHS DD	224
X2	TTSSEEEKEDRNTNVNAMHQV TDSHTPCHKDVASSEPFSGQVNSQLNCSVNGSSSYHS DD	300
c	TTSSEEEKEDRNTNVNAMHQV TDSHTPCHKDVASSEPFSGQVNSQLNCSVNGSSSYHS DD	282
X5	TTSSEEEKEDRNTNVNAMHQV TDSHTPCHKDVASSEPFSGQVNSQLNCSVNGSSSYHS DD	260

a	DDDDDDDDDDNIDDDGDNDHEALGVGDNSIPTIYFSHTVEPKRVRINTGKNTTLKFSE	360
X3	DDDDDDDDDDNIDDDGDNDHEALGVGDNSIPTIYFSHTVEPKRVRINTGKNTTLKFSE	312
X1	DDDDDDDDDDNIDDDGDNDHEALGVGDNSIPTIYFSHTVEPKRVRINTGKNTTLKFSE	342
X4	DDDDDDDDDDNIDDDGDNDHEALGVGDNSIPTIYFSHTVEPKRVRINTGKNTTLKFSE	284
X2	DDDDDDDDDDNIDDDGDNDHEALGVGDNSIPTIYFSHTVEPKRVRINTGKNTTLKFSE	360
c	DDDDDDDDDDNIDDDGDNDHEALGVGDNSIPTIYFSHTVEPKRVRINTGKNTTLKFSE	342
X5	DDDDDDDDDDNIDDDGDNDHEALGVGDNSIPTIYFSHTVEPKRVRINTGKNTTLKFSE	320

a	KKEEAKRKRKNSTGSGHSAQELPTIRTPADIYRAFVDVVNGEYVPRKSILKSRSENSVC	420
X3	KKEEAKRKRKNSTGSGHSAQELPTIRTPADIYRAFVDVVNGEYVPRKSILKSRSENSVC	372
X1	KKEEAKRKRKNSTGSGHSAQELPTIRTPADIYRAFVDVVNGEYVPRKSILKSRSENSVC	402
X4	KKEEAKRKRKNSTGSGHSAQELPTIRTPADIYRAFVDVVNGEYVPRKSILKSRSENSVC	344
X2	KKEEAKRKRKNSTGSGHSAQELPTIRTPADIYRAFVDVVNGEYVPRKSILKSRSENSVC	420
c	KKEEAKRKRKNSTGSGHSAQELPTIRTPADIYRAFVDVVNGEYVPRKSILKSRSENSVC	402
X5	KKEEAKRKRKNSTGSGHSAQELPTIRTPADIYRAFVDVVNGEYVPRKSILKSRSENSVC	380

a	SDTSESSAAEFDDRRGVLRSISCEEATCSDTSESILEEFPQENQKKLLPLSVTPEAFSGT	480
X3	SDTSESSAAEFDDRRGVLRSISCEEATCSDTSESILEEFPQENQKKLLPLSVTPEAFSGT	432
X1	SDTSESSAAEFDDRRGVLRSISCEEATCSDTSESILEEFPQENQKKLLPLSVTPEAFSGT	462
X4	SDTSESSAAEFDDRRGVLRSISCEEATCSDTSESILEEFPQENQKKLLPLSVTPEAFSGT	404
X2	SDTSESSAAEFDDRRGVLRSISCEEATCSDTSESILEEFPQENQKKLLPLSVTPEVLRV	480
c	SDTSESSAAEFDDRRGVLRSISCEEATCSDTSESILEEFPQENQKKLLPLSVTPEVLRV	462
X5	SDTSESSAAEFDDRRGVLRSISCEEATCSDTSESILEEFPQENQKKLLPLSVTPEVLRV	440
	***** . : .	
a	VIEKEFVSPSLTPPPAIAHPALPTIPERKEVLLEASEETGKR V SKFKAARLQQKD	535
X3	VIEKEFVSPSLTPPPAIAHPALPTIPERKEVLLEASEETGKR V SKFKAARLQQKD	487
X1	VIEKEFVSPSLTPPPAIAHPALPTIPERKEVLLEASEETGKR V SKFKAARLQQKD	517
X4	VIEKEFVSPSLTPPPAIAHPALPTIPERKEVLLEASEETGKR V SKFKAARLQQKD	459
X2	GYSRNLA-----PLNVIL-----	493
c	GYSRNLA-----PLNVIL-----	475
X5	GYSRNLA-----PLNVIL-----	453

Figure 6: AA sequence alignment for human URI1 isoforms. This shows that the AA sequence between isoforms varies in the C- and N termini. Asterisks below AA residues indicate sequence identity for all URI1 isoforms. The methionine residue in green font indicates an alternative start codon which may have resulted in the lower molecular weight URI1 band observed in Figure 3A. The AA residues in red font correspond to the approximate position of the epitope that the CST URI1 antibody uses to detect URI1.

Mouse URI1

For mouse URI1, the NCBI database depicts 4 different URI1 isoforms. The molecular weight of URI1 isoforms X1, X2, X3, and URI1 are 60, 54, 50 and 59, respectively (summarized in Table 11). BLAST shows that the AA sequence of these isoforms vary in their N-terminus due to mRNA translation starting later into the protein compared to other URI1 isoforms and dissimilarities in AA residues between AA sequences (Figure 7).

The epitope recognized by the CST URI antibody in mouse surrounds AA 517 of URI1 isoform X1. Therefore, all listed URI1 isoforms would be detected by the CST URI1 antibody. Also, the Thermofisher URI1 antibody recognizes the AA 403-426 epitope of URI1, so theoretically, this antibody would recognize all 4 URI1 isoforms as well.

The CST URI1 antibody recognized only one band close to the 48 kDa molecular weight marker. This band may represent URI1 isoform X3 since its calculated molecular weight is 50 kDa and this band is close to the 48 kDa molecular weight marker in the Western blot.

The Thermofisher URI1 antibody detected 3 URI1 bands. One of the 3 bands was close to the 63 molecular weight marker and this band may represent URI1 isoform X1 because its calculated molecular weight is 60 kDa. The band detected by this antibody that is close to the 48 kDa molecular weight marker is likely the same band detected by the CST URI1 antibody.

Lastly, an additional band was detected close to the 35 kDa molecular weight marker but none of the listed URI1 isoforms are reported to have a similar molecular weight. Interestingly, the URI1 over-expression lysate Western blot also showed a band with a similar molecular weight. Like human URI1, there are several methionine residues dispersed throughout the URI1 AA sequence, which represent locations where mRNA translation may

commence. The use of an alternate start codon may account for the presence of the ~35 kDa URI1 band. Further investigation into alternate start codons shows that if URI1 mRNA translation had started from Methionine 154 of URI1 isoform X1, the protein produced would be 42 kDa which corresponds approximately to the ~35 kDa band observed Figure 4B. Therefore, the ~35 kDa band observed in Figure 4B may be the result of the use of an alternative start codon.

Mouse URI1 Isoform AA sequence Alignment

URI1	-----MEPPSEPEPEPQPLAEASAAAPLRAPEVARLREEQEKVVTNCQEKIQH	48
X2	-----MS	2
X1	MLSTFFSTTLLLFASSELFCAHVIFFPESHDPQRAP-----VYVLVVTNCQEKIQH	53
X3	-----	0
URI1	WEKVDNDYSALQERLRTLDPDKLSYDVMVFFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSA	108
X2	WEKVDNDYSALQERLRTLDPDKLSYDVMVFFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSA	62
X1	WEKVDNDYSALQERLRTLDPDKLSYDVMVFFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSA	113
X3	-----MVPFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSA	34

URI1	KQAVGLVEHRKEHVRKTIDDFKKVLKNFESRVEFTEDLQKMSDAAGDFVDIREEEKSDFE	168
X2	KQAVGLVEHRKEHVRKTIDDFKKVLKNFESRVEFTEDLQKMSDAAGDFVDIREEEKSDFE	122
X1	KQAVGLVEHRKEHVRKTIDDFKKVLKNFESRVEFTEDLQKMSDAAGDFVDIREEEKSDFE	173
X3	KQAVGLVEHRKEHVRKTIDDFKKVLKNFESRVEFTEDLQKMSDAAGDFVDIREEEKSDFE	94

URI1	FKGKQRIAHKPHSKPKTSDIFEADFENGVPKNTFDADELWARLEELERQEELLGELESK	228
X2	FKGKQRIAHKPHSKPKTSDIFEADFENGVPKNTFDADELWARLEELERQEELLGELESK	182
X1	FKGKQRIAHKPHSKPKTSDIFEADFENGVPKNTFDADELWARLEELERQEELLGELESK	233
X3	FKGKQRIAHKPHSKPKTSDIFEADFENGVPKNTFDADELWARLEELERQEELLGELESK	154

URI1	PDTVIANGEDRVSSSEEKEGADTGVNVVSPVTDSSAASSCKRRAGNAGLPNGQVNSLNYS	288
X2	PDTVIANGEDRVSSSEEKEGADTGVNVVSPVTDSSAASSCKRRAGNAGLPNGQVNSLNYS	242
X1	PDTVIANGEDRVSSSEEKEGADTGVNVVSPVTDSSAASSCKRRAGNAGLPNGQVNSLNYS	293
X3	PDTVIANGEDRVSSSEEKEGADTGVNVVSPVTDSSAASSCKRRAGNAGLPNGQVNSLNYS	214

URI1	VNGSNSYHSNKDDDEEEEDDDDDDEDDDNESDHAI SADNSIPTIYFSHTVEPKRVRINT	348
X2	VNGSNSYHSNKDDDEEEEDDDDDDEDDDNESDHAI SADNSIPTIYFSHTVEPKRVRINT	302
X1	VNGSNSYHSNKDDDEEEEDDDDDDEDDDNESDHAI SADNSIPTIYFSHTVEPKRVRINT	353
X3	VNGSNSYHSNKDDDEEEEDDDDDDEDDDNESDHAI SADNSIPTIYFSHTVEPKRVRINT	274

URI1	GKNTTLKFSEKKEEAKRKRKSGAGSHATHELPAIKSPADIYRVFVDVNGEYVPRKSILK	408
X2	GKNTTLKFSEKKEEAKRKRKSGAGSHATHELPAIKSPADIYRVFVDVNGEYVPRKSILK	362
X1	GKNTTLKFSEKKEEAKRKRKSGAGSHATHELPAIKSPADIYRVFVDVNGEYVPRKSILK	413
X3	GKNTTLKFSEKKEEAKRKRKSGAGSHATHELPAIKSPADIYRVFVDVNGEYVPRKSILK	334

URI1	SRSRENSVCSDTSESSAADVEDRRGLLRSTSSEEAVATEAGGSSLDELQENHPKKPLPSG	468
X2	SRSRENSVCSDTSESSAADVEDRRGLLRSTSSEEAVATEAGGSSLDELQENHPKKPLPSG	422
X1	SRSRENSVCSDTSESSAADVEDRRGLLRSTSSEEAVATEAGGSSLDELQENHPKKPLPSG	473
X3	SRSRENSVCSDTSESSAADVEDRRGLLRSTSSEEAVATEAGGSSLDELQENHPKKPLPSG	394

URI1	VSEAFSGTVIEKEFLSPSLAPYSAIAHHALPTIPERKEVPSEVSEEPTRVSKFRAARLQ	528
X2	VSEAFSGTVIEKEFLSPSLAPYSAIAHHALPTIPERKEVPSEVSEEPTRVSKFRAARLQ	482
X1	VSEAFSGTVIEKEFLSPSLAPYSAIAHHALPTIPERKEVPSEVSEEPTRVSKFRAARLQ	533
X3	VSEAFSGTVIEKEFLSPSLAPYSAIAHHALPTIPERKEVPSEVSEEPTRVSKFRAARLQ	454

URI1	QRS	531
X2	QRS	485
X1	QRS	536
X3	QRS	457

Figure 7: AA sequence alignment for mouse URI1 isoforms. Asterisks below AA residues indicate sequence identity for all URI1 isoforms. This shows that the AA sequence between isoforms varies in the N-terminus. The AA residues in red font corresponds to the approximate position of the epitope that the CST URI1 antibody uses to detect URI1. The AAs in blue font correspond to the epitope used to detect URI1 by the Thermofisher URI1 antibody. The methionine residue in green font indicates an alternative start codon which may have resulted in the ~35 kDa URI1 band observed in Figure 4B.

Rat URI1

Three rat URI1 isoforms have been identified by the NCBI database. The molecular weights of URI1 isoforms X1, X2, and URI1 are 51, 42, and 59 kDa, respectively (summarized in Table 11). Using BLAST to align these rat URI1 isoforms showed that the AA sequences of these URI1 isoforms vary only at the N-terminus due to mRNA translation starting later into the protein compared to other URI1 isoforms.

The CST URI1 antibody would theoretically detect three of these URI1 isoforms in H4IIE cell lysates since the C-terminus of rat URI1 isoforms are identical. Interestingly, detection of URI1 bands was also dependent on growing conditions of H4IIE cells. When H4IIE cells were starved until quiescent, only one band near the 35 kDa molecular weight marker was observed. However, after quiescent H4IIE cells were treated with 100 μ M of OA for at least 4 hours, there was an additional band close to the 75 kDa molecular weight marker. The CST URI1 antibody datasheet predicts URI1 to be 79 kDa.

When comparing the observed bands in Western blots to the listed URI1 isoforms in the NCBI database, rat URI1 isoform X2 seems to be the only URI1 isoform represented in the Western blots, since the calculated molecular weight of this isoform is 42 kDa and a band in H4IIE cell URI1 Western blots is situated above the 35 kDa molecular weight marker. The remaining 2 URI1 isoforms are 51 and 59 kDa and there were no bands in the Western blots close to these molecular weights. It is possible that the reason for detecting the ~79 kDa URI1 band and not the 51 and 59 kDa bands is that these URI1 isoforms undergo post-translational modification which result in a protein that is 79 kDa .

Rat URI1 Isoform AA sequence Alignment

X2	-----	0
X1	-----	0
URI1	MEPPSEPEPEPQPFAEASAAAPLRAPEVARLREEQEKVVTNCQEKIQHWEKVDNDYSALQ	60
X2	-----	0
X1	-----MVPFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHRKE	46
URI1	ERLRTLDPDKLSYDIMVFPFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHRKE	120
X2	-----MSDAAGDFVDIREEEKSDFEFKKGQRIAHKPH	32
X1	HVRKTIDDFKKVLKNFESRVEFTEDLQKMSDAAGDFVDIREEEKSDFEFKKGQRIAHKPH	106
URI1	HVRKTIDDFKKVLKNFESRVEFTEDLQKMSDAAGDFVDIREEEKSDFEFKKGQRIAHKPH *****	180
X2	SKPKTSDIFEAEFENGIKPQGTFDDELWARLEELERQEELLGELKSKPDTVIANGEDTV	92
X1	SKPKTSDIFEAEFENGIKPQGTFDDELWARLEELERQEELLGELKSKPDTVIANGEDTV	166
URI1	SKPKTSDIFEAEFENGIKPQGTFDDELWARLEELERQEELLGELKSKPDTVIANGEDTV *****	240
X2	SSEEEKEDEDTGNNVSPVTDSSAPSSCKRHGGNAGLPNGQTSSLNYSVNGSNSYHSNKE	152
X1	SSEEEKEDEDTGNNVSPVTDSSAPSSCKRHGGNAGLPNGQTSSLNYSVNGSNSYHSNKE	226
URI1	SSEEEKEDEDTGNNVSPVTDSSAPSSCKRHGGNAGLPNGQTSSLNYSVNGSNSYHSNKE *****	300
X2	DEEEEEDEDDDEDDDDSDHVSADNSIPTIYFSHTVEPKRVRINTGKNTTLKFSEK	212
X1	DEEEEEDEDDDEDDDDSDHVSADNSIPTIYFSHTVEPKRVRINTGKNTTLKFSEK	286
URI1	DEEEEEDEDDDEDDDDSDHVSADNSIPTIYFSHTVEPKRVRINTGKNTTLKFSEK *****	360
X2	KEEAKRKRKSGAASHSAHELPAIRSPADIYRVFVDVNGEYIPRKSILKSRSENSVCSD	272
X1	KEEAKRKRKSGAASHSAHELPAIRSPADIYRVFVDVNGEYIPRKSILKSRSENSVCSD	346
URI1	KEEAKRKRKSGAASHSAHELPAIRSPADIYRVFVDVNGEYIPRKSILKSRSENSVCSD *****	420
X2	TSessaADVDIDRRAPLRSPSSEETEAGSSTLEELQENHPKKPLPSGVSEAFSGTVIEKE	332
X1	TSessaADVDIDRRAPLRSPSSEETEAGSSTLEELQENHPKKPLPSGVSEAFSGTVIEKE	406
URI1	TSessaADVDIDRRAPLRSPSSEETEAGSSTLEELQENHPKKPLPSGVSEAFSGTVIEKE *****	480
X2	FLSPSLAPHSAIAHHALPTIPERKEVLSEASEEPAKR ^V SKFRAARLQQRN	382
X1	FLSPSLAPHSAIAHHALPTIPERKEVLSEASEEPAKR ^V SKFRAARLQQRN	456
URI1	FLSPSLAPHSAIAHHALPTIPERKEVLSEASEEPAKR ^V SKFRAARLQQRN *****	530

Figure 8: AA sequence alignment for rat URI1 isoforms. Asterisks below AA residues indicate sequence identity for all URI1 isoforms. This shows that the AA sequence between isoforms varies in the N-terminus. The AA in red font corresponds to the approximate position of the epitope that the CST URI1 antibody uses to detect URI1.

Conclusions from Analysis of URI1 Isoforms Across Different Species Using the NCBI Database

The URI1 antibody characterization experiments shown in this section suggest that URI1 is subjected to post-translational modification, has multiple start codons and/or undergoes alternative splicing to produce several URI1 isoforms and that the CST URI1 antibody specifically binds to URI1. The presence of multiple post translational modifications has been previously reported by Mita et al (164). The URI1 bands which were detected varied in their molecular weights. While a distinct pattern across several Western blots was demonstrated, URI1 bands of a particular molecular weight were detected in other sample types (for example, a ~35 kDa band was observed in human, mouse, and rat lysates). Therefore, these Western blots establish that the expression profile of URI1 is not completely understood and synthesis of certain URI1 isoforms is determined by the species and the conditions of the cell. Research into the significance of these differences is warranted. Wan et al. (168) had also observed truncated forms of URI1 in HEK293 cells, and as long as these truncated forms of URI1 contained a particular biological domain, the URI1 isoform was also able to mediate a biological effect.

Results Section B: Liver Tissue Experiments

Male *db/db* (n=18) and lean C57BL/6 (n=28) mice were acquired when 6 weeks old, and after a 7-day acclimatization period, they were randomly assigned to different experimental diet groups. *Db/db* mice are an accepted model of MetS and reliably develop hepatic steatosis (195). A total of 16 mice (8 *db/db* and 8 lean C57BL/6 mice) were placed into the CTL diet group while the other 30 mice (10 *db/db* and 20 lean C57BL/6 mice) were placed into the t10,c12 CLA diet (0.4% w/w). In this study, *db/db* mice were used as an experimental model of hepatic steatosis to investigate the effects of t10,c12 CLA on hepatic steatosis. Lean mice were used as healthy controls.

The mice were on diets for 4 weeks, after which they were weighed and euthanized by decapitation. Livers were dissected, flash frozen in liquid nitrogen and stored at -80°C. Lysates were made from livers for TG concentration assays and Western blotting experiments, as described in the Triacylglyceride Concentration Assay and Protein Extraction from Liver Tissue sections from the Methods Chapter.

Liver Weight

Relative liver weights were measured to assess whether diet or genotype had discernible effects on the liver. Changes in liver weight often represent differences of hepatic lipid content. Since *db/db* mice act as a model of hepatic steatosis, it is expected that their relative liver weights will be greater than lean controls due to greater hepatic lipid content.

Differences of relative liver weights between CTL and t10,c12 CLA diets may indicate whether t10,c12 CLA improves or aggravates hepatic steatosis. Relative liver weights (liver weight / 100 g body weight) were used for data analysis to normalize liver weights to total body weight (BW).

At the end of the 4-week dietary intervention, the livers from *db/db* mice fed the t10,c12 CLA diet weight were the heaviest and the livers of lean mice fed the CTL diet were the least heavy (Figure 9A). *Db/db* mice fed the CTL or t10,c12 CLA diet had relative liver weights that were 1.7- and 2.1-fold greater, respectively, than lean mice fed the same diets (6.97 ± 0.54 vs. 4.08 ± 0.06 g and 13.26 ± 0.46 vs. 6.19 ± 0.56 g, respectively). The relative liver weight of t10,c12 CLA fed lean mice was 1.5-fold greater than CTL diet-fed lean mice (6.19 ± 0.56 vs. 4.08 ± 0.06 g, respectively) and statistically equivalent to *db/db* mice fed the CTL diet (6.19 ± 0.56 vs. 6.97 ± 0.54 , respectively). Lastly, the relative liver weight of *db/db* mice fed the t10,c12 CA diet was 2-fold greater than *db/db* mice fed CTL diet (13.26 ± 0.456 vs. 6.97 ± 0.54 g, respectively).

Liver Triacylglyceride Concentration

To determine whether differences in relative liver weights were due to differences in hepatic lipid content, liver TG content was measured using a colorimetric assay. TG concentration is expressed as mg TG/mg protein. Firstly, the hepatic TG concentrations of *db/db* mice fed the CTL or t10,c12 CLA diet were 6.6- and 1.8-fold greater, respectively, than lean mice fed the same diets (0.447 ± 0.071 vs. 0.068 ± 0.008 and 0.617 ± 0.073 vs. 0.338 ± 0.041 mg TG/mg protein, respectively) (Figure 9B). TG concentrations of lean mice fed the t10,c12 CLA diet were 5-fold greater compared to lean mice fed the CTL diet (0.338 ± 0.041 vs. 0.0678 ± 0.008

mg TG/mg protein, respectively) but were statistically equivalent to *db/db* mice fed either diet (0.338±0.041 vs. 0.447±0.071 and 0.617±0.073 mg TG/mg protein, respectively). TG concentrations of *db/db* mice fed t10,c12 CLA were the most elevated, but statistically equivalent to *db/db* mice fed the CTL diet (0.617±0.073 vs. 0.447±0.071 mg TG/mg protein, respectively). TG concentrations of lean mice fed the CTL diet were significantly less than all other groups (0.068±0.008 vs 0.338±0.041, 0.447±0.071, and 0.617±0.073 mg TG/mg protein).

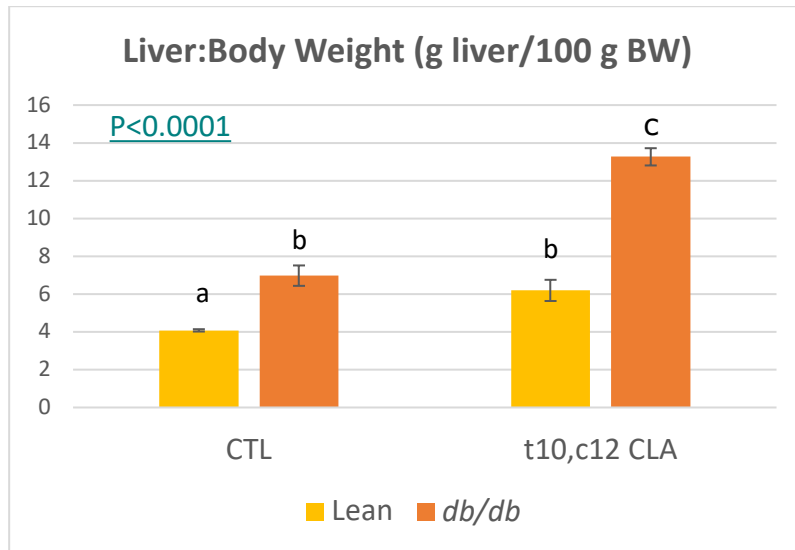
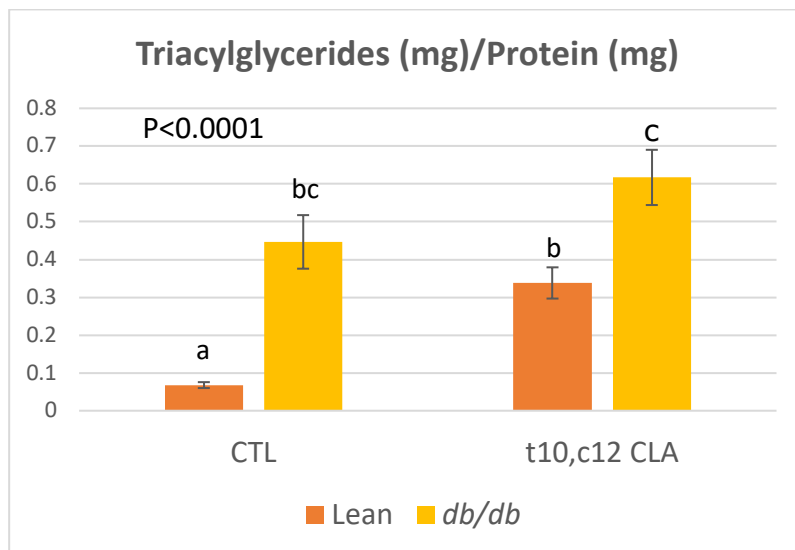
A**B**

Figure 9: Relative liver weights (A) and TG concentration (B) of lean and *db/db* mice fed the CTL or t10,c12 CLA diet. Values are expressed as means $\pm$ SEM with N = 7-12. Different letters indicate significant differences ($p < 0.05$) among the means. Data were analyzed by non-parametric testing. P values for the Kruskal-Wallis test are indicated in the upper left corner of the figures.

URI1 Protein Levels

Investigations into relative liver weight and hepatic lipid concentration revealed that the *db/db* genotype and the t10,c12 CLA diet resulted in increased hepatic lipid accumulation.

Research by Gomes et al. (169) suggests that the protein URI1 promotes hepatic lipid accumulation. Measurements of relative hepatic URI1 protein levels were carried out by Western blotting to assess the relationship between hepatic URI1 protein levels and hepatic lipid accumulation. The Western blotting used the CST URI1 antibody which detects the 48 kDa URI1 band in mouse liver lysates.

Both the t10,c12 CLA diet and the *db/db* genotype were associated with elevated URI1 protein levels as there were statistically significant main effects for both the diet and genotype (Figures 10A and 10B). Specifically, the t10,c12 CLA diet increased hepatic URI1 protein levels 4-fold compared to mice fed the control diet (diet means: 2.97 ± 0.032 vs 0.73 ± 0.12 arbitrary units (AU), respectively). The *db/db* genotype increased hepatic URI1 1.7-fold compared to lean mice (genotype means: 2.49 ± 0.44 vs 1.43 ± 0.25 AU, respectively).

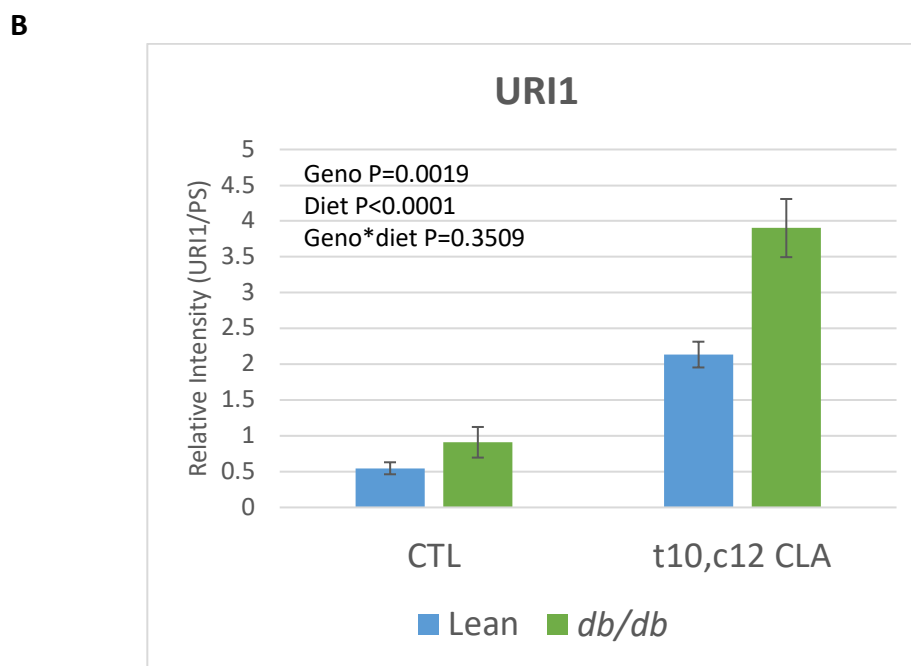
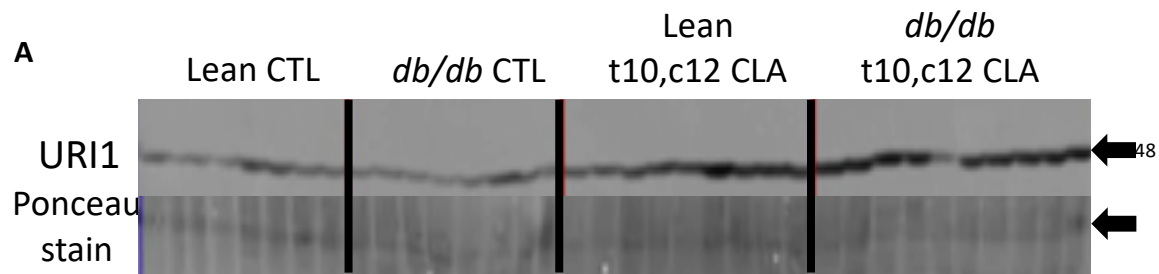


Figure 10: URI1 Western blot using livers from lean and *db/db* mice fed CTL or t10,c12 CLA diet and the CST URI1 antibody (A). Black arrows on the Western blot indicate the locations of molecular weight markers. Black arrows on the PS indicate the band used for normalization. Relative protein levels of hepatic URI1 (B). Densitometry was used to quantify the intensity of the URI1 band shown. Data were normalized to total relative protein levels (measured by PS). Values are expressed as means $\pm$ SEM, N=8-10. Data were analyzed using two-way ANOVA and p values for the main effects and interaction are indicated in the upper left corner of the figure.

Correlation Analyses

Both hepatic lipid accumulation and hepatic URI1 protein levels were increased by the t10,c12 CLA diet and the *db/db* genotype (Figure 10). The association between hepatic lipid accumulation and URI1 protein levels was analyzed by correlation analysis to assess how closely these variables are related. This inquiry may indicate whether these two variables are influenced by the same factors or if there is a causal relationship.

Several statistically significant correlations were revealed through correlation analysis (summarized in Table 12). Most importantly, there was a statistically significant correlation between hepatic URI1 protein levels and relative liver weights (Figure 11). This shows that the data are consistent with X and Y being impacted by the same factor. Further experimentation is required to delineate whether this relationship is incidental or causal. All correlations were analyzed using Spearman's rank correlation coefficient.

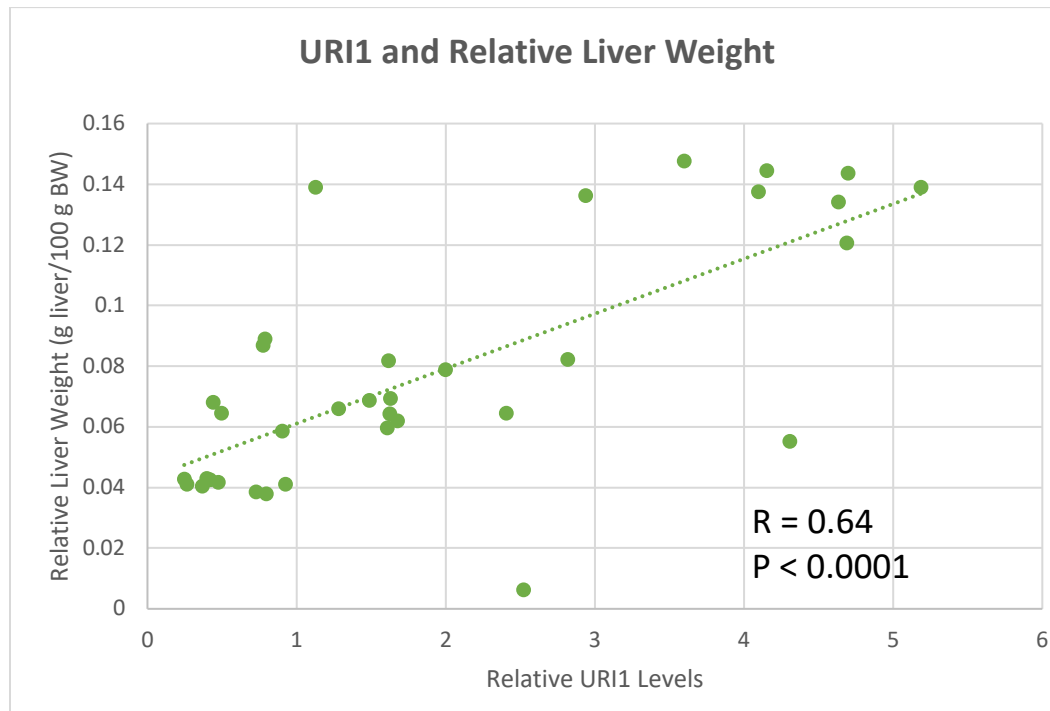


Figure 11: Hepatic URI1 protein levels plotted against relative liver weight. Data points represent individual mice and their respective hepatic URI1 protein levels and relative liver weight, N=35. Data were analyzed using Spearman's rank correlation coefficient.

Table 12: Summary of Correlation Analyses.¹

Correlation	N	R Value	P value
URI1 and Liver Weight	35	0.64	<0.001
URI1 and TG Concentration	34	0.61	0.0001
TG Concentration and Liver:Body Weight	69	0.78	<0.0001
TG Concentration and Liver Weight	69	0.8	<0.0001

¹A perfect association between 2 variables is indicated when the correlation coefficient, R, is equal to 1 and no association is indicated when $R=0$. Correlations are considered significant at $p<0.05$. All data were analyzed using Spearman's rank correlation coefficient.

Lipid Metabolism Proteins

Three different proteins were used as molecular markers to explore different aspects of hepatic lipid accumulation. Relative hepatic protein levels of these molecular markers were measured by Western blot.

Hepatic *de novo* lipogenesis was evaluated by measuring levels of FAS. FAS is a cytosolic enzyme which catalyzes the synthesis of PA from acetyl-coA and malonyl-CoA. FAS is an important enzyme for regulating *de novo* biosynthesis of long chain FAs (236). Western blotting showed a statistically significant interaction effect (genotype×diet) where lean mice fed the t10,c12 CLA diet had elevated levels of FAS compared to CTL diet-fed lean mice, CTL diet-fed *db/db* mice, and t10,c12 CLA-fed *db/db* mice (258±36 vs. 95±34, 150±32, and 106±25 AU, respectively) (Figures 12A and 13A).

Hepatic FFA uptake was evaluated by measuring the protein levels of FAT/CD36 and FABP1. These two proteins facilitate the uptake and intracellular transport of long chain FFAs in hepatocytes and other cell types (65,71). FAT/CD36 Western blots show statistically significant main effects of both diet and genotype, where both the *db/db* and the t10,c12 CLA diet genotype resulted in 4- and 2-fold increased FAT/CD36 hepatic protein levels compared to lean and CTL diet-fed mice, respectively (genotype means: 29.2±4.0 vs 7.3±1.1 AU and diet means: 22.1±4.1 vs 11.2± 2.6 AU, respectively) (Figures 12B and 13B). The FABP1 Western blot showed a statistically significant main effect from only the diet, where the t10,c12 CLA diet resulted in 1.7-fold increased FABP1 levels compared to CTL diet (diet means: 156±15 vs 92±9 AU, respectively) (Figures 12C and 13C).

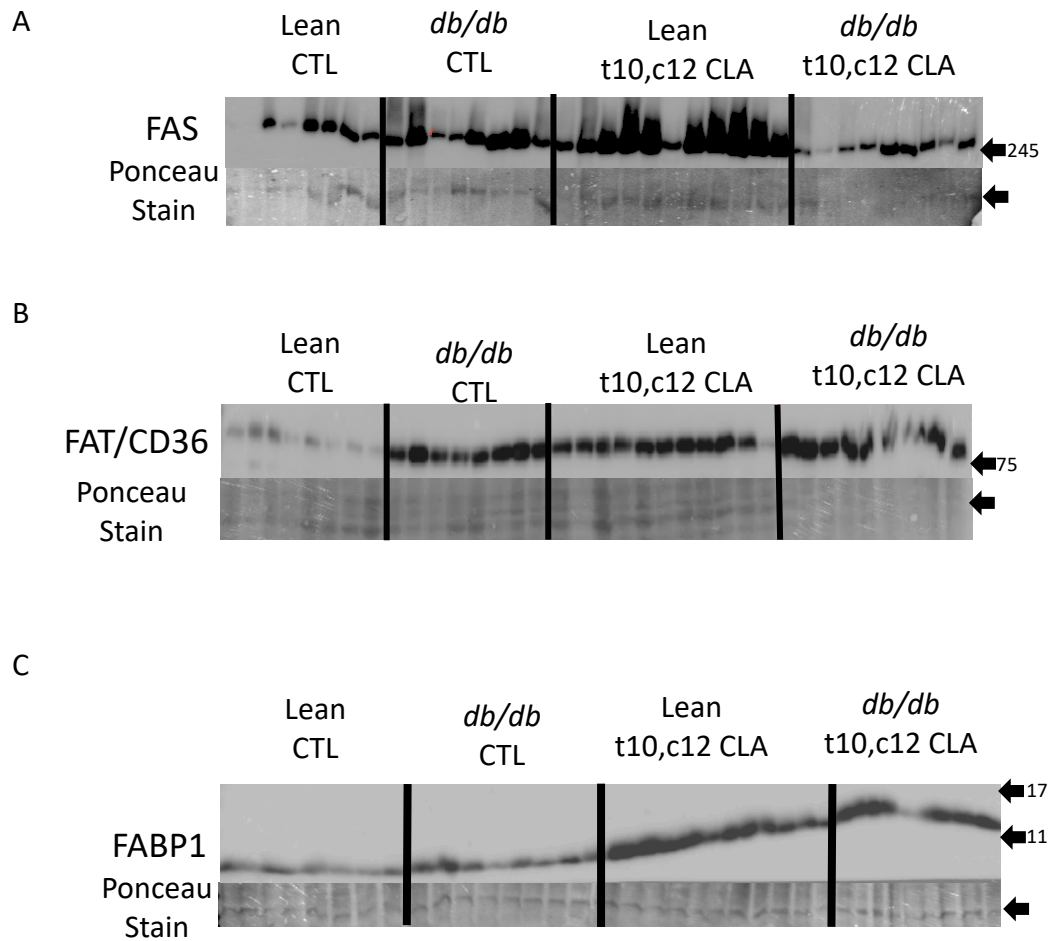
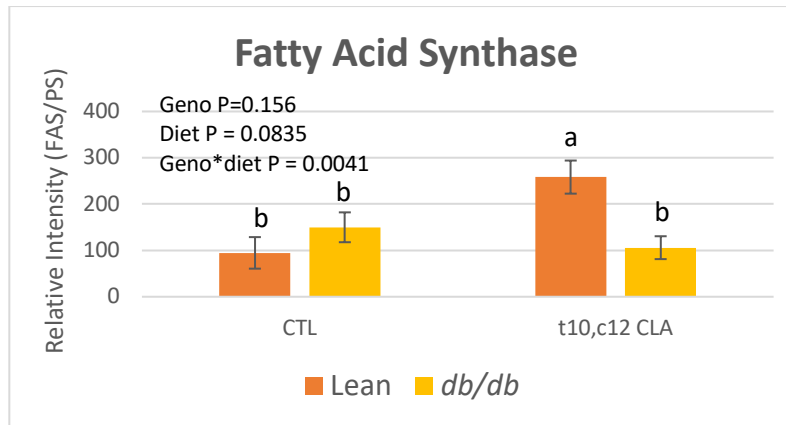
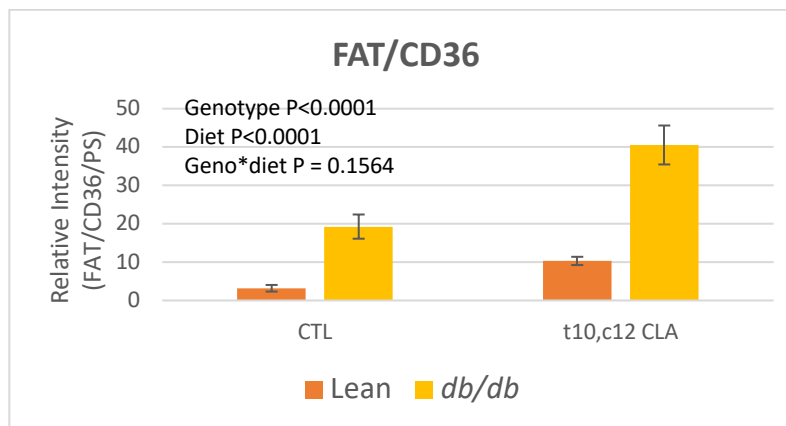


Figure 12: Western blots of lipid metabolism proteins using livers from lean and *db/db* mice fed the CTL or t10,c12 CLA diet. Western blots of FAS (**A**), FAT/CD36 (**B**), and FABP1 (**C**). Black arrows on the Western blots indicate the locations of molecular weight markers. Black arrows on the PS indicate the band used for normalization.

A



B



C

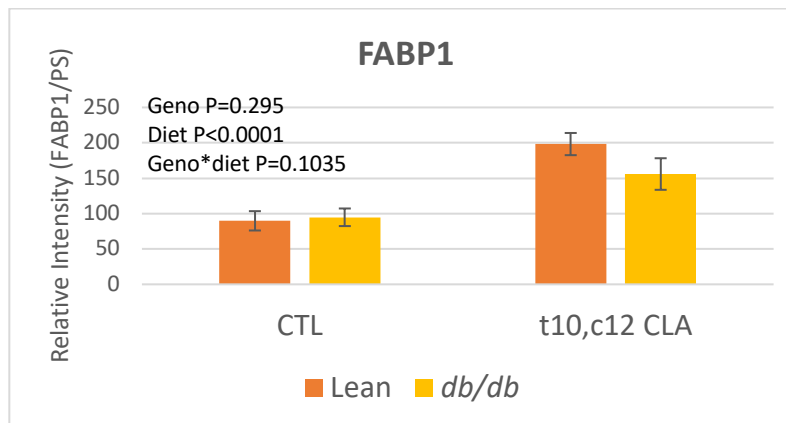


Figure 13: Relative levels of lipid metabolism proteins in livers of t10,c12 CLA-fed and CTL diet-fed lean and *db/db* mice. Hepatic levels of FAS (A), FAT/CD36 (B), and FABP1 (C). Values are expressed as means $\pm$ SEM, N=8-11. Densitometry was used to quantify the intensity of the FAS, FAT/CD36, and FABP1 bands. Data were normalized to total relative protein levels (measured by PS). Data were analyzed by using two-way ANOVA and p values for the main and interaction effects are indicated in the upper left corner of the figure. Different letters indicate significant differences ($p < 0.05$) among the means when there was a significant interaction.

Cell Signaling Proteins

Several cellular processes in the liver are affected by NAFLD. These cellular processes were investigated by measuring relative levels of relevant proteins by Western blot.

AMPK serves as a sensor of cellular energy status by sensing ATP and ADP levels. If needed, AMPK can promote catabolic processes and inhibit anabolic processes to create ATP after AMPK phosphorylation (123). In the liver, liver kinase B1 (LKB1) is the main upstream kinase which activates AMPK by phosphorylating AMPK at the AA residue, Threonine (T)172, in the α -subunit (132). AMPK phosphorylation levels were measured to determine if AMPK activation was different between experimental groups. Data analysis of the Western blot showed a statistically significant main effect where mice fed the t10,c12 CLA diet had increased phosphorylated AMPK (p-AMPK) levels 1.6-fold compared to mice fed the CTL diet (diet means: 71.5 ± 10.0 vs 43.0 ± 8.6 , respectively) (Figures 14A and 15A). Furthermore, there was a trend ($P=0.069$) for the genotype $\times$ diet interaction such that t10,c12 CLA-fed *db/db* mice had elevated p-AMPK levels compared to lean and *db/db* mice fed the CTL diet.

JNK protein belongs to a family of mitogen activated protein kinases which is important for mediating cellular responses to stress, and it is activated upon phosphorylation (113). JNK is phosphorylated by a 3-tiered signaling cascade, consisting of various mitogen activated protein (MAP) kinase kinase kinases (MAP3K), MAP kinase kinases (MAP2K) and MAP kinases (MAPK). MAPKs phosphorylate JNK at AA residues T127 and Tyrosine (Y)185 to facilitate its activation (114). JNK has been revealed to be involved in hepatic lipid accumulation in experimental models of NAFLD (237). There are several JNK isoforms which have molecular weights of 46 and 55 kDa. Western blotting, measuring bands at both of these molecular weights, revealed that

there is a significant interaction effect where lean mice fed the CTL diet had significantly lower levels of phosphorylated JNK (p-JNK) compared to CTL diet-fed *db/db* mice, t10,c12 CLA-fed lean mice, and t10,c12 CLA-fed *db/db* mice (0.18 ± 0.05 vs 0.91 ± 0.12 , 0.74 ± 0.16 , and 1.08 ± 0.21 , respectively) (Figures 14B and 15B).

mTOR coordinates cell growth in accordance with nutrient levels and growth factors. Phosphorylation of proteins involved in the mTOR pathway promotes anabolic processes and inhibits catabolic processes (238). The pathophysiological effects of nutrient overloading that lead to NAFLD are dependent on the mTOR pathway (118). Among the proteins involved in the mTOR signaling cascade that are targets of phosphorylation is S6 Ribosomal Protein (S6RP). A key effector for the promotion of anabolic processes by mTOR is S6K1. S6K1 phosphorylates and activates many substrates, including S6RP at its serine (S)235/236 residue (117). To gauge the extent of mTOR activation, the phosphorylation levels of S6RP were measured by Western blotting. It was shown that mice fed the t10,c12 CLA diet had 2.51-fold higher levels of phosphorylated S6 Ribosomal protein (p-S6RP) compared to mice fed the CTL diet (diet means: 19.2 ± 1.7 vs 7.7 ± 1.3 , respectively) (Figures 14C and 15C).

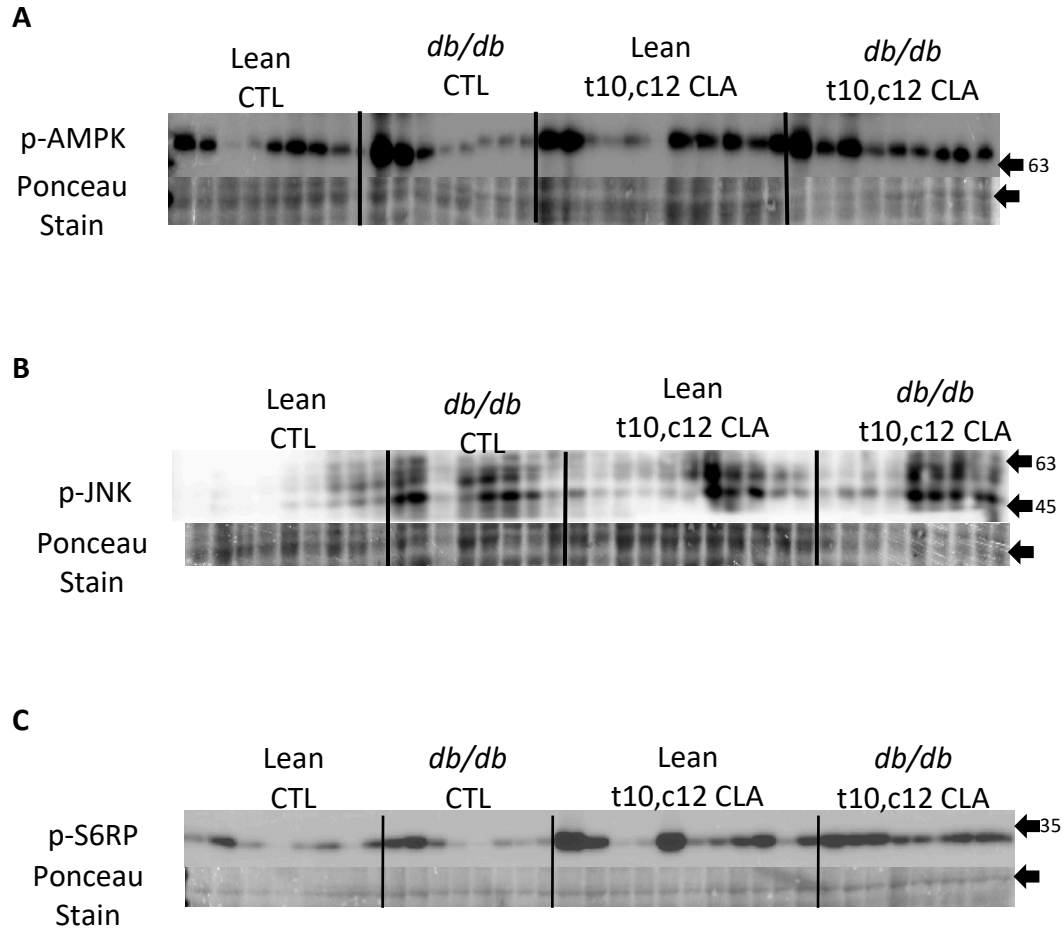


Figure 14: Western blots of cell signaling proteins using livers from lean and *db/db* mice fed CTL or t10,c12 CLA diet. Western blots of p-AMPK (T172) (**A**), p-JNK (T183/Y185) (**B**), and p-S6RP (S235/236) (**C**). Black arrows on the Western blots indicate the locations of molecular weight markers. Black arrows on the Western blots indicate the location of molecular weight markers. Black arrows on the PS indicate the band used for normalization.

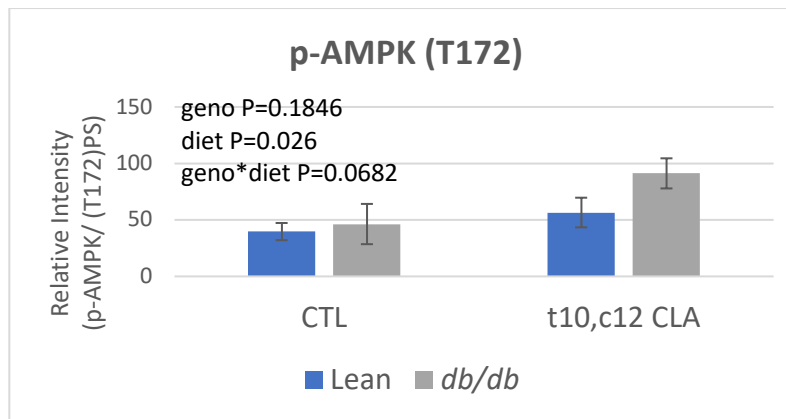
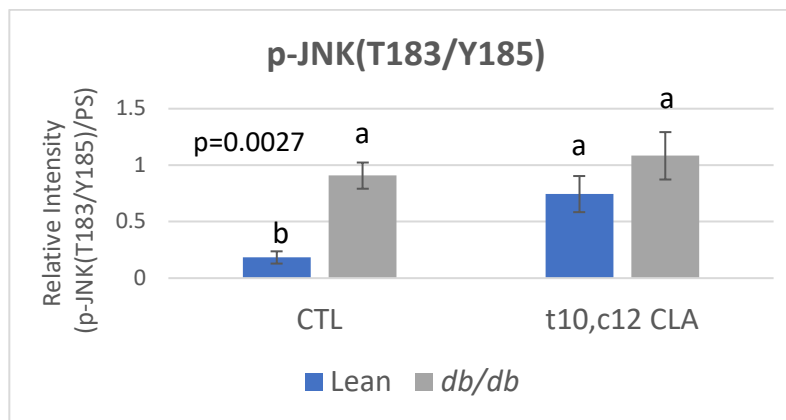
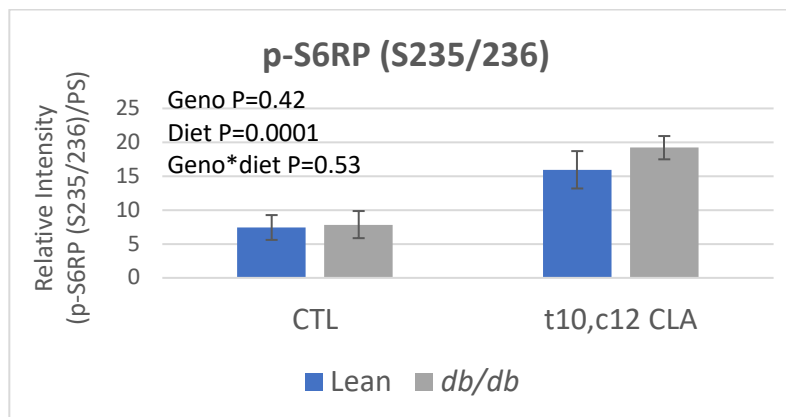
A**B****C**

Figure 15: Relative levels of cell signaling proteins in livers of t10,c12 CLA-fed and CTL diet-fed lean and *db/db* mice. Hepatic levels of p-AMPK (T172) (A), p-JNK (T127/Y185) (B), p-S6RP (S235/236) (C). Densitometry was used to quantify the intensity of the p-AMPK, p-JNK, and p-S6RP bands. Data were normalized to total relative protein levels (measured by PS). Values are expressed as means $\pm$ SEM, N=8-11. Data in A and C were analyzed using two-way ANOVA. Data in B were analyzed by non-parametric testing. P values for the main and interaction effects or the Kruskal-Wallis test are indicated in the upper left corner of the figures. Different letters above the bars indicate significant differences ($p < 0.05$) among the means.

Results Section C: *In Vitro* Experiments with H4IIE Cells

Rat H4IIE hepatoma cells were used as an *in vitro* model for studying the molecular mechanisms of TG accumulation. H4IIE cells have been shown to grow and respond to stimuli in a physiologically relevant manner (197). The treatment of H4IIE cells with FFAs conjugated with BSA simulates the uptake of FFAs by hepatocytes during fasting which are in the circulation due to adipose tissue lipolysis (3). Uptake of FFAs from adipose tissue lipolysis by hepatocytes is physiologically relevant for individuals with NAFLD since it has been shown that 59% of the TG content of livers from people with NAFLD was derived from FFAs from adipose tissue (200). In response to FFA treatments, hepatocytes facilitate the safe storage of FFAs in the form of hepatic lipid droplets, which mainly consist of neutral TGs (61). BODIPY is a commercial fluorescent dye which reliably stains neutral lipids in lipid droplets which form in hepatocytes in response to FFA treatments (201). BODIPY was used to visualize and measure neutral lipid accumulation in H4IIE cells in response to FFA treatments.

Fatty Acid Induced Neutral Lipid Accumulation in H4IIE Cells

OA (100 μ M) was used to induce lipid droplet formation in H4IIE cells. OA was used because it is the FA that is the most effective for inducing lipid droplet formation *in vitro* (203) and it is the FA most used for this purpose. A total of 15 coverslips with quiescent H4IIE cells were incubated in 100 μ M of OA for 0-, 1-, 4-, 8-, or 24-hours (3 coverslips for each time point). After, coverslips were stained with BODIPY and Hoechst 33342 (a nuclear stain) and then

imaged using an epifluorescence microscope. Images generated were analyzed by ImageJ (227). The number of lipid droplets per nucleus (LD/nucleus) in an image was determined to evaluate the amount of neutral lipid accumulation had occurred in response to treatments.

Statistical analysis revealed that cells incubated with OA for 24 hours had significantly more LD/nucleus compared to all other groups (Figures 16A and 16B). H4IIE cells incubated with OA for 1 hour contained significantly more LD/nucleus than the control 0-hour group. The 0-, 4-, and 8-hour groups were all statistically equivalent.

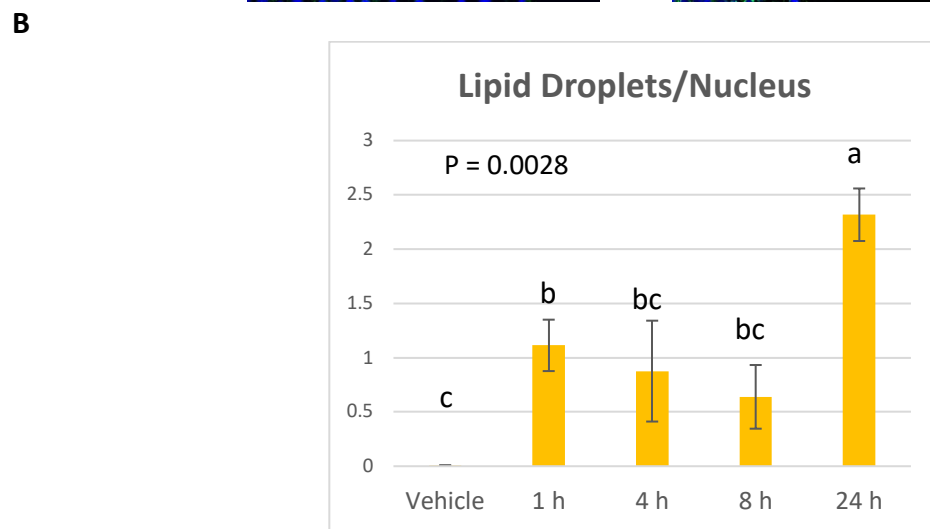
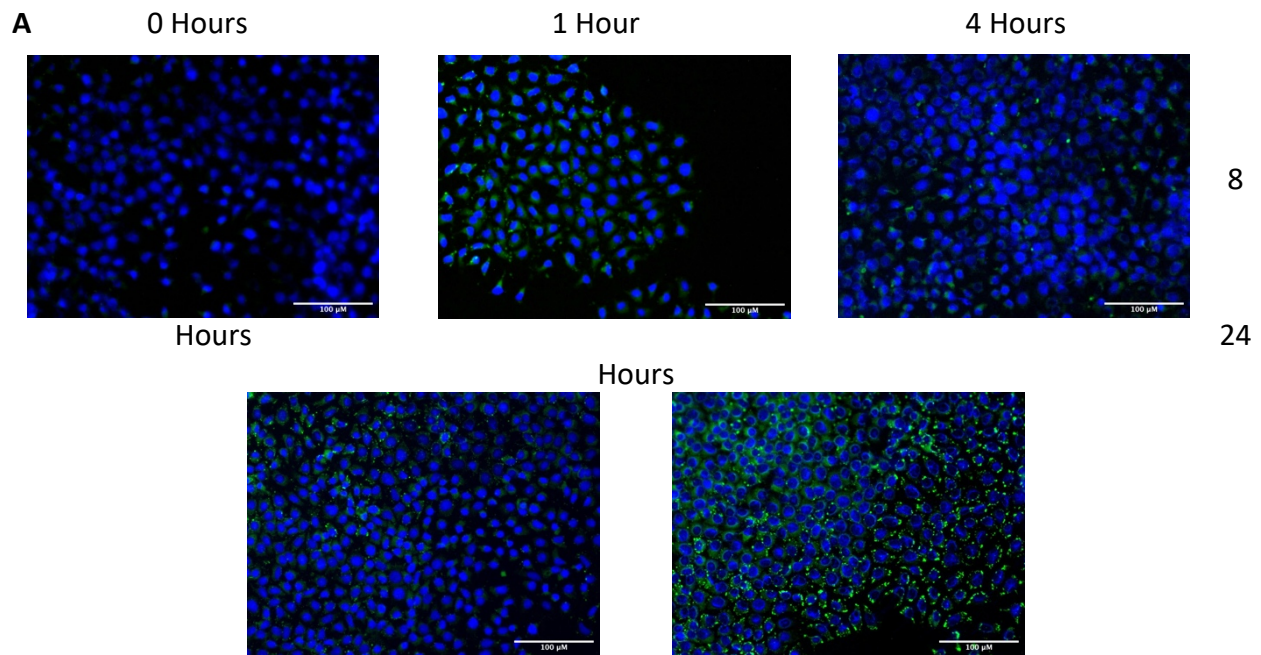


Figure 16: Representative images of H4IIE cells incubated with 100 μ M OA from 0 to 24 hours and stained with the neutral lipid stain, BODIPY (green), and the nuclei stain, Hoechst 33342 (blue) (A) and expressed as LD/nucleus (B). Values are expressed as mean $\pm$ SEM, N=3. Data were analyzed using one-way ANOVA and Duncan's multiple range test. The p value for the main effect is indicated in the upper left corner of the figure and different letters indicate significant differences ($P < 0.05$) among the means.

URI1 Protein Levels in H4IIE Cells After Incubation in Oleic Acid

As indicated previously, *in vitro* experiments show that neutral lipids accumulate over time in H4IIE cells after OA treatment. Furthermore, it was shown in mouse livers that URI1 protein levels are correlated with NAFLD severity. On this account, URI1 protein levels were measured by Western blotting in H4IIE cells after OA treatment to investigate whether URI1 protein levels change in association with neutral lipid accumulation *in vitro*.

Western blotting of H4IIE cell lysates using the CST URI1 antibody revealed 2 distinct bands, one at ~79 kDa and another at ~35 kDa. Of the two bands observed, the ~79 kDa band of URI1 is widely discussed in the literature and has been revealed to be biologically active since it possesses binding domains to RPB5, PP1 γ , and OGT (156,160,163). For the reasons outlined in Results Section A, the ~79 kDa URI1 band was used for analysis (Figure 17A).

Quiescent H4IIE cells were treated with 100 μ M of OA for 1-, 2-, 4-, 8-, and 24-hours. After 24 hours of incubating H4IIE cells with 100 μ M of OA, there were significantly higher levels of URI1 at 4-, 8-, and 24 hours compared to the 0-hour, 1-hour, and 2-hour timepoints. URI1 levels at 24 h were greater than those at 4 hours (Figures 17A and 17B). Thus, URI1 expression is upregulated in response to a 4- to 24-hour treatment with OA. This finding mirrors the results that showed the highest values for LD/nucleus in H4IIE cells are obtained after 24 hours of OA treatment. Similarly, in mouse tissue, URI1 levels are highest in livers with elevated hepatic lipid accumulation (Figure 10).

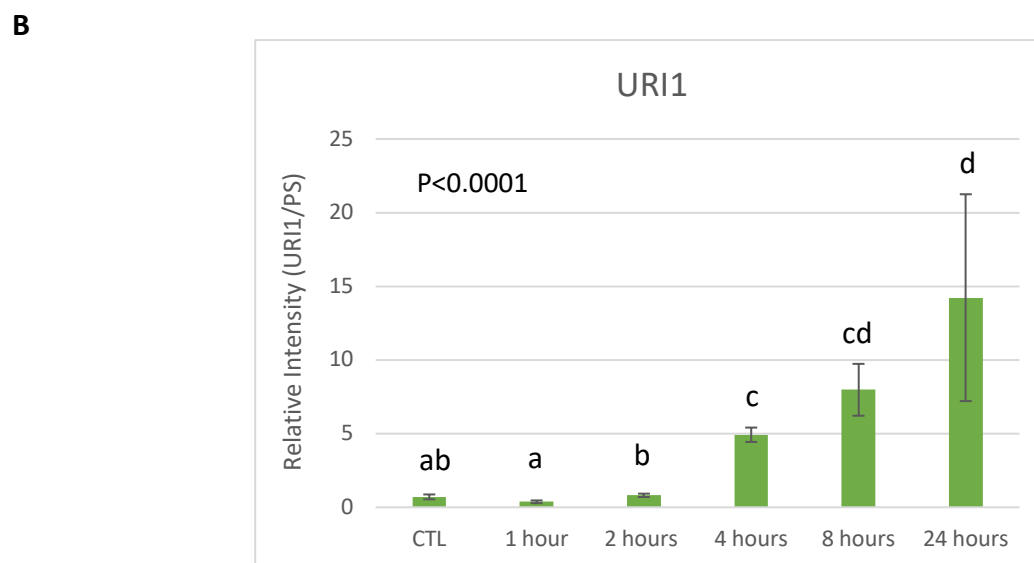
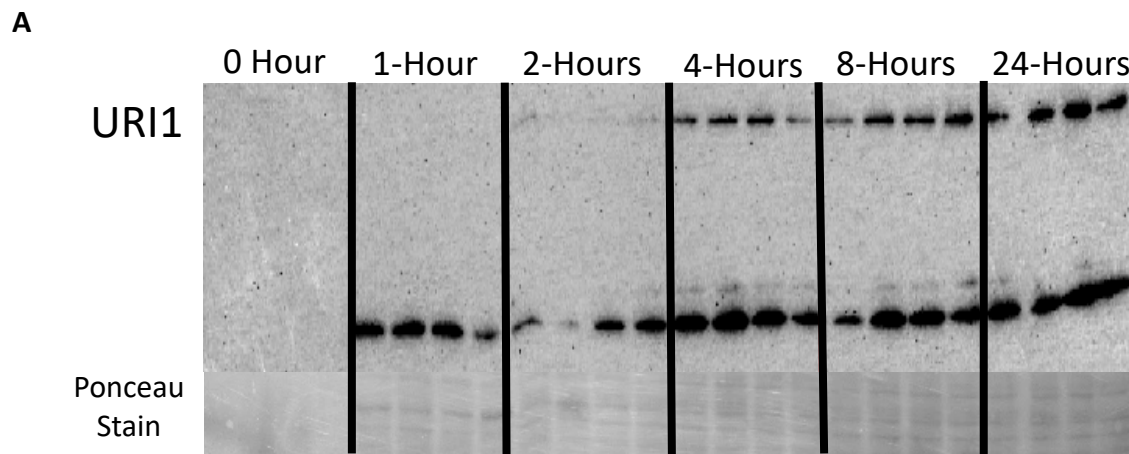


Figure 17: URI1 Western blot of H4IIE cells after OA treatment for 0-24 hours (A) and relative protein levels of URI1 (~79 kDa) (B). Densitometry was used to quantify the intensity of the URI1 band. Data were normalized to total relative protein levels (measured by PS). Values are expressed as mean $\pm$ SEM, N=3-4. Data were analyzed using one-way ANOVA and Duncan's multiple range test. The p value for the main effect is indicated in the upper left corner of the figure and different letters indicate significant differences ($P<0.05$) among the means.

Effects of Different Fatty Acids on S6RP Phosphorylation

The mTOR pathway is an important mediator of hepatic lipid accumulation and was previously observed to be elevated in mice fed a t10,c12 CLA diet (118). Whether different FAs directly activate mTOR was investigated *in vitro*. Different FAs, t10,c12 CLA, c9,t11 CLA, OA, and PA (100 μ M each), were used to treat quiescent H4IIE cells for 24 hours, and then Western blotting was employed to measure p-S6RP levels. This analysis revealed that only the OA treatment resulted in significantly elevated p-S6RP levels (Figures 18A and 19A) .

Next, OA-induced S6RP phosphorylation was measured over time. Quiescent H4IIE cells were incubated with 100 μ M of OA for 0-, 1-, 2-, 4-, 8-, and 24-hours. p-S6RP levels were highest after 2 hours (Figures 18B and 19B) and declined after this timepoint. p-S6RP was significantly elevated after 1-, 2-, 4-, and 8-hours compared to the 0-hour timepoint and p-S6RP levels were statistically equivalent at the 0- and 24-hour timepoints. Only p-S6RP levels at the 2-hour timepoint were significantly elevated compared to the 8-hour timepoint.

Curiously, 24-hour t10,c12 CLA treatment did not augment S6RP phosphorylation (Figures 18A and 19A). Go et al. (176), had reported that incubating HepG2 cells with t10,c12 CLA (50 or 100 μ M) for 3 hours resulted in an increase in phosphorylated mTOR associated proteins. These specific experimental conditions were replicated to investigate if a similar outcome would occur in H4IIE cells. Cells were also treated with LA to assess if the effects of t10,c12 CLA are FA isomer specific. Insulin (100 nM) was used as a positive control and to investigate possible synergistic effects from combination treatments of insulin and either lipid. Total S6RP (t-S6RP) levels were used to normalize p-S6RP levels.

After incubating H4IIE cells for 3 hours with t10,c12 CLA or LA (100 μ M), Western blotting showed that t10,c12 CLA significantly increased p-S6RP/t-S6RP levels compared to the vehicle and LA (Figures 18C and 19C). However, while insulin significantly increased p-S6RP/t-S6RP levels, no FA treatments with insulin showed a significant difference from insulin alone. Furthermore, when assessing the effects of t10,c12 CLA on t-S6RP/PS levels, t10,c12 CLA and treatments including insulin resulted in a significant reduction of t-S6RP/PS levels. This result may indicate how FA treatment further increases mTOR activation. The extent of mTOR activation is directly proportional to p-S6RP/t-S6RP and FA treatment may also augment mTOR activation by reducing t-S6RP.

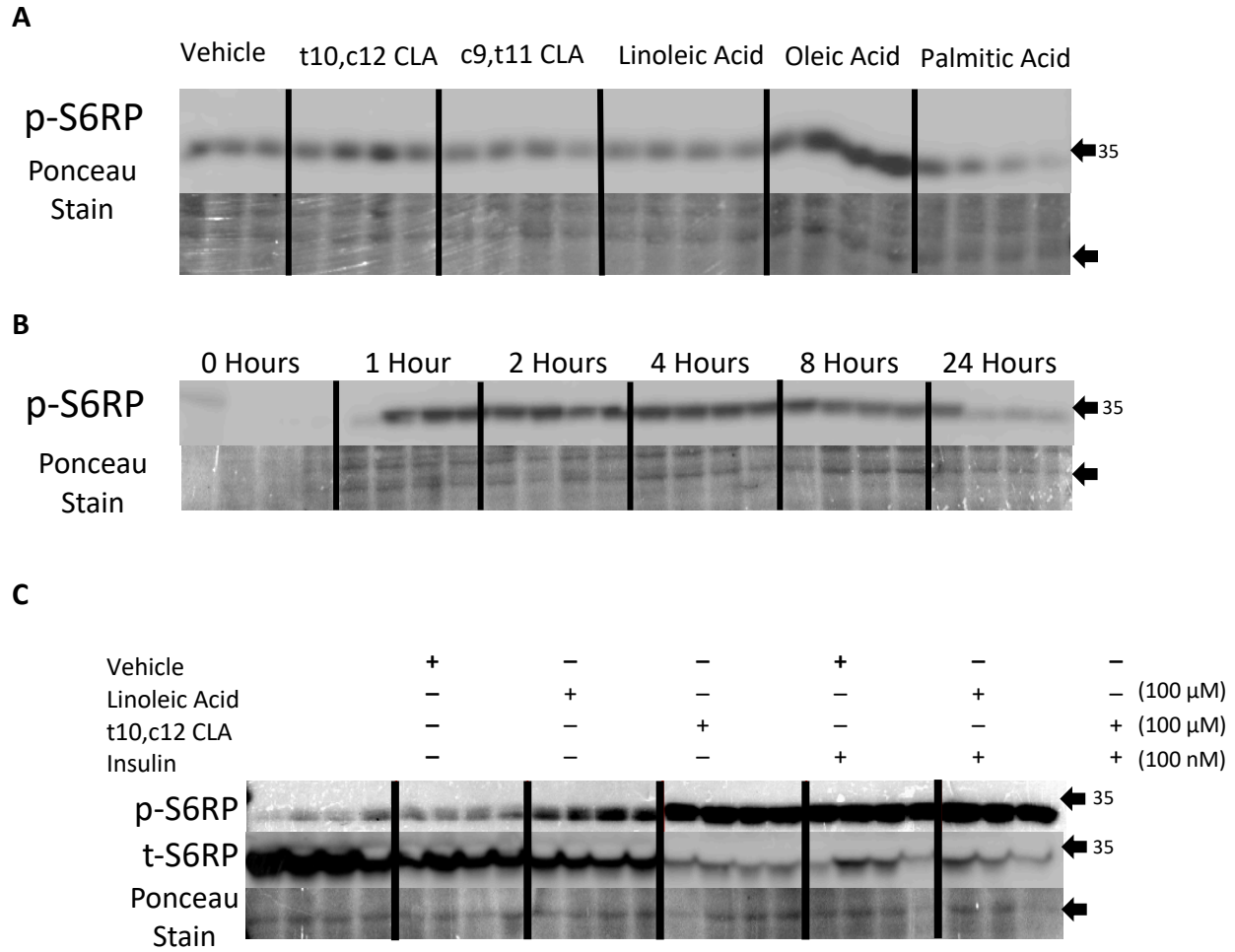


Figure 18: Western blots showing the effects of different FFAs on p-S6RP (S235/236) levels after 24 hours (A), a time course experiment of the effect of OA treatment on p-S6RP (S235/236) levels from 0 to 24 hours (B), and the effect of a 3 hour treatment of LA or t10,c12 CLA, with/without insulin on p-S6RP (S235/236)/t-S6RP levels (C). Black arrows on the Western blots indicate the locations of molecular weight markers. Black arrows on the ponceau stain indicate the band used for normalization.

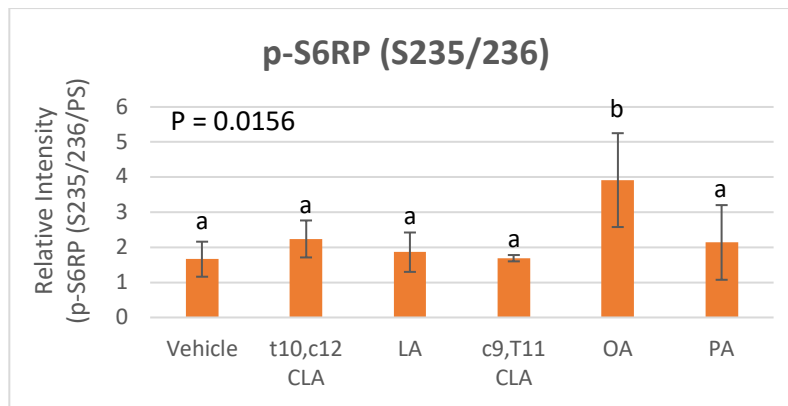
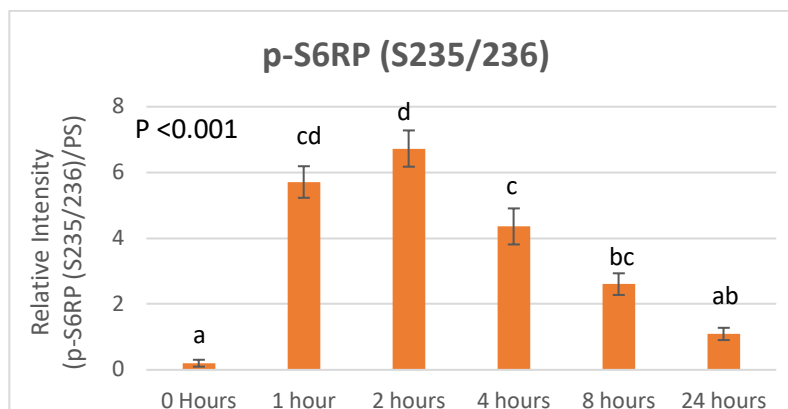
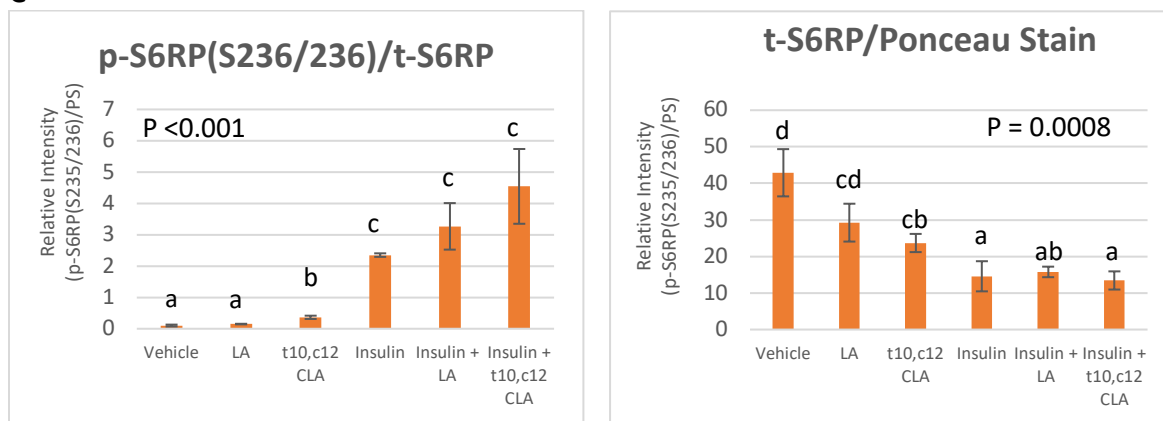
A**B****C**

Figure 19: Relative p-S6RP (S235/236) levels in H4IIE cells after treatments with different fatty acids. Relative p-S6RP (S235/236) levels after 24 hour treatment with different FFAs (**A**), after OA treatment over time (**B**), and relative p-S6RP (S235/236)/t-S6RP and t-S6RP/PS levels in H4IIE cells after a 3 hour t10,c12 CLA treatment with/without insulin (**C**). Densitometry was used to quantify the intensity of the bands shown. Data were normalized to total relative protein levels (measured by PS) or t-S6RP. Values are expressed as means $\pm$ SEM, N=3-4. Data were analyzed using one-way ANOVA and Duncan's multiple range test. The p value for the main effect is indicated in the upper left corner of the figures, and different letters indicate significant differences ($P < 0.05$) among the means.

Effect of *trans*-10, *cis*-12 Conjugated Linoleic Acid on Lipid Metabolism Proteins *In Vitro*

Experiments have revealed that FABP1 and FAT/CD36 are proteins relevant to hepatic lipid accumulation (68,73). Furthermore, in this thesis, t10,c12 CLA-induced hepatic lipid accumulation also coincided with upregulated protein levels of FABP1 and FAT/CD36 in lean and *db/db* mice. Thus, H4IIE cells were treated with t10,c12 CLA (100 μ M) and Western blotting was employed to assess the direct effects of t10,c12 CLA on the proteins of interest. Cells were treated with LA (100 μ M) to assess if the effects of t10,c12 CLA are isomer specific. H4IIE cells were also treated with OA (100 μ M) since it is a prominent inducer of neutral lipid accumulation in these cells. H4IIE cells were also treated with OA in combination with t10,c12 CLA or LA to investigate synergistic effects. The duration of all treatments was 3 hours.

Western blotting showed that t10,c12 CLA and OA were able to significantly increase FABP1 levels compared to the vehicle and LA (Figures 20A and 20C). There were no significant differences between OA treatment on its own compared to OA combined with other FAs. There was also no significant difference between t10,c12 CLA treatment on its own compared to the t10,c12 CLA combined with OA treatment (Figures 20A and 20C). This evidence indicates that t10,c12 CLA and OA can individually upregulate levels of FABP1 in H4IIE cells.

Western blotting also showed that there were no statistically significant differences in FAT/CD36 protein levels between different single fatty acid treatments (Figures 20B and 20D). However, H4IIE cells treated with a combination of OA and LA had significantly increased levels of FAT/CD36 protein levels compared to the vehicle control group, the LA group, and the t10,c12 CLA group (Figures 20B and 20D). Since FAT/CD36 levels were only significantly

increased by combination treatments where the total FFA concentration would be 200 μmol , these results suggest that FAT/CD36 levels are increased as a result of increased concentrations of total FFA.

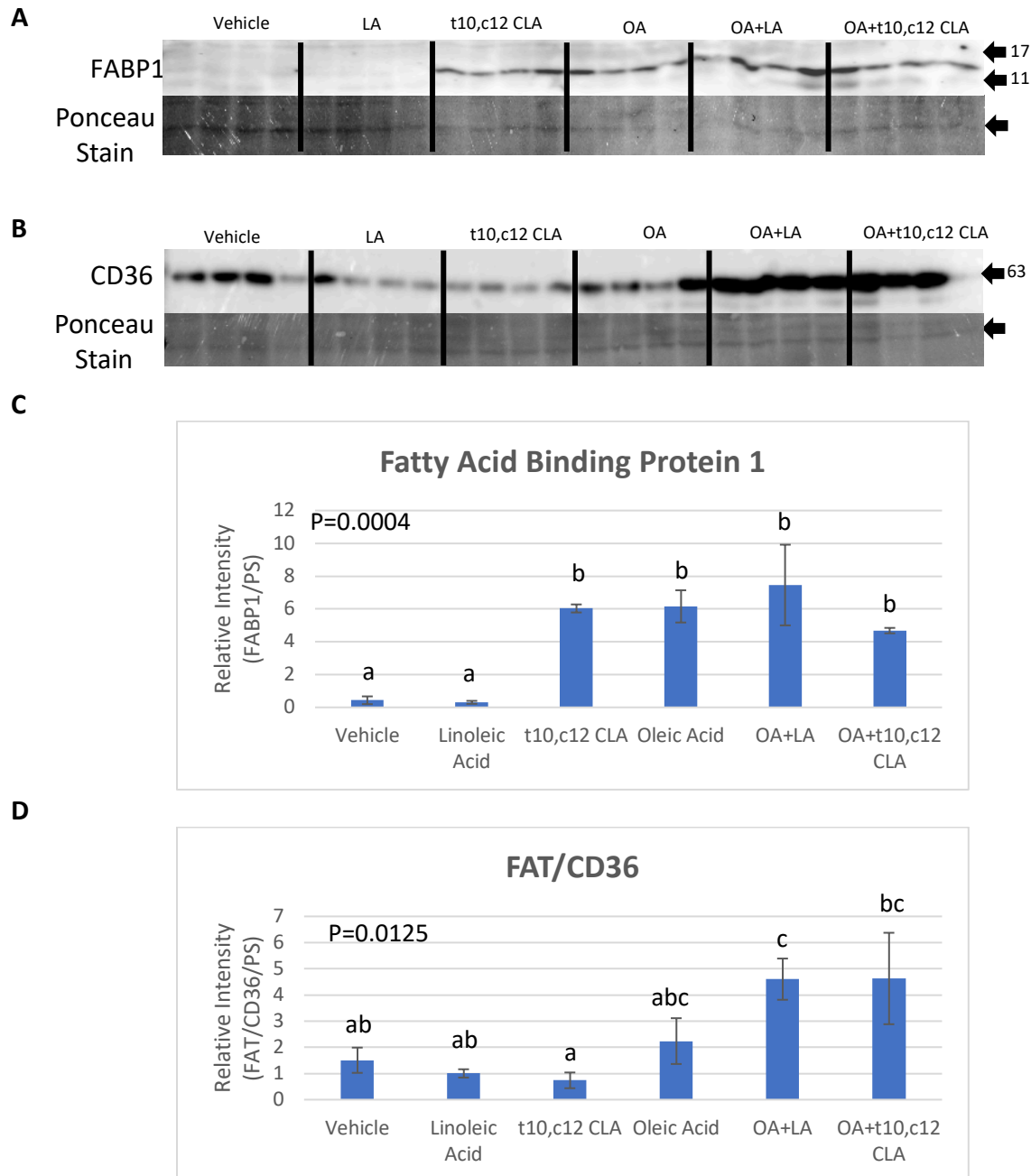


Figure 20: Western blots showing the effects of incubating H4IIE cells with LA, t10,c12 CLA, and/or OA for 3 hours on FABP1 (A) and FAT/CD36 (B) levels and relative protein levels of FABP1 (C) and FAT/CD36 (D). Black arrows on the Western blots (A, B) indicate the locations of molecular weight markers. Black arrows on the PS indicate the band used for normalization. Densitometry was used to quantify the intensity of the bands. Data were normalized to total relative protein levels (measured by PS) (C, D) and are expressed as means $\pm$ SEM, N=4. Data were analyzed using one-way ANOVA and Duncan's multiple range test. P values for the main effect are indicated in the upper left corners of figures C and D, and different letters indicate significant differences ($P < 0.05$) among the means.

Results Section D: Generation of a *Uri1* KO H4IIE Cell Line

Previous results have highlighted that URI1 protein levels correlate with hepatic steatosis severity in mouse livers. Furthermore, it was observed that URI1 protein levels increased over time in H4IIE cells treated with 100 μ M of OA, peaking 24 hours after treatment. In parallel, BODIPY staining of H4IIE cells showed that neutral lipid accumulation peaked after 24 hours of treatment with 100 μ M of OA. From these results, URI1 protein levels and hepatic lipid accumulation appear to be linked. To identify whether this relationship is causal, a CRISPR/Cas9 approach was used to generate *Uri1* KO H4IIE cells. If there are differences in lipid droplets in *Uri1* KO H4IIE cells compared to WT H4IIE cells in response to 100 μ M OA treatment, this would indicate there is a causal relationship between hepatic lipid accumulation and URI1.

To create these *Uri1* KO H4IIE cells, 2 different sgRNA strands which target the *Uri1* gene were used (205). The *Uri* gene maps to chromosome 1 and is made up of 11 exons (Figures 21A and 21 B). The first sgRNA targeted the 4th exon in the *Uri1* gene (designated *sgUri1-1*) and the second sgRNA targeted the 10th exon in the *Uri1* gene (designated *sgUri1-2*) (Figure 21B). The 19 variable nucleotide sequences of the 3' end of sgRNAs are shown in Table 7 in the Synthetic Guide RNA Plasmid Expansion section in the Methods chapter. Two sgRNA guides were employed to increase the chance of creating a *Uri1* KO H4IIE clone. The 3' end of the sgRNA is complimentary to gDNA within the *Uri1* gene. sgRNA will hybridize with complimentary DNA within the *Uri1* gene, create a complex with the Cas9 endonuclease, and then Cas9 will induce a DSB in the *Uri1* gene (Figure 21C). From here, the NHEJ repair mechanism repairs the

DSB. However, this process is error prone, and often leads to indels. If these indels result in a frameshift in the gene, it may create a PTC. These PTCs are detected by the NMD mRNA surveillance pathway. If PTCs are detected by this pathway, mRNAs harbouring a PTC are degraded. The overall result is that this allele will no longer be able to produce the protein, essentially knocking out the gene. However, NHEJ-induced indels may result in an in-frame alteration (an addition or deletion of a number of base pairs equal to 3 or any product of 3), thus resulting in the addition/removal of codons (a set of 3 consecutive nucleotides which code for a single AA residue). This results in the protein's AA sequence being altered due to the addition/removal of codons but still be translated. The change of AA sequence may or may not affect the protein's functionality.

There are four possible outcomes from a CRISPR/Cas9-induced DSB. They are the following:

1. First is a homozygous gene KO where both alleles attain a PTC. This results in zero expression of the gene of interest.
2. Second is a heterozygous gene KO where a PTC is only present in one allele and the other allele is unaffected. This results in only one allele expressing the gene of interest and thus half the normal protein level.
3. Third is a complex heterozygous KO. In this thesis, compound heterozygous KO refers to when one allele attains a PTC and the other allele has an indel that does not introduce a PTC. This results in no expression of the gene of interest from one allele, and the other allele expressing a protein with an altered AA sequence. The change in AA sequence may or may not affect the protein's functionality. Complex

heterozygous KOs may also refer to when CRISPR/Cas9 mediates distinctive gene edits in both alleles, and each edit introduces PTCs and complete KO of the gene of interest. Lastly, complex heterozygous KO may also refer to when CRISPR-Cas9 gene edits result in distinctive in-frame gene indels for each allele that do not introduce PTCs. This results in aberrant protein expression from each allele. However, hereafter, complex heterozygous KO in this thesis will refer to only the first definition outlined.

4. Lastly, the DSB may cause mutations which do not introduce PTCs in either allele but cause mutations that alter the protein's AA sequence. H4IIE clones with these mutations will be referred to as mutants.

The *Uri1* KO H4IIE cells were generated using a 2-step process which involved constitutive expression of sgRNA (achieved by lenti-viral based transduction) and transient expression of the Cas9 endonuclease (facilitated by nucleofection). This process increases the chance of CRISPR/Cas9-induced gene edit through constitutive sgRNA expression while minimizing the risk of off-target CRISPR/Cas9-induced DSBs which is associated with excessive Cas9 expression.

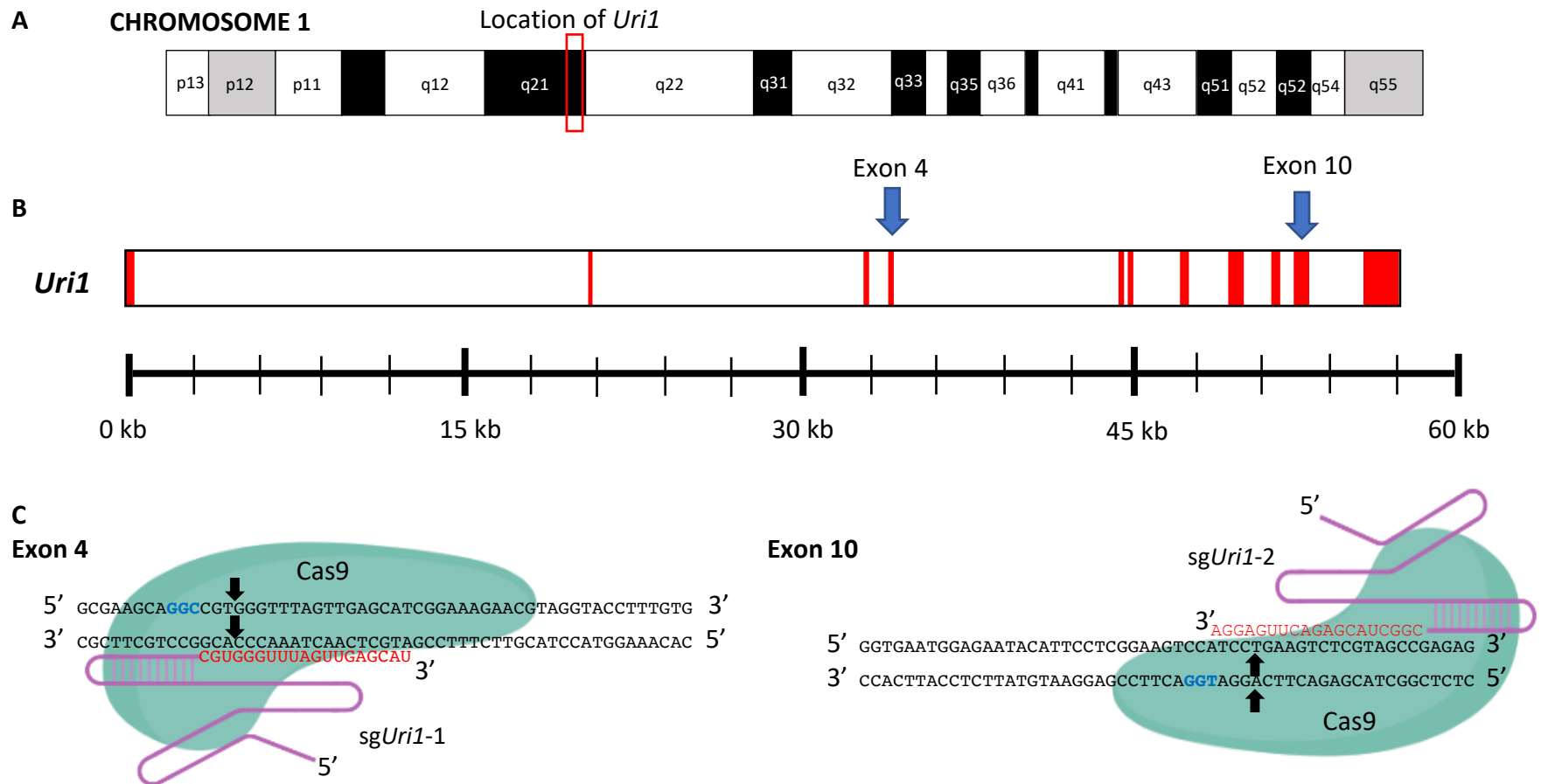


Figure 21: Hybridization sites of *sgUri1-1* and *sgUri1-2* used for CRISPR/Cas9-induced *Uri1* gene editing. The *Uri1* gene localizes to chromosome 1q21 (A). Exons 4 and 10 (exons are represented by red boxes) of the *Uri1* gene were targeted by the *sgUri1-1* and *-2*, respectively (B). Both sgRNAs contained a variable 19 nucleotide sequence (red text) at the 3' end which is complementary to a region in the *Uri1* gene. The 5' end of the sgRNA facilitates the sgRNA-Cas9 complex formation and brings the Cas9 endonuclease near the target site in the *Uri1* gene. Cas9 will then induce a DSB 3 bp upstream from the PAM site (blue coloured text) which is indicated by the black arrows (C).

Generation and Isolation of *Uri1* KO H4IIE Cells

Both the *sgUri1-1* and *sgUri1-2* plasmids contained the BFP gene within the expression cassette to allow for the detection and isolation by FACS of successfully transduced H4IIE cells. These sgRNAs with BFP plasmids were delivered to H4IIE cells using lentiviral transduction and FACS was used to isolate BFP expressing H4IIE ($\text{H4IIE}^{\text{BFP}+}$) cells. H4IIE cells which did not undergo transduction were used to establish gating parameters to create a threshold of BFP expression required for isolation by FACS. According to the number of cells which were sorted and the number of cells which were returned, the transduction rate was 0.03%.

Plasmids containing Cas9 endonuclease with green fluorescence protein (GFP) were delivered to $\text{H4IIE}^{\text{BFP}+}$ cells using nucleofection, an electroporation-based transfection method. Another round of FACS was used to isolate H4IIE cells expressing both BFP and GFP. H4IIE cells which did not undergo transduction or nucleofection were used to establish gating parameters to create a threshold of simultaneous BFP and GFP expression required for isolation by FACS. After nucleofection, FACS-isolated H4IIE cells that simultaneously express both BFP and GFP ($\text{H4IIE}^{\text{BFP}+/\text{GFP}+}$) were collected for further screening experiments. The simultaneous expression of BFP and GFP indicate that these cells express the sgRNA targeted to *Uri1* and the Cas9 endonuclease, and may have experienced edits in the *Uri1* gene by these 2 components. The transfection rate of Cas9/GFP using nucleofection was 42%.

Clonal populations were obtained from $\text{H4IIE}^{\text{BFP}+/\text{GFP}+}$ cells by seeding a single $\text{H4IIE}^{\text{BFP}+/\text{GFP}+}$ cell into wells of a 96 well plate and allowing the single cell to expand into a clonal population. All the wells of two 96 well plates were seeded such that 1 in every 2 wells contained 1 cell with a total 100 μL of complete media. In total, 32 clonal populations of

H4IIE^{BFP+/GFP+} cells were established. Next, these 32 H4IIE^{BFP+/GFP} clonal populations were screened for URI1 protein expression by Western blotting (Figures 22 and 23).

Identification of Candidate *Uri1* KO Clonal Populations by Western Blotting

Western blotting was used to determine whether H4IIE^{BFP+/GP+} cells had less or no *Uri1* expression. Initial screening experiments used quiescent H4IIE^{BFP+/GFP+} cells. Under these conditions, only the ~35 kDa URI1 band was detected. Using this band to identify *Uri1* KO H4IE clone candidates, 21 clonal populations exhibited reduced or absent URI1 (~35 kDa band) levels (Figure 22). Of these clones, 13 of them were subjected to further screening by DNA sequencing.

The only condition which enabled proper analysis of the 79 kDa URI1 band in H4IIE cells was after serum starvation to quiescence and treatment with 100 μ M OA. These conditions were replicated in clones 2A3, 1B8 and 2F7 in triplicate to see if this band would be present after OA treatment. When loading the gel, one lane in between different clone samples was left empty to ensure that contamination between samples did not occur. The result of this experiment was that the URI1 protein levels of clones 2A3 and 1B8 were half of those in WT and zero expression in clone 2F7 (Figure 23A, and 23B). In the presence of OA treatment for 24 hours, these 3 clones showed a statistically significant reduction of URI1 protein, assessed by the 79 kDa band or the ~35 kDa band, compared to the WT control.

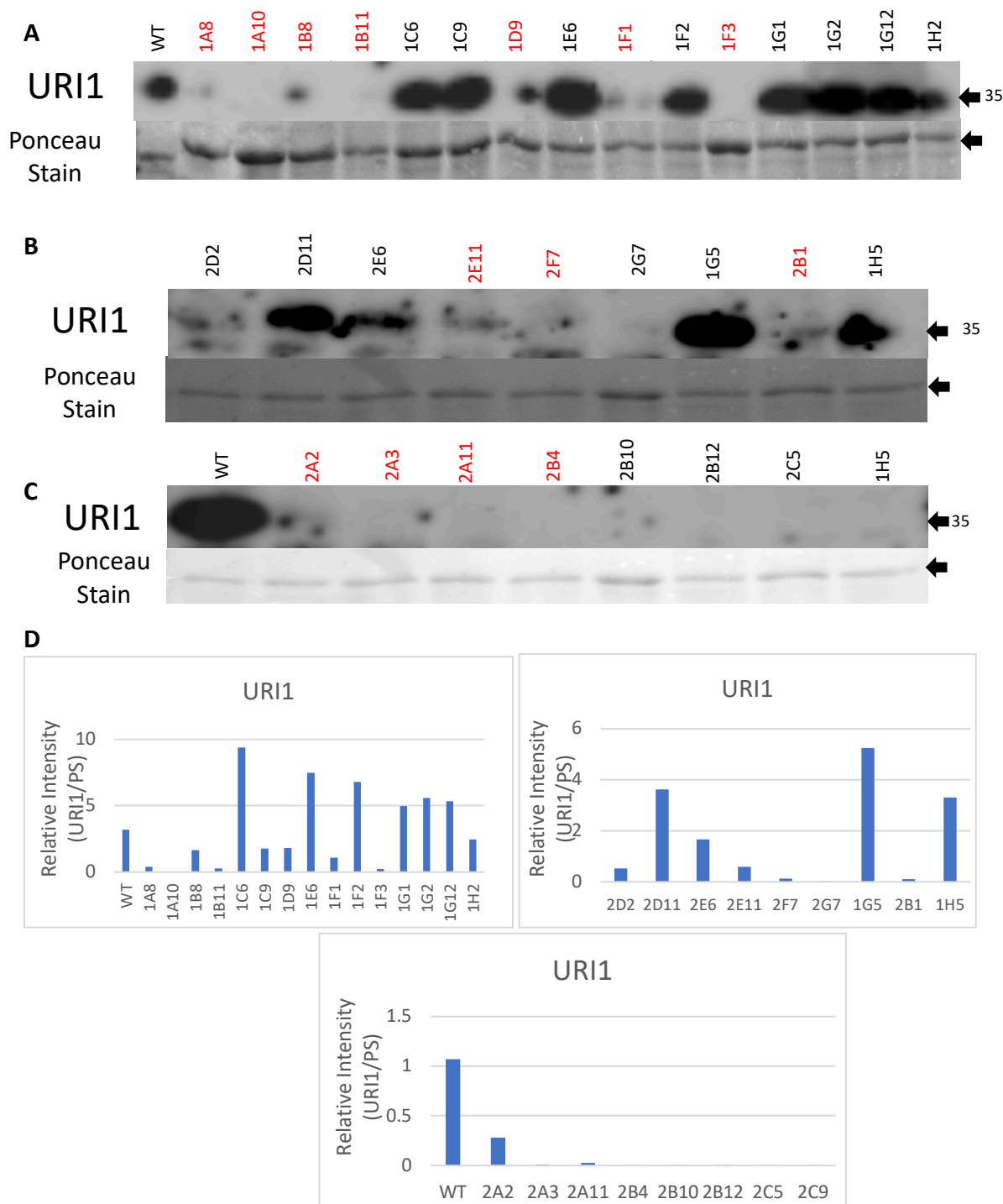


Figure 22: URI1 KO H4IIE Clone Screening Western blots using quiescent H4IIE^{BFP+/GFP+} clones and relative protein levels of URI1. Representative blots of URI1 protein levels in 32 H4IIE^{BFP+/GFP+} clones (A,B, and C). Clones in red font were subjected to DNA sequencing. Black arrows on the Western blots indicate the locations of molecular weight markers. Black arrows on the PS indicate the band used for normalization. Relative protein levels of UR1 (~35 kDa band) (D). Densitometry was used to quantify the intensity of the bands. Data were normalized to total relative protein levels (measured by PS).

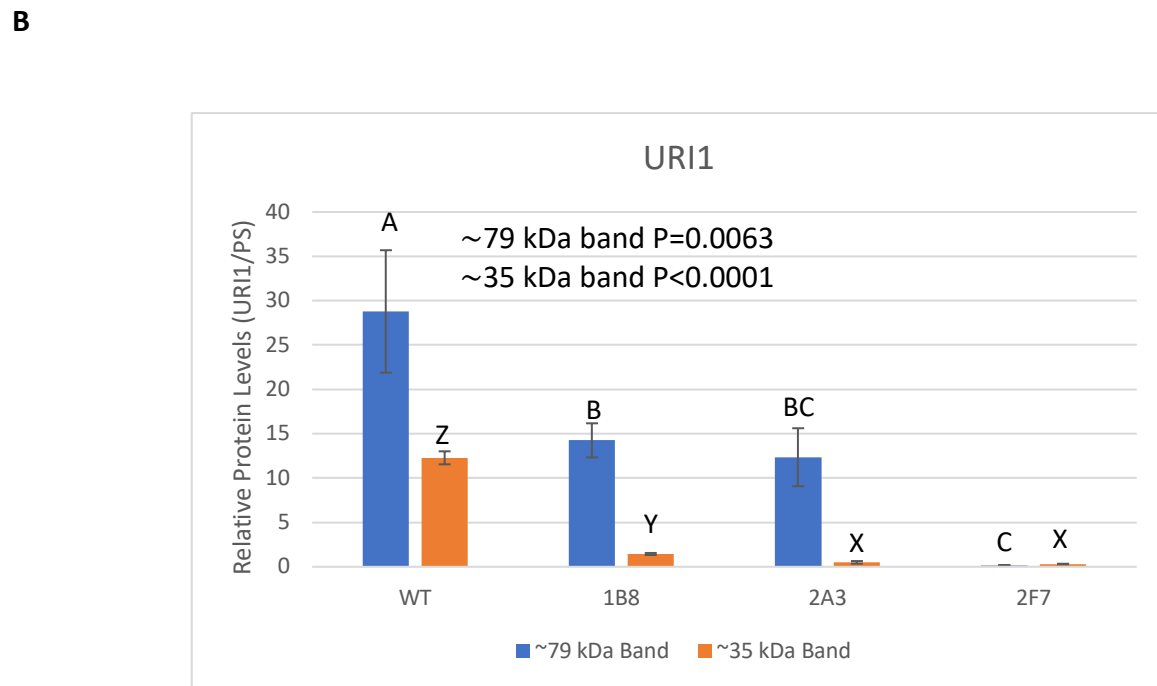
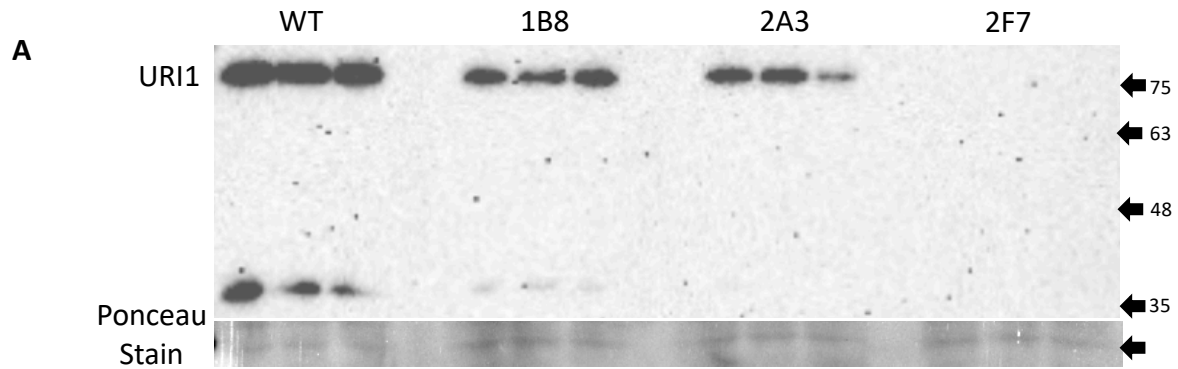


Figure 23: URI1 KO H4IIE Clone Screening Western blots using OA treated H4IIE^{BFP+/GFP+} clones and relative protein levels of URI1. Western blots from H4IIE^{GFP/BFP+} clones treated with OA (100 μ M) for 24 hours showing both the ~79 kDa and ~35 kDa URI1 bands (**A**). Black arrows on the Western blots indicate the locations of molecular weight markers. Black arrows on the ponceau stain indicate the band used for normalization. Relative protein levels of the 79 kDa URI1 band and the ~35 kDa URI1 band (**B**). Values are expressed as means $\pm$ SEM, N=3. Data were analyzed using one-way ANOVA and Duncan's multiple range test for both the ~79 and ~35 bands. P values for the main effect are indicated in the upper left corner of panel B. Different letters indicate significant differences (P<0.05) among the means.

Validation of *Uri1* KO H4IIE Cells by DNA Sequencing

Clones with diminished or absent URI1 protein levels were expanded for DNA extraction with phenol chloroform. After, PCR was used to amplify the DNA of the 2 exons in the *Uri1* gene which were targeted by the sgRNAs. This was done to amplify this specific DNA for future sequencing to investigate if there was a CRISPR/Cas9-induced DNA edit (Figure 24A).

PCR amplification was carried out in a total volume of 20 μ L. Five μ L of the PCR product was run on a 1.5% agarose gel with a 1 kilobase DNA ladder to validate the PCR product (by comparing the predicted PCR product size with its actual size). Examples of PCR product validation are shown in Figure 24B. The rest of the PCR product was purified using the QIAquick PCR Purification Kit. Then, the DNA concentration and purity were determined using a Nano-Drop Spectrophotometer. DNA samples were mixed with nuclease free water and the forward primer in accordance with the requirements of TCAG DNA Sequencing/Synthesis Facility for sample preparation and Sanger sequencing. Sequencing results were returned as chromatograms.

In the chromatograms for clone 1F1, there was no indication of a CRISPR/Cas9-induced DSB for either exon 4 or exon 10 (Figure 25). Also, the Western blot showed some URI1 (~35 kDa) expression for this clone. Therefore, it is likely that there was no CRISPR/Cas9 induced DSB in clone 1F1, making it a WT H4IIE clone. Furthermore, when the sequence was aligned with the reference *Uri1* gene sequence from NCBI using the Clustal Omega multiple sequence alignment tool, both sequences had 100% alignment (230). This is strong evidence that there were no

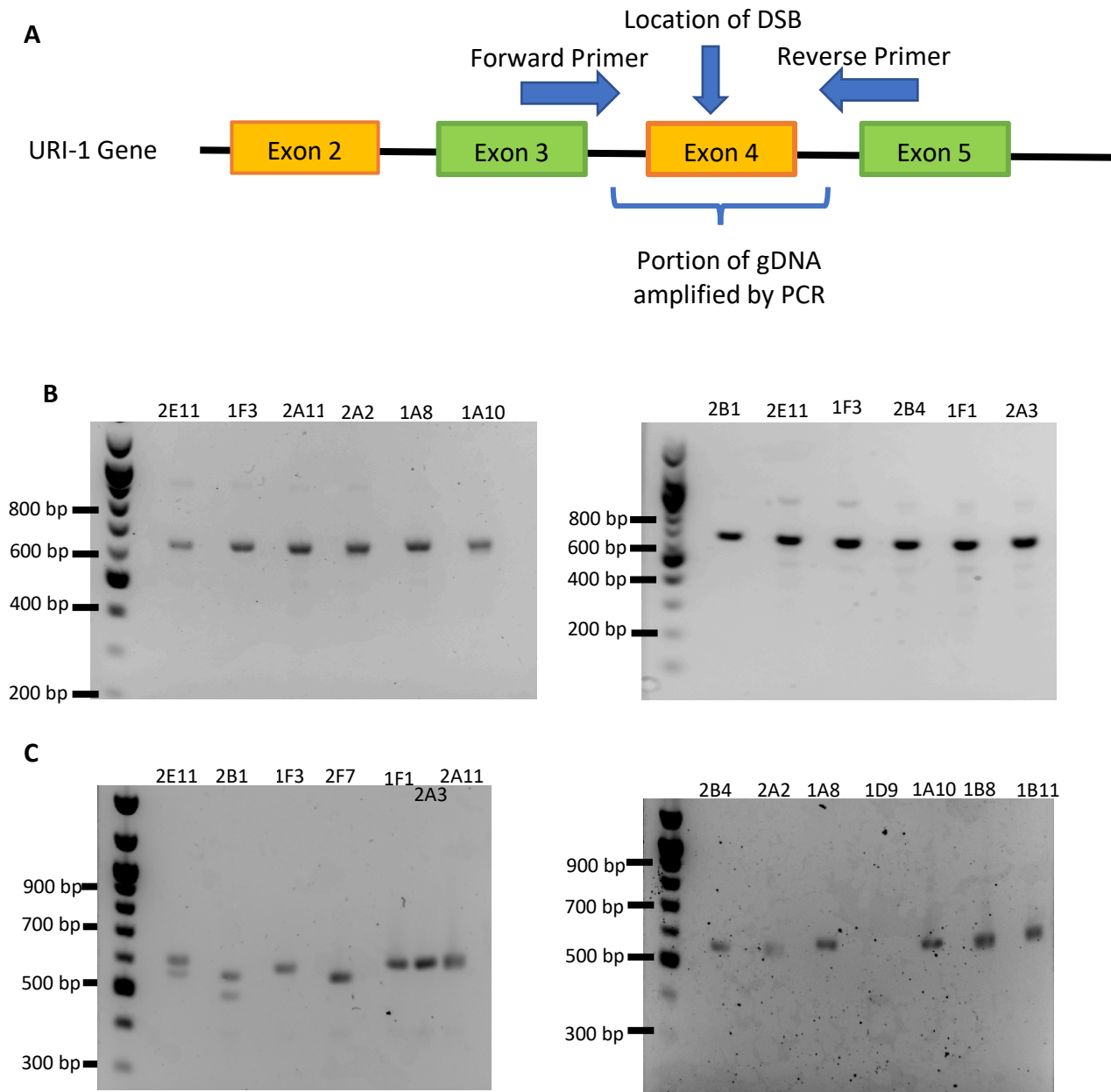


Figure 24: Depiction of PCR reactions and PCR gel electrophoresis. Depiction of forward and reverse PCR primers binding to introns flanking the exon where the CRISPR/Cas9 endonuclease theoretically induced a DSB (exon 4) and the portion of gDNA which is amplified by PCR (**A**). This would also be true for exon 10 (not shown). Purified PCR products from different H4IIE^{GFP+/BFP+} clones of exon 4 (**B**) and exon 10 (**C**) run on a 1.5% agarose gel. The predicted size of the exon 4 and exon 10 PCR products were 666 and 599 bp, respectively.

edits in the *Uri1* gene in the cells used prior to delivery of the sgRNA strands and Cas9 endonuclease to the cells.

The chromatogram of exon 4 in clone 2A3 showed double peaks approximately where the Cas9 endonuclease was targeted and this strongly suggests a DSB had occurred (Figure 26). Next, the sequencing result was uploaded into CRISP-ID (232). The nucleotide sequence of exon 4 of *Uri1* from the NCBI database was used as the reference sequence. CRISP-ID was then able to resolve the 2 different alleles resulting from the DSB and the sequential NHEJ repair mechanism. CRISP-ID revealed a 14 bp deletion in allele 1. The other allele had one single nucleotide polymorphism (SNP) in allele 2.

Next, the resultant sequences were translated into AA sequences using the ExPASy translate tool (233). The URI1 protein in WT H4IIE cells is 530 AAs long and 59.3 kDa. In clone 2A3, allele 1 would produce a 122 AA long truncated URI1 protein that is 13.9 kDa since a PTC is situated in exon 4. However, this protein would likely not be produced due to NMD. The second allele produces a URI1 protein that is nearly identical to the WT URI1 protein. There was a single AA switch from glutamic acid to aspartic acid in allele 2. Since one *Uri1* allele was knocked out and there was an alteration to the AA sequence of the other allele, clone 2A3 would be a compound heterozygous *Uri1* KO H4IIE clone. It should be noted that glutamic and aspartic acids have similar properties and would likely not alter the protein's functionality.

When evaluating the exon 10 chromatogram from the same clone, there were no indications that a DSB had occurred. Furthermore, when the sequence was aligned with the reference sequence, it showed a near perfect alignment. Therefore, no DSBs had occurred in exon 10 of the *Uri1* gene in clone 2A3.

Thus, clone 2A3 had half the expression of the ~79 kDa URI1 band in the presence of OA treatment. Sequencing results showed that there was a PTC in one allele and a SNP in the other allele in exon 4. This clone would likely express *Uri1* from only one allele, which contains a SNP, therefore making this clone a complex heterozygous *Uri1* KO H4IIE clone.

When assessing the clone 2F7 sequencing results, the chromatograms received for exon 10 showed no double peaks and had no indications that a DSB had occurred (Figure 28). However, alignment of the sequence with the reference sequence showed that there was a 44 bp deletion in both alleles. A 44 bp deletion would result in an out-of-frame deletion. There were no CRISPR-Cas9 induced gene edits in exon 4.

Next the *Uri1* gene sequence from clone 2F7 was translated into an AA sequence using the Expasy translate tool (233). The translation contained a PTC that would make a 414 AA long truncated URI1 protein that is 47.1 kDa. However, the protein would likely not get translated due to NMD. This clone showed zero expression of the ~79 kDa URI1 band in response to OA treatment. A 44 bp deletion occurred in both alleles and created PTCs. Therefore, clone 2F7 is a homozygous *Uri1* KO H4IIE clone.

This process was applied to all the clones which were subjected to sequencing. The process for identifying clone 1F1 as a WT H4IIE cell was explained in Figure 25 to show how chromatograms and DNA and AA sequence alignments appear in WT cells and to contrast this against clones with CRISPR/Cas9-induced edits. The process of identifying clones 2A3, 1B8, and 2F7 (Figures 26, 27, and 28, respectively) was explained thoroughly, because these clones were selected for further experimentation. A summary of Western blot and DNA sequencing results for the other clones is shown in Table 13.

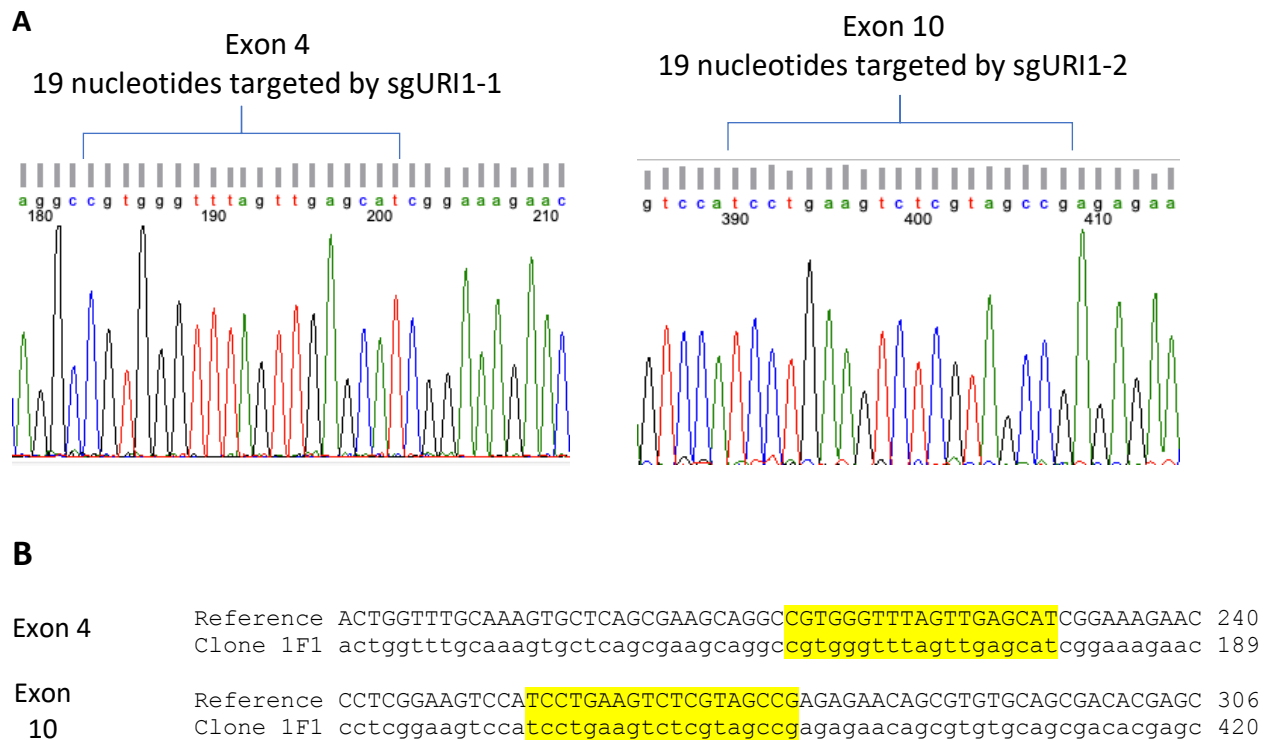
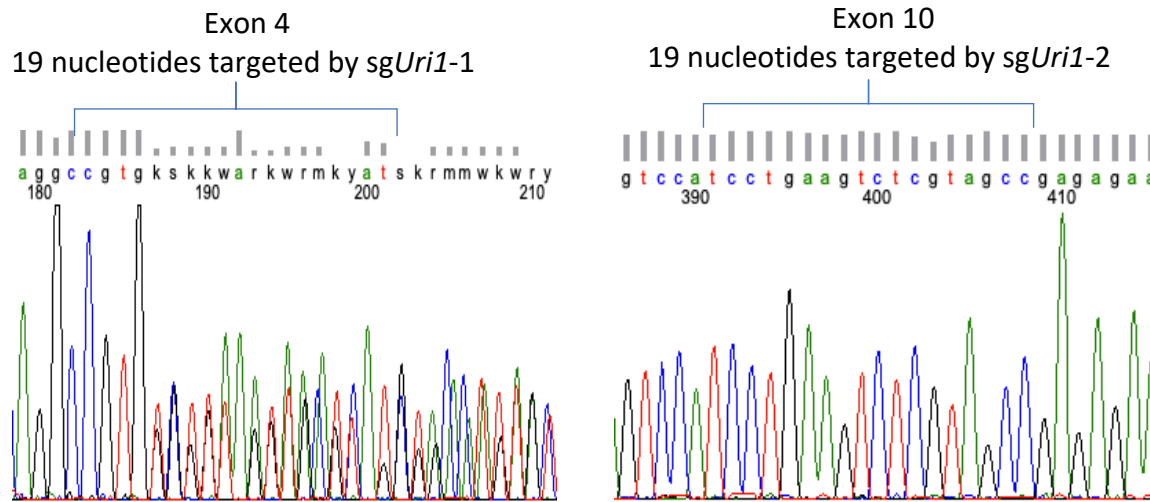


Figure 25: Chromatograms of sequencing results from clone 1F1 (A). DNA alignment between reference DNA and clone 1F1 DNA at the regions where CRISPR/Cas9 was targeted (B). Regions highlighted in yellow correspond to the region where the sgRNA strands were targeted.

A



B

	Reference	5'	TTTGCAAAGTGCTCAGCGAAGCAGGC	CGTGGGTTTAGTTGAGCAT	TCGAAAGAAC	3'
Exon 4	2A3 Allele 1	5'	TTTGCAAAGTGCTCAGCGAAGCAGGC	CGTG-----	TCGAAAGAAC	3'
	2A3 Allele 2	5'	TTTGCAAAGTGCTCAGCGAAGCAGGC	CGTGGGTTTAGTTGA	CATTCGAAAGAAC	3'
Exon 10	Reference	5'	CCTCGGAAGTCCA	TCCTGAAGTCTCGTAGCCG	AGAGAACAGCGTGTGCAGCGACA	3'
	Clone 2A3	5'	cctcgaagtcca	tctgaagtctcgtagccg	agagaacagcgtgtgcagcgaca	3'

C

	Reference	PFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVEHRKEHVRKTIDDFK	130
Protein not produced	2A3 Allele 1	PFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSAKQAV	122
	2A3 Allele 2	PFGPLAFMPGKLVHTNEVTVLLGDNWFAKCSAKQAVGLVDHRKEHVRKTIDDFK	130

Figure 26: Chromatograms of sequencing results from clone 2A3 (A). DNA alignment between reference DNA and clone 2A3 DNA at the regions where CRISPR/Cas9 was targeted (B). Regions highlighted in yellow correspond to the regions where the sgRNAs were targeted. Regions replaced with red hyphens indicate where a deletion indel has occurred. The AA residue highlighted in green indicates where a SNP has occurred. AA sequence alignment using the URI1 protein translated from the reference Uri1 gene sequence and the *Uri1* alleles from clone 2A3 (C). AA residues highlighted in red correspond to residues which differ from the reference AA sequence. The 14 bp deletion which occurred in 2A3 Allele 1 would result in a frameshift mutation and a PTC. This would ultimately lead to the resultant mRNA being degraded by NMD and no protein being produced and/or the production of a truncated URI1 protein.

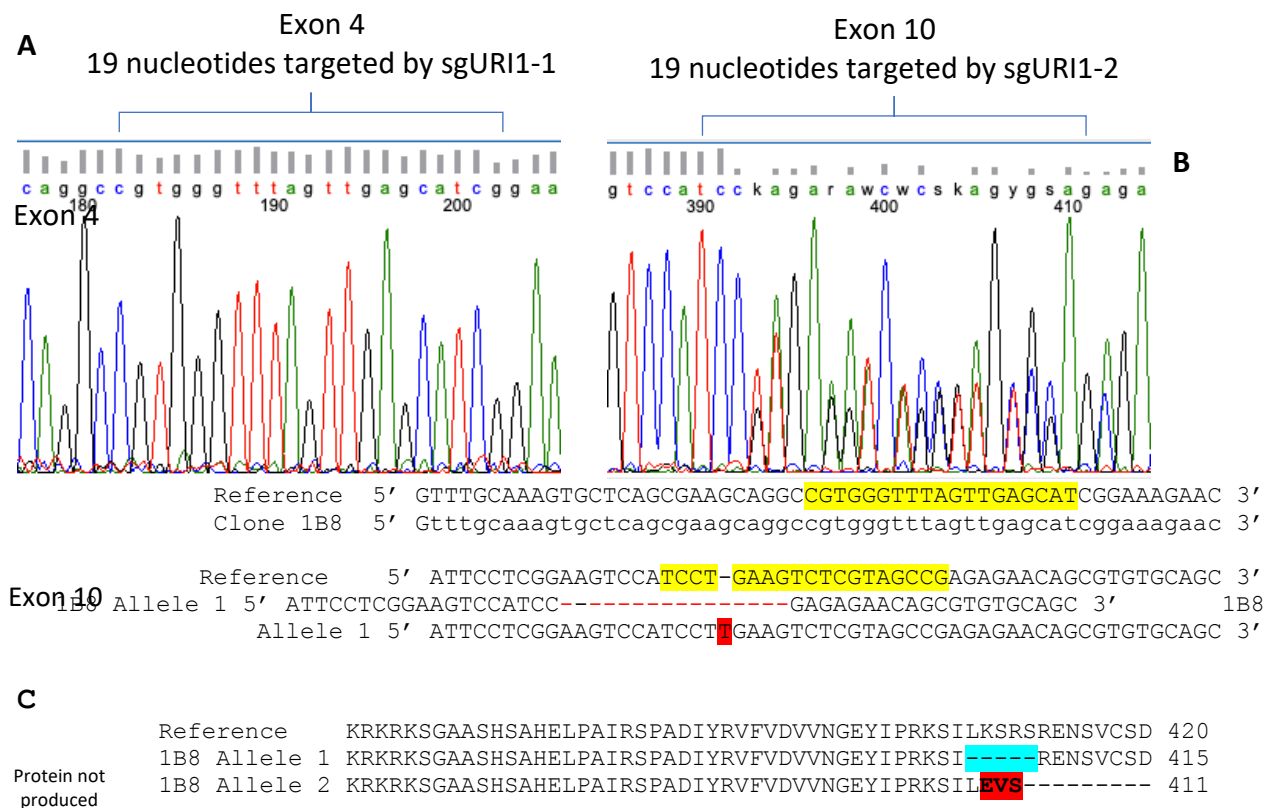


Figure 27: Chromatograms of sequencing results from clone 1B8 (A). DNA alignment between a portion of the reference DNA and clone 1B8 DNA at the regions where CRISPR/Cas9 was targeted (B). Regions highlighted in yellow correspond to the regions where the sgRNA was targeted. Regions highlighted in red refer to addition mutations. Regions replaced with red hyphens identify a single base insertion. AA sequence alignment from a portion of the URI1 protein translated from the reference Uri1 gene sequence and from the *Uri1* alleles from clone 1B8 (C). AA residues highlighted in red blue correspond to residues which differ from the reference AA sequence. Hyphens highlighted in blue indicate where an in-frame deletion mutation occurred. The single base insertion which occurred in clone 2A3 allele 2 would result in a frameshift mutation and a PTC. This would ultimately lead to the resultant mRNA being degraded by NMD and no protein being produced and/or the production of a truncated URI1 protein.

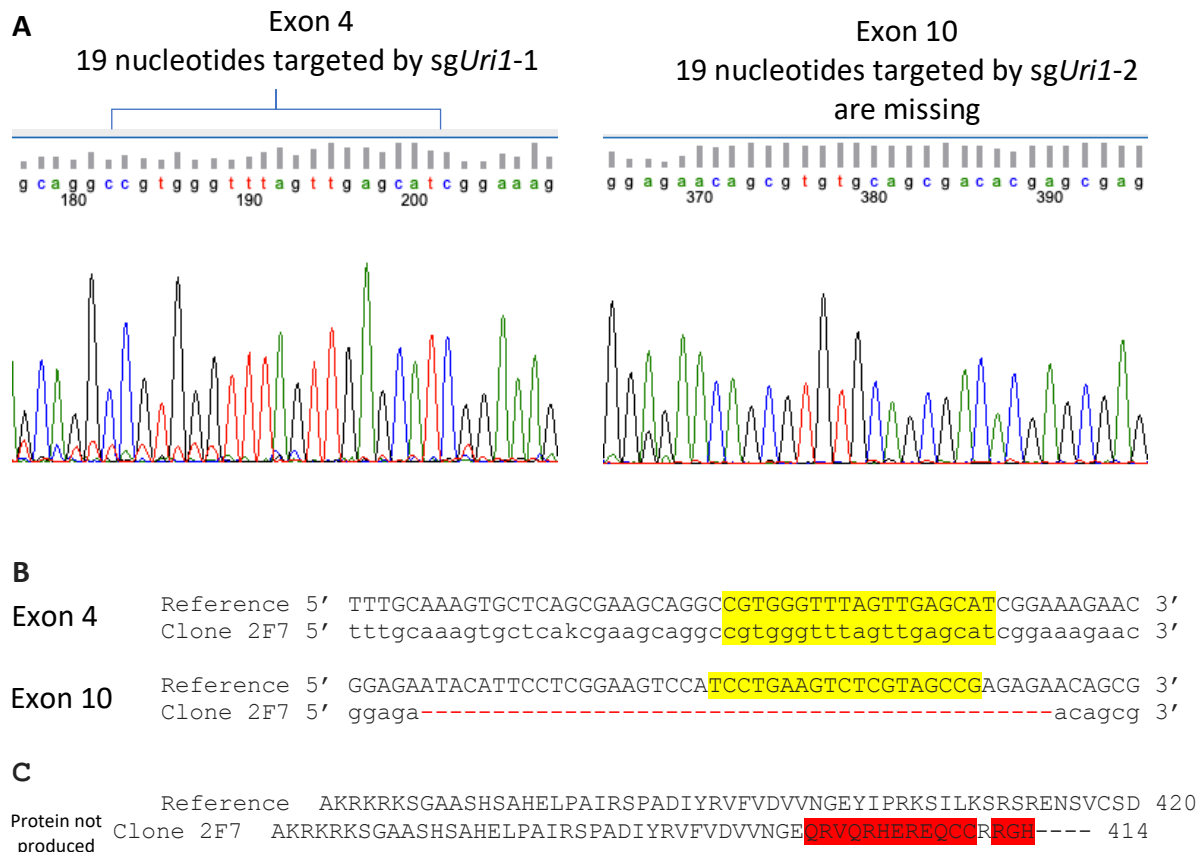


Figure 28: Chromatograms of sequencing results from clone 2F7 (A). DNA alignment between a portion of the reference DNA and clone 1B8 DNA at the regions where CRISPR/Cas9 was targeted (B). Regions highlighted in yellow correspond to the regions where the sgRNAs were targeted. Regions replaced with red hyphens indicate where a 44 bp deletion occurred. DNA alignment shows the same deletion mutation occurring in both alleles. AA sequence alignment from a portion of the URI1 protein translated from the reference *Uri1* gene sequence and from the *Uri1* alleles from clone 1B8 (C). AA residues highlighted in red correspond to residues which differ from the reference AA sequence. The homozygous 44 bp deletion mutation would result in a frameshift mutation and a PTC in both alleles. This would ultimately lead to the resultant mRNA being degraded by NMD and no protein being produced and/or the production of a truncated URI1 protein.

Table 13: Summary of URI1 Western blot and Sequencing Results from 14 H4IIE^{BFP+/GFP+} Clones.¹

Clone	URI1 Protein Level Relative to Control (~35 kDa Band)	URI1 Protein Level Relative to Control (~79 kDa Band)	Exon 4 Allele 1	Exon 4 Allele 2	Exon 10 Allele 1	Exon 10 Allele 2	Status
1F1	0.33	NA	No edit	No edit	No edit	No edit	WT
2A3	0.01	0.423	14 bp deletion	1 SNP	No edit	No edit	Compound <i>Uri1</i> ^{+/-}
1B8	0.52	0.50	No edit	No edit	15 bp deletion	1 bp insertion	Compound <i>Uri1</i> ^{+/-}
2F7	0.08	0.01	No edit	No edit	44 bp deletion [‡]	44 bp deletion [‡]	<i>Uri1</i> ^{-/-}
2B1	0.07	NA	No edit	No edit	Unknown	Unknown	Unknown
2E11	0.36	NA	No edit	No edit	99 bp deletion	40 bp deletion	Compound <i>Uri1</i> ^{+/-}
1F3	0.07	NA	No edit	No edit	12 bp deletion [‡]	12 bp deletion [‡]	Mutant
2A11	0.02	NA	No edit	No edit	12 bp deletion	1 bp insertion	Compound <i>Uri1</i> ^{+/-}
2B4	0.00	NA	No edit	No edit	24 bp deletion [‡]	24 bp deletion [‡]	Mutant
2A2	0.26	NA	No edit	No edit	44 bp deletion	27 bp deletion	Compound <i>Uri1</i> ^{+/-}
1A8	0.12	NA	No edit	No edit	3 bp deletion	1 bp insertion	Compound <i>Uri1</i> ^{+/-}
1D9	0.56	NA	No edit	No edit	182 bp deletion [‡]	182 bp deletion [‡]	Mutant
1A10	0.01	NA	3 bp insertion	1 SNP	Unknown	unknown	Unknown
1B11	0.08	NA	No edit	No edit	12 bp deletion	1 bp insertion	Compound <i>Uri1</i> ^{+/-}

¹NA means investigation into whether the particular band is present in Western blots was not undertaken. The ‡ indicates identical mutations which occurred in both alleles. Clones with the 'unknown' designation are clones whose sequencing results could not be resolved by CRISP-ID. *Uri1*^{+/-} and *Uri1*^{-/-} designations represents heterozygous and homozygous *Uri1* KO clones, respectively.

Clones 2A3, 1B8, and 2F7 were selected for the following experiments, which examined cell growth characteristics and lipid droplet formation, because this set of clones was diverse in how the *Uri1* gene was ablated. The heterozygous *Uri1* deletion in Clone 2A3 was caused by a DSB in exon 4, while in clone 1B8, the heterozygous *Uri1* deletion had occurred through a DSB in exon 10. Clone 2F7 was the only clone which was identified to have a homozygous *Uri1* deletion.

Hereafter, clone 1B8 will be referred to as *Uri1*^{+/-} 1, clone 2A3 will be referred to as *Uri1*^{+/-} 2, and clone 2F7 will be referred to as *Uri1*^{-/-}. No other clones were included in the next experiments, although they should be examined in the future to determine if they exhibit unique characteristics, or share similarities with clones having comparable alterations. Multiple *Uri1* KO H4IIE clones were included in the following experiments to relate the type of alteration with URI1 function. If more than one clone had similar characteristics which were unique from WT H4IIE cells, it would show that these clones exhibit these characteristics because of *Uri1* genetic ablation and not because of clone-to-clone heterogeneity.

It is evident that the ~35 kDa URI1 band is an unreliable predictor of the type of CRISPR/Cas9-induced mutation. Several of these clones had zero expression of this URI1 band but sequencing results in many of them showed no introduction of PTCs into the *Uri1* gene (i.e., clones 1F3 and 2B4, as shown in Table 13). However, most of the clones with reduced levels of the ~35 kDa URI1 band had some sort of *Uri1* gene edit. Thus, reduced expression of the ~35 kDa URI1 band may help indicate which clones had *Uri1* gene edits but not the type of mutation which had occurred. However, it is also possible that CRISPR/Cas9 gene editing in H4IIE cells

was extremely prevalent since 13 of 14 H4IIE^{BFP+/GFP+} clones sequenced contained a *Uri1* gene edit.

Using the ~79 kDa URI1 band to predict the nature of the CRISPR/Cas9-induced mutation was much better. However, this URI1 band is only detectable when H4IIE cells are exposed to specific conditions (24 h OA treatment of quiescent cells) and only 3 of these clones were screened this way.

Interestingly, only 2 of the putative KO clones exhibited alterations within exon 4 of the *Uri1* gene. The only clones with a mutation at this location were *Uri1*^{+/-} 2 and clone 1A10. Only *Uri1*^{+/-} 2 experienced a DSB in exon 4 which led to a PTC or truncated URI1 protein.

Of the 14 clones sequenced, the *Uri1*^{-/-} clone was the only one identified to harbour a homozygous *Uri1* KO. Since this type of mutation was so rare among H4IIE^{BFP+/GFP+} cells, despite high prevalence of CRISPR/Cas9 gene edits among H4IIE^{BFP+/GFP+} clones, it may indicate the importance of this protein for H4IIE cell viability.

Lastly, several clones experienced in-frame deletions. Due to the low likelihood of multiple in-frame indels occurring, it may indicate that at least some *Uri1* expression is important for survival.

In conclusion, one homozygous and several compound heterozygous *Uri1* KO H4IIE cell lines were successfully generated and validated using the CRISPR/Cas9 gene editing system. *Uri1* KO and WT H4IIE cells were then used in the following experiments. Identifying differences in characteristics between WT and *Uri1* KO H4IIE cells helps to elucidate the role *Uri1* normally fulfills in healthy cells. Results from these experiments can also enable us to postulate the

consequences of high expression of *Uri1* which may be brought on by certain pathological conditions.

Results Section E: Experimentation with *Uri1* KO H4IIE Cells

Real Time Cell Analysis

The generation of CRISPR/Cas9 *Uri1* KO H4IIE cells is an intensive process. It is important to assess whether this process had effects on H4IIE cell characteristics that are not related to *Uri1* gene editing. Real time cell analysis (RTCA) was used to assess the growth characteristics of *Uri1* KO H4IIE clones and compare them to WT H4IIE cells. This experiment provides insight into whether the process of introducing a CRISPR/Cas9-induced genetic deletion of *Uri1* in H4IIE cells had discernable effects on the characteristics of H4IIE cells.

RTCA measures the impedance of the conductance of media caused by proliferating adherent cells and is a measure of cell growth. Conductance impedance is reported as the unitless parameter, CI, where $CI = (\text{impedance at time point } n - \text{impedance in the absence of cells}) / \text{nominal impedance value}$. CI is directly proportional to cell confluence.

One thousand cells of each H4IIE clone (WT, *Uri1*^{+/-} 1, *Uri1*^{+/-} 2, and *Uri1*^{-/-}) were seeded in the E-plate 16 in quadruplicate. After docking the E-plate onto the RTCA DP Station, the RTCA control unit was programmed to perform 1000 CI measurements every 15 minutes (Figure 29).

The maximal CI (when cells have become confluent) reached for WT, *Uri1*^{+/-} 1, *Uri1*^{+/-} 2, and *Uri1*^{-/-} H4IIE cells was 1.16±0.10, 1.13±0.05, 1.28±0.03, and 1.06±0.04, respectfully. There were no statistically significant differences among these values. *Uri1*^{+/-} 2 cells reached confluency quicker compared to other cell types. WT, *Uri1*^{+/-} 1, *Uri1*^{+/-} 2, and *Uri1*^{-/-} H4IIE cells reached confluency after 99.6±5.1, 98.0±3.4, 77.8±2.5, and 97.4±3.1 hours, respectively. One-

way ANOVA showed that the time to reach confluency in *Uri1*^{+/-} 2 H4IIE cells was significantly less compared to all other cell types. These results are summarized in Table 14.

CI values were analyzed in 10-hour increments by using one-way ANOVA. From 30 hours to 80 hours, *Uri1*^{+/-} 2 H4IIE cells had significantly increased CI values compared to all other cell types at the same timepoints (Figure 29).

Analyzing the rate of change (ROC) of cell growth when cells were 50% confluent was used to assess the speed at which these cells grow. To identify the ROC of CI when cells were 50% confluent, curve fitting was used to construct a curve from a mathematical formula that best fits the data points of each respective cell type. Tenth order polynomial formulas were used as curve fitting formulas. Only data points between 0 and 125 hours were used because after 125 hours, CI values become erratic and difficult to model. Also, after 125 hours 100% confluency had been reached and cells had started to detach. The formulas must have R² values that are ≥0.999. The mean R² values for the curve fitting formulas of WT, *Uri1*^{+/-} 1, *Uri1*^{+/-} 2, and *Uri1*^{-/-} H4IIE cell RTCAs were 0.9998±4.79×10⁻⁵, 0.9996±9.46×10⁻⁵, 0.9992±2.25×10⁻⁴, and 0.9997±9.13×10⁻⁵, respectively. The timepoints at which cells were 50% confluent were identified. The derivatives of these formulas were used to find the ROC when cells were 50% confluent. The ROCs at 50% confluency for WT, *Uri1*^{+/-} 1, *Uri1*^{+/-} 2, and *Uri1*^{-/-} H4IIE cells were 0.0186±1.75×10⁻³, 0.0194±1.8×10⁻³, 0.0247±1.3×10⁻³, and 0.0153±1.08×10⁻³ CI/hour, respectively. One-way ANOVA showed that the ROC at 50% confluency of *Uri1*^{+/-} 2 H4IIE cells was faster compared to all other cell types. These results are summarized in Table 14.

Due to the observed differences in growth parameters, the growth of each cell line was visually monitored. Five thousand WT, *Uri1*^{+/-} 1, *Uri1*^{+/-} 2, and *Uri1*^{-/-} H4IIE cells were each seeded into separate wells of a 96 well plate in 100 µL of complete media. Photos were taken every day for 4 days for each well.

There were no apparent differences between WT, *Uri1*^{+/-} 1, and *Uri1*^{-/-} H4IIE cells (Figures 31 and 32). In contrast, *Uri1*^{+/-} 2 H4IIE cells appeared to grow faster compared to the other cell lines. Furthermore, *Uri1*^{+/-} 2 H4IIE cells appeared to grow very close together. This may indicate that these cells have lost contact inhibition of proliferation and possess characteristics similar to cancerous cells (239). Since this was only observed in one *Uri1* KO clone, it is difficult to ascertain whether these changes in their growth characteristics were a result of *Uri1* gene edits or due to clone-to-clone heterogeneity in H4IIE^{BFP+/GFP+} cells.

In summary, *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE clones exhibited similar growth characteristics compared to WT H4IIE cells. *Uri1*^{+/-} 2 H4IIE cells grow faster compared to all other cell types. *Uri1*^{+/-} 2 H4IIE cells had a DSB within exon 4 of *Uri1*, while the other *Uri1* KO H4IIE cells had a DSB within exon 10 of *Uri1*. Interestingly, DSBs within exon 4 of *Uri1* were very rare, compared to DSBs within exon 10 of *Uri1* among H4IIE^{BFP+/GFP+} cells. Therefore, the difference in characteristics between *Uri1*^{+/-} 1 and 2 clones may be due to CRISPR/Cas9 edits in exon 4 resulting in reduced cell viability, as evidenced by its scarcity, and, in the event that this cell type survives, these cells acquire a rare phenotype. Additionally, the difference in characteristics may also been due to CRISPR/Cas9 off-target effects.

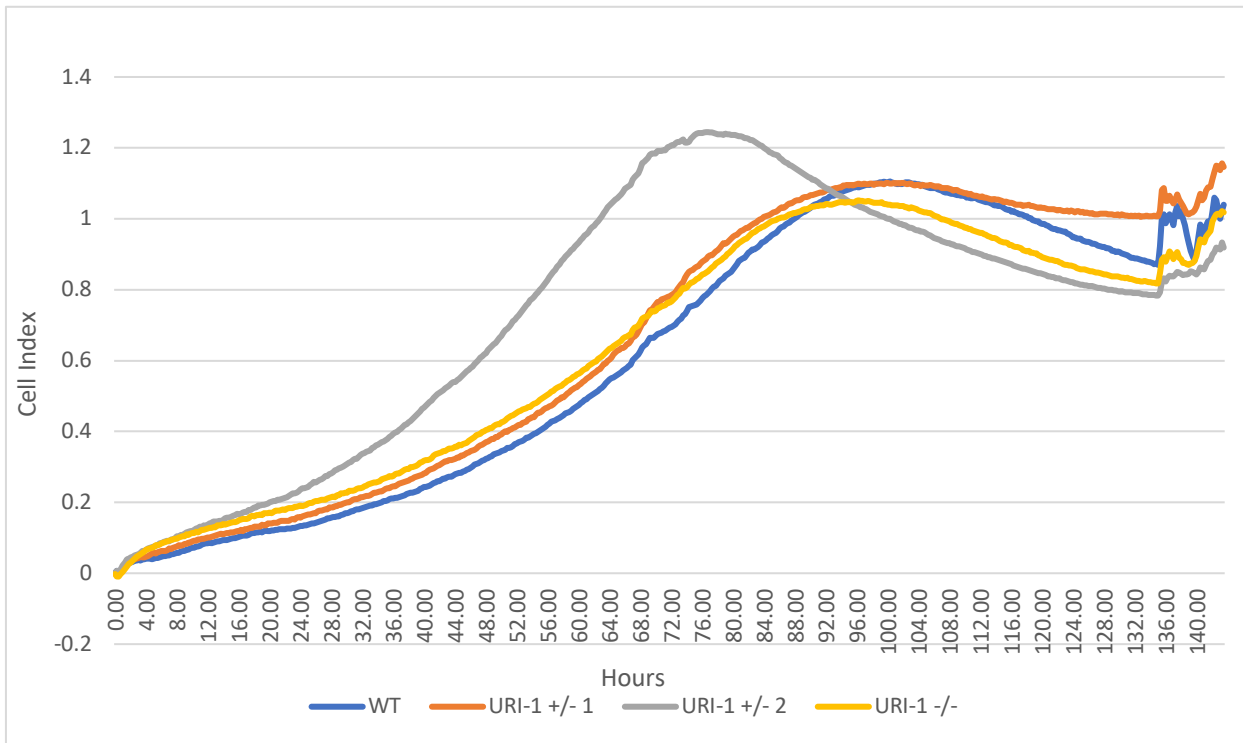


Figure 29: RTCA proliferation curves for WT, *Uri1*^{+/-} 1, *Uri1*^{+/-} 2, and *Uri1*^{-/-} H4IIE cells. Values are presented as means where N=4.

A

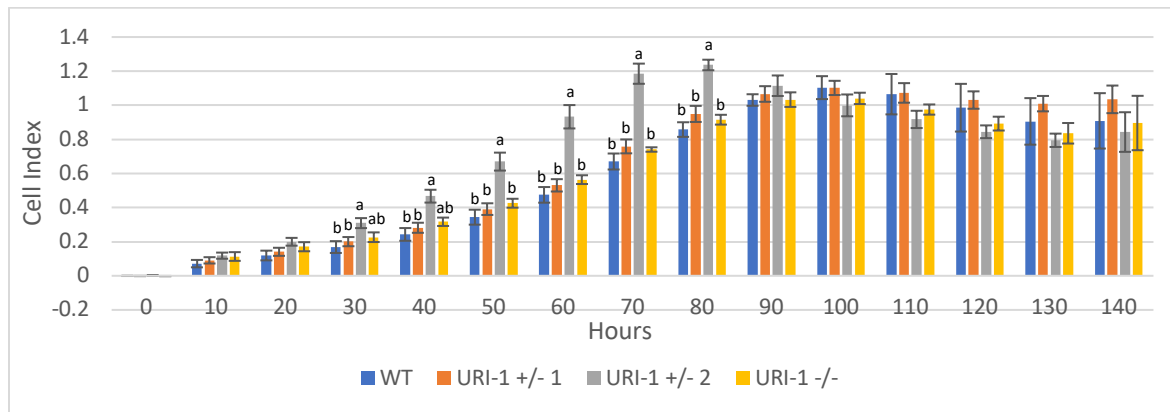


Figure 30: CI values of WT, *Uri1*^{+/- 1}, *Uri1*^{+/- 2}, and *Uri1*^{-/-} H4IIE cells in 10-hour increments between 0 and 140 hours of seeding (A). Values are presented as means $\pm$ SEM where N=4. Data with a 10-hour interval were analyzed using one-way ANOVA and Duncan's multiple range test. Different letters within a 10-hour interval indicate significant differences (P<0.05). An absence of letters indicates no statistical differences.

B

Table 14: Growth Characteristic Parameters in WT, *Uri1*^{+/- 1}, *Uri1*^{+/- 2}, and *Uri1*^{-/-} H4IIE cells Assessed by RTCA.¹

	Wild Type	<i>Uri1</i> ^{+/- 1}	<i>Uri1</i> ^{+/- 2}	<i>Uri1</i> ^{-/-}
Max CI	1.16 $\pm$ 0.097	1.13 $\pm$ 0.053	1.284 $\pm$ 0.033	1.062 $\pm$ 0.038
Hours to reach confluency	99.56 $\pm$ 5.11 ^b	98 $\pm$ 3.43 ^b	77.81 $\pm$ 2.5 ^a	97.37 $\pm$ 3.05 ^b
Curve Fitting Formula R² value	0.9998 $\pm$ 4.79 $\times$ 10 ⁻⁵	0.9996 $\pm$ 9.46 $\times$ 10 ⁻⁵	0.9992 $\pm$ 2.25 $\times$ 10 ⁻⁴	0.9997 $\pm$ 9.13 $\times$ 10 ⁻⁵
ROC at ½ Max CI (CI/hour)	0.0186 $\pm$ 1.75 $\times$ 10 ⁻³ ^b	0.0194 $\pm$ 1.8 $\times$ 10 ⁻³ ^b	0.0247 $\pm$ 1.3 $\times$ 10 ⁻³ ^a	0.0153 $\pm$ 1.08 $\times$ 10 ⁻³ ^b

¹CI is the unitless parameter used to report the impedance of the conductance caused by proliferating adherent cells. CI is directly proportional to cell confluence. ROC (rate of change) of CI is the speed CI changes overtime. Values are presented as means $\pm$ SEM where N=4. Data were analyzed using one-way ANOVA and Duncan's multiple range test. Different letters indicate differences among the means (P<0.05).

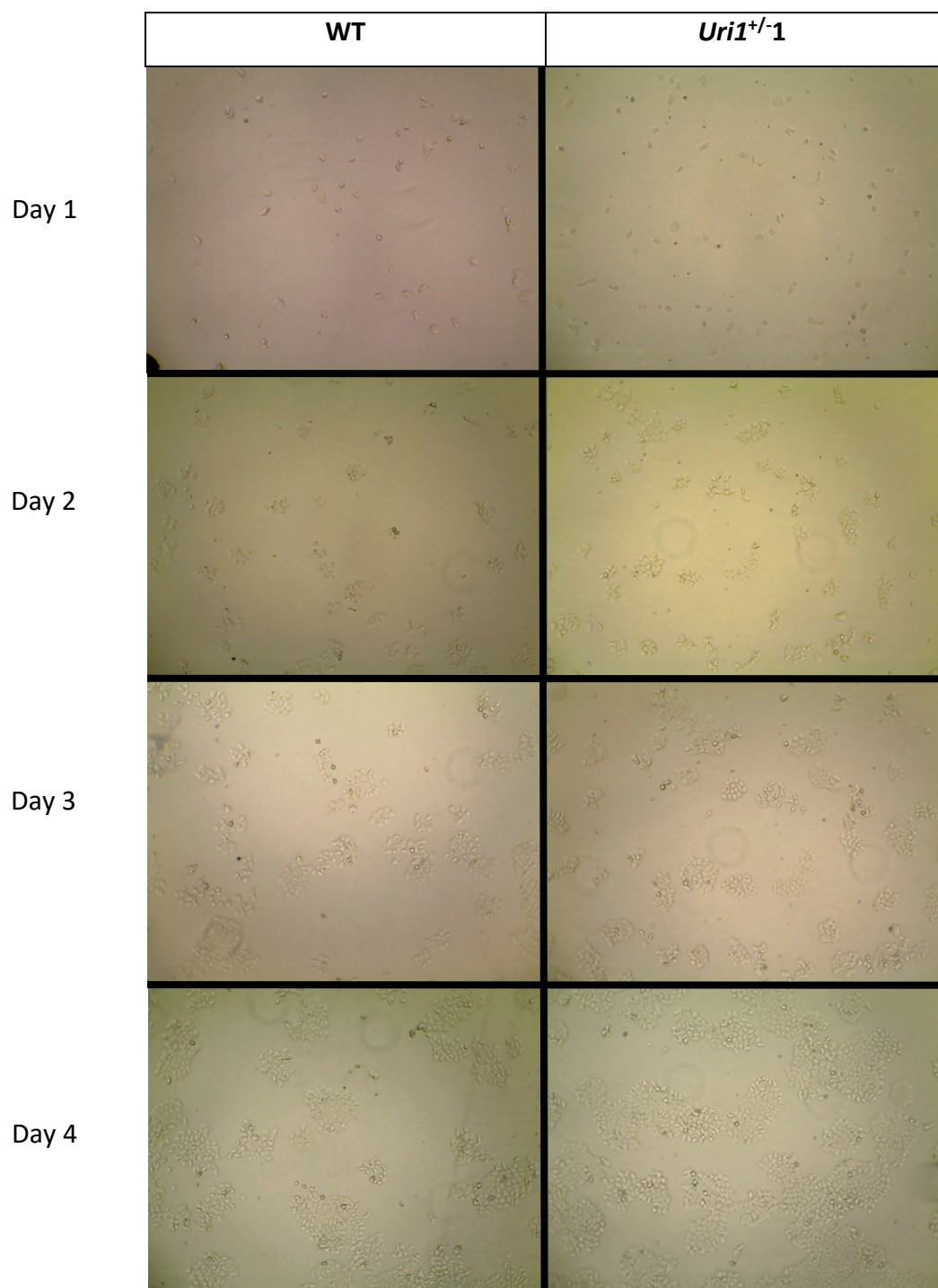


Figure 31: Visualization of WT and *Uri1*^{+/-} 1 H4IIE cell growth over 4 days.

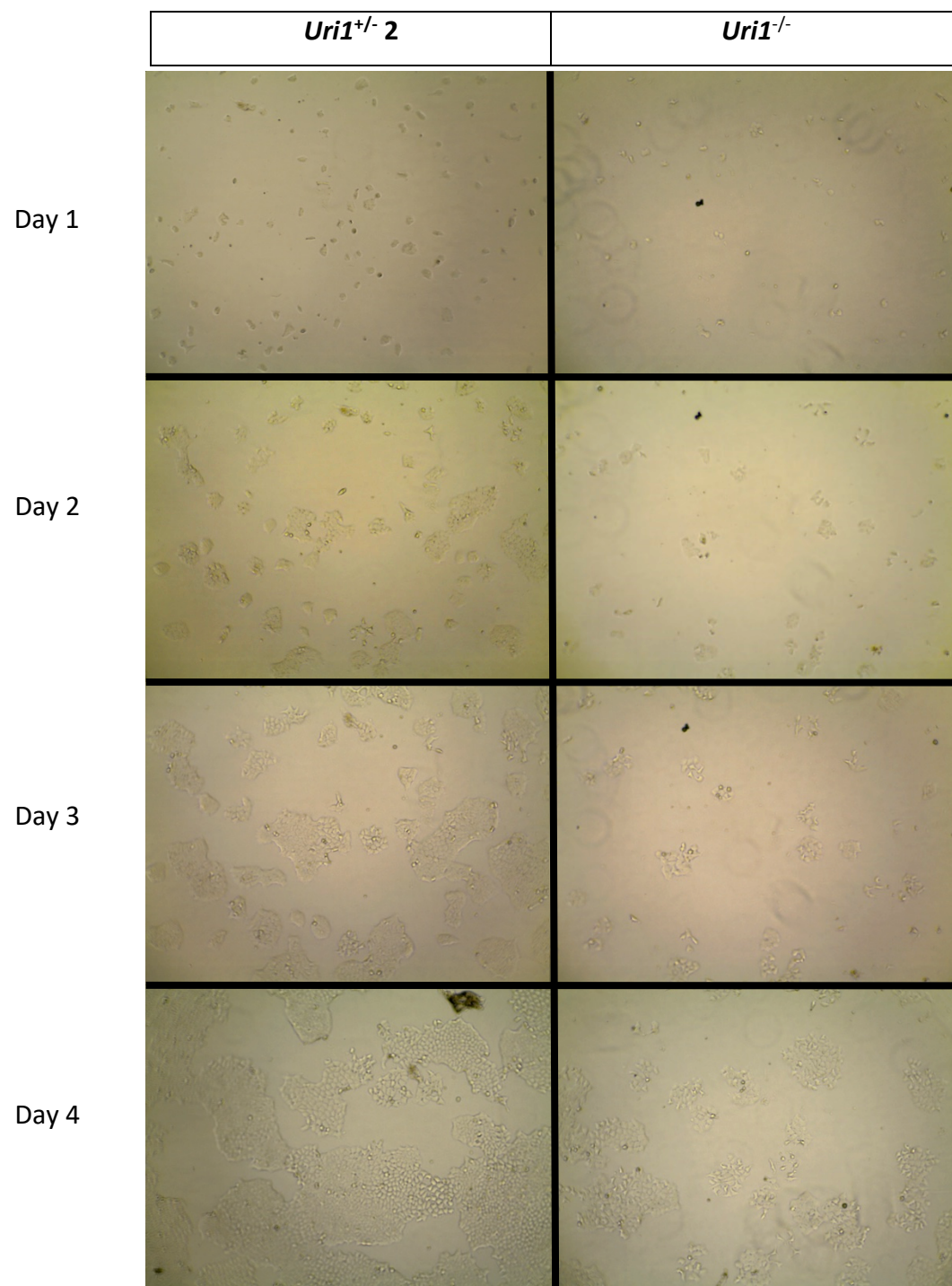


Figure 32: Visualization of *Uri1*^{+/-} 2 and *Uri1*^{-/-} H4IIE cell growth over 4 days.

Neutral Lipid Accumulation in *Uri1* KO H4IIE Cells

After establishing and characterizing the *Uri1* KO H4IIE clones, BODIPY staining was used to assess neutral lipid accumulation in response to OA treatment. WT H4IIE cells and the three selected *Uri1* KO H4IIE clones were seeded in 6 well plates with coverslips, allowed to become quiescent, and treated with OA or vehicle for 8-hours. After treatment, cells were fixed, stained with BODIPY and Hoechst 33342, and then imaged using an epifluorescence microscope. The generated images were then analyzed with ImageJ software. LD/nucleus in each image was determined to evaluate the extent neutral lipid accumulation in response to treatments. Results were then analyzed by two-way ANOVA with genotype and treatment as main effects.

BODIPY staining revealed *Uri1*^{+/-} 1 H4IIE cells accumulate less neutral lipid in comparison to WT H4IIE cells (Figure 33A and 33B). Two-way ANOVA showed that there was a statistically significant main effect of genotype, where the *Uri1*^{+/-} 1 genotype resulted in reduced neutral lipid accumulation compared to WT (genotype means: 0.59±0.18 vs 1.46±0.27 LD/nucleus, respectively). There was a trend (p=0.06) for the genotype × treatment interaction such that OA treatment induced LDs in WT, but not *Uri1*^{+/-} 1, H4IIE cells.

An opposite result was observed in *Uri1*^{+/-} 2 H4IIE cells. BODIPY staining showed that *Uri1*^{+/-} 2 H4IIE cells accumulated more neutral lipid compared to WT cells (Figure 34A and 34B). Non-parametric statistical analysis showed that *Uri1*^{+/-} 2 H4IIE cells treated with OA contained more LD/nucleus compared to WT cells treated with OA (2.00±0.22 vs 0.74±0.53 LD/nucleus, respectively).

Uri1^{-/-} H4IIE cells showed similar results to *Uri1*^{+/-} 1 H4IIE cells. There was a statistically significant main effect for both genotype and treatment for LD/nucleus (Figure 35A and 35B). *Uri1*^{-/-} H4IIE cells had fewer LD/nucleus compared to WT cells (genotype means: 0.70±0.12 vs 1.61±0.34 LD/nucleus, respectively), and OA treated cells had more LD/nucleus compared to vehicle treated cells (treatment means: 1.47±0.28 vs 0.65±0.16 LD/nucleus, respectively).

There is notable variation in the LD/nucleus observed in the WT vehicle and OA treated conditions observed in the *Uri1*^{+/-} 2 BODIPY staining experiment compared to the *Uri1*^{+/-} 1 and *Uri1*^{-/-} BODIPY staining experiments. The LD/nucleus in the WT vehicle and OA treated conditions in the *Uri1*^{+/-} 2 BODIPY staining experiment were 0.1 and 0.74, respectively while in *Uri1*^{+/-} 1 BODIPY staining experiment the LD/nucleus in the WT vehicle and OA treated conditions were 0.92 and 2.01, respectively. The LD/nucleus in the WT vehicle and OA treated conditions in *Uri1*^{+/-} 1 and *Uri1*^{-/-} BODIPY staining experiments were comparable. The reason for variation is that exposure times used for the different experiments were different. The exposure time for the *Uri1*^{+/-} 2 BODIPY staining experiments was lower since BODIPY fluorescence in *Uri1*^{+/-} 2 H4IIE cells was strong enough such that background BODIPY fluorescence would have been included in LD/nucleus analysis if comparable exposure times were used. To maintain consistency within the experiment, the same exposure time was used for WT cells which do not exhibit as strong BODIPY fluorescence. The exposure time in WT vehicle and OA treated conditions in the *Uri1*^{+/-} 2 BODIPY staining experiment was lower compared to *Uri1*^{+/-} 1 and *Uri1*^{-/-} BODIPY staining experiments which accounts for the variability observed in the LD/nucleus measurements.

In summary, *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells exhibited a reduction in neutral lipid accumulation compared to WT controls. However, *Uri1*^{+/-} 2 H4IIE cells had greater neutral lipid accumulation. Using *Uri1*^{+/-} 2 H4IIE cells may not be the best model for investigating neutral lipid accumulation in *Uri1* KO H4IIE cells in the context of hepatic steatosis since *Uri1*^{+/-} 2 H4IIE possess aberrant characteristics and do not resemble WT H4IIE cells. *Uri1*^{+/-} 2 H4IIE cells exhibit more characteristics of cancerous cells compared to WT H4IIE cells and it has been reported that HCC cells have increased lipid content and upregulated expression of lipogenic genes compared to non-HCC cells (240). This may be why *Uri1*^{+/-} 2 H4IIE cells experience increased neutral lipid accumulation compared to WT and other *Uri1* KO H4IIE clones. However, they may be a useful model for examining the altered metabolic characteristics of HCC cells.

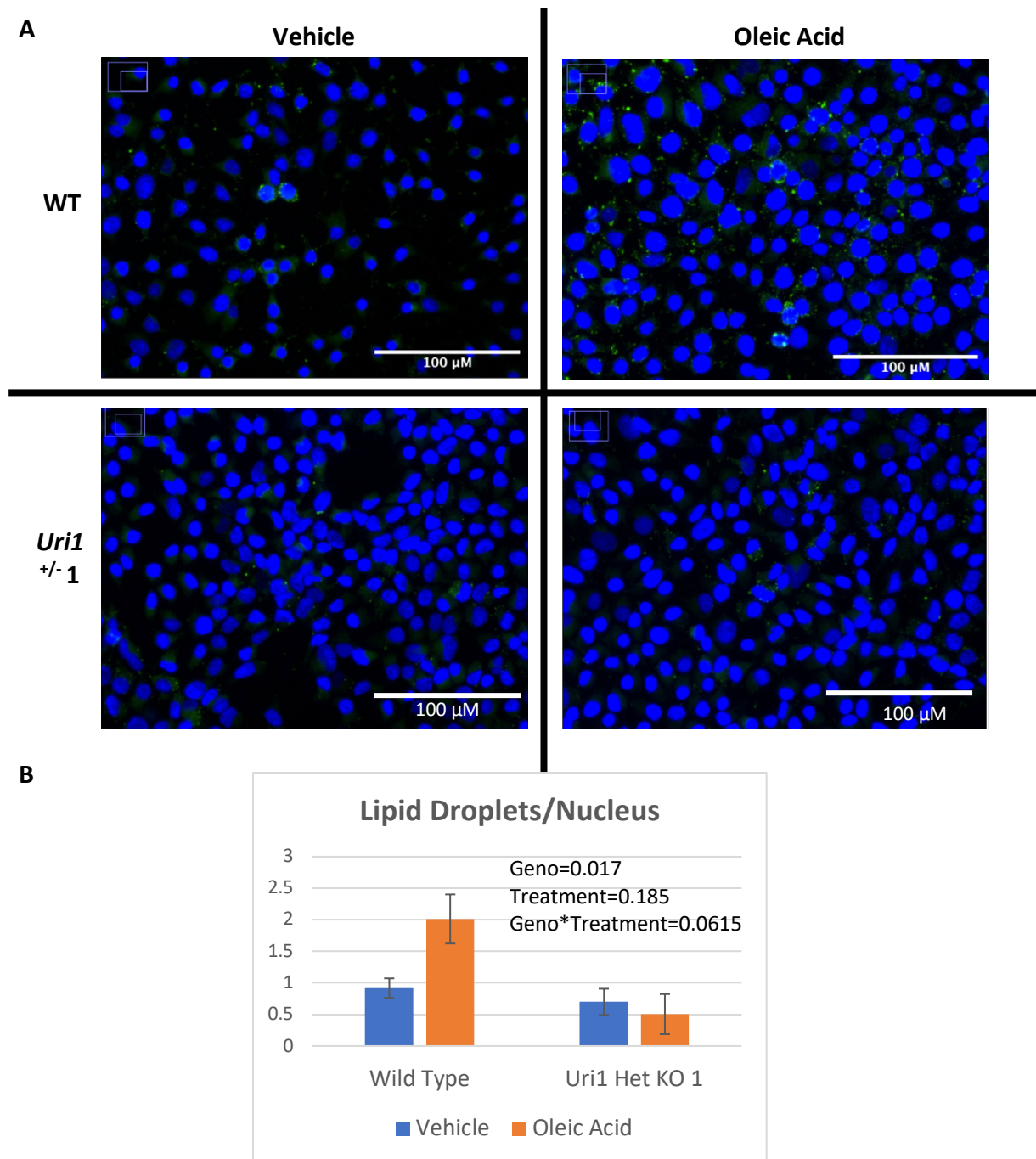


Figure 33: BODIPY staining in OA treated WT and *Uri1*^{+/-} 1 H4IIE cells. Representative images of WT and *Uri1*^{+/-} 1 H4IIE cells treated with vehicle or OA for 8 hours (A). Bars in the bottom right corners of the images are 100 μm. Number of lipid droplets per nucleus (B). Values are presented as means ± SEM where N=4-5. Data were analyzed by two-way ANOVA and p values for the main and interaction effects are indicated in the upper right corner of the figure.

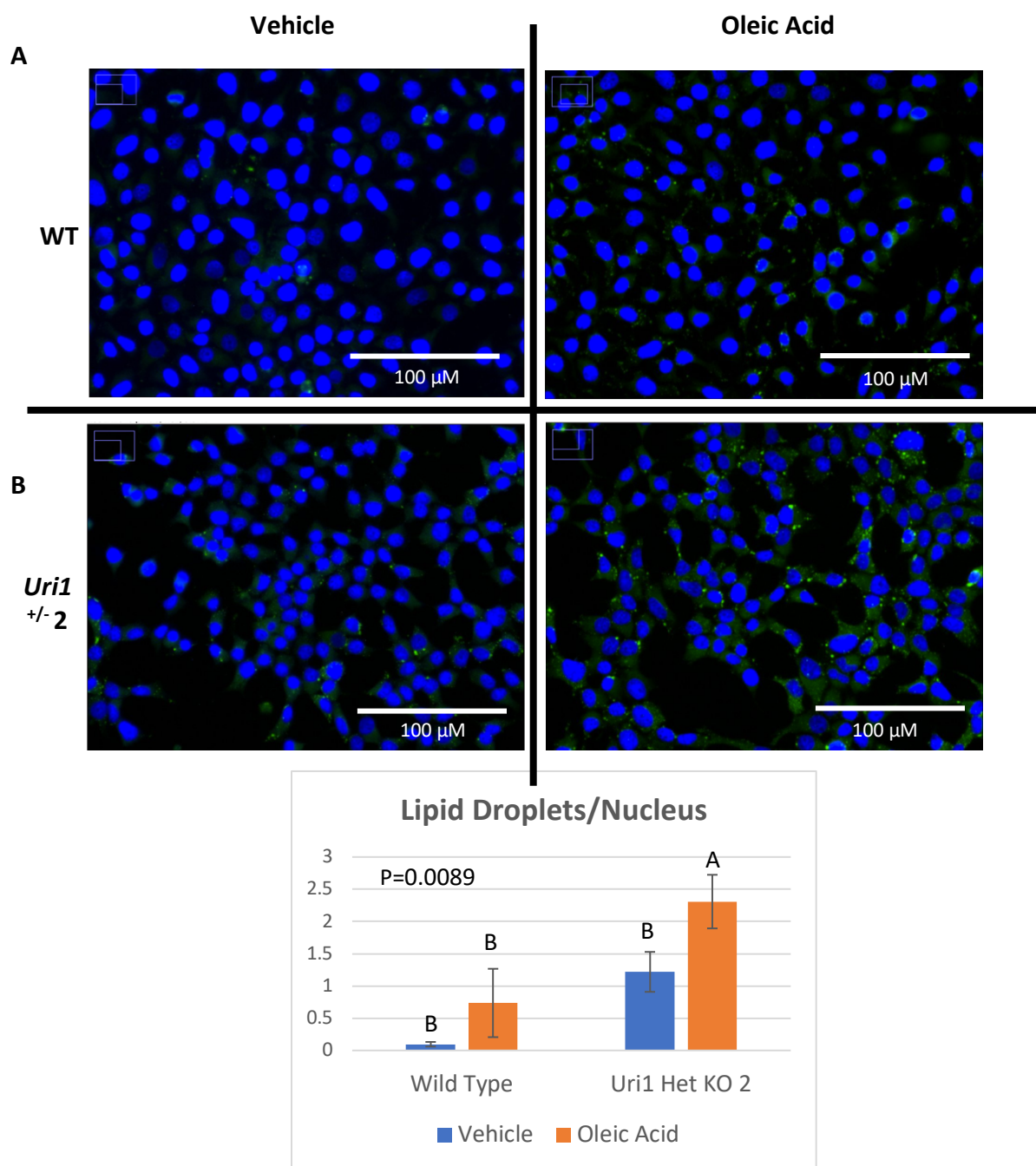


Figure 34: BODIPY staining in OA treated WT and *Uri1*^{+/- 2} H4IIE cells. Representative images of WT and *Uri1*^{+/- 2} H4IIE cells treated with vehicle or OA for 8 hours (A). Bars in the bottom right corners of the images are 100 μM. Number of lipid droplets per nucleus (B). Values are presented as means ± SEM, N=4-5. Panel B was analyzed by non-parametric statistics and different letters indicate significant differences (P<0.05) among the means. The p value for the Kruskal-Wallis test is in the upper left corner of the figure.

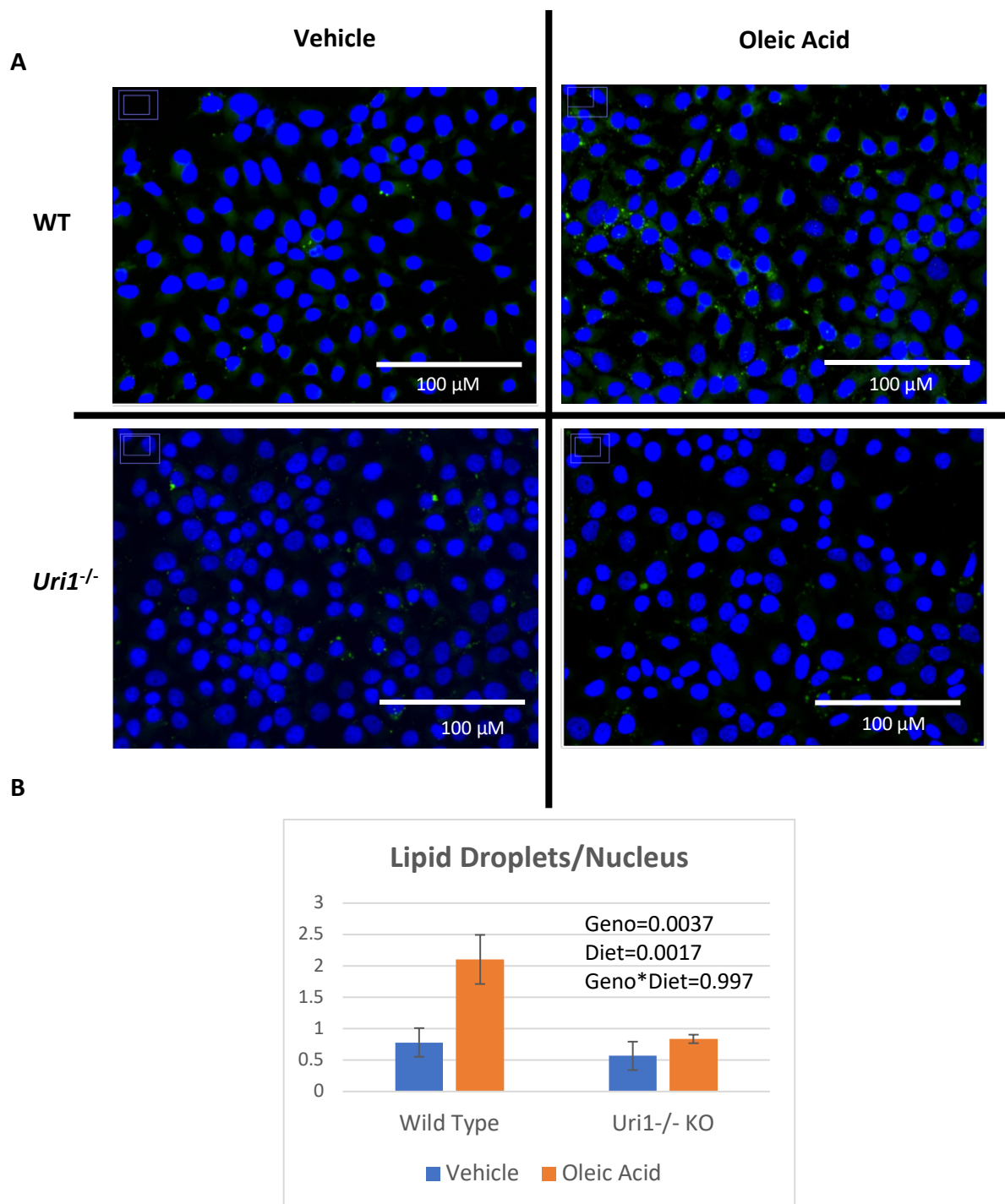


Figure 35: BODIPY staining in OA treated WT and *Uri1*^{-/-} H4IIE cells. Representative images of WT and *Uri1*^{-/-} H4IIE cells treated with vehicle or OA for 8 hours (**A**). Bars in the bottom right corners of the images are 100 μ m. Number of lipid droplets per nucleus (**B**). Values are presented as mean $\pm$ SEM, N=3-5. Data were analyzed by two-way ANOVA and p values for the main and interaction effects are indicated in the upper right corner of the figure.

Discussion

Summary of Findings

The research in this thesis, through the use of both *in vitro* and *in vivo* models, provides evidence that t10,c12 CLA aggravates NAFLD and identifies various cellular processes that are relevant to t10,c12 CLA-induced hepatic steatosis. The same approach has also helped to establish URI1 as a protein that contributes to the development of hepatic steatosis. This finding is strongly supported by experiments with *Uri1* KO H4IIE cells.

Specifically, this study shows that a diet with t10,c12 CLA (0.4% w/w) causes significant hepatic lipid accumulation in lean mice and aggravates hepatic steatosis in *db/db* mice. Leptin signaling abnormalities in *db/db* mice lead to uncontrolled appetite and hyperphagia, and result in obesity-induced hepatic steatosis (195). Cellular processes associated with t10,c12 CLA-induced hepatic lipid accumulation were upregulation of hepatic *de novo* lipogenesis, FA uptake, mTOR activation, and AMPK phosphorylation. This study benefitted from the combined use of lean C57BL/6 WT and genetically obese *db/db* mice that allowed for identification of the mechanisms by which t10,c12 CLA promotes hepatic steatosis that are distinct from obesity. Additional *in vitro* experiments using H4IIE rat hepatoma cells and t10,c12 CLA have revealed that FABP1 and mTOR activation may mediate t10,c12 CLA-induced hepatic lipid accumulation.

Importantly, measuring hepatic URI1 protein levels by Western blotting showed that URI1 protein levels were positively correlated with relative liver weight and hepatic TG concentration during t10,c12 CLA- and obesity-induced hepatic steatosis. *In vitro* experiments were then utilized to further validate this concept. H4IIE cells had the highest levels of neutral

lipid accumulation after treatment with 100 μ M OA for 24 hours compared to earlier timepoints. In parallel, URI1 protein levels were also elevated the most 24 hours after treatment with 100 μ M OA.

The initial *in vitro* and *in vivo* experiments revealed a strong correlation between *Uri1* protein expression and hepatic lipid accumulation. To determine whether this relationship is incidental or causal, *Uri1* KO H4IIE cells were generated using the CRISPR/Cas9 gene editing system. *Uri1* KO H4IIE cells were then subjected to OA treatment experiments and neutral lipid accumulation was assessed by BODIPY staining.

CRISPR/Cas9-mediated KO of the *Uri1* gene in H4IIE cells was achieved for the first time, as evidenced by Western blotting and sequencing data. CRISPR/Cas9 was very effective at inducing *Uri1* gene edits since 13 of 14 sequenced H4IIE^{GFP+/BFP+} clones had gene edits at locations in the *Uri1* gene targeted by Cas9. Interestingly, several H4IIE^{GFP+/BFP+} clones experienced in-frame deletions which allow for expression of *Uri1* but with an altered AA sequence. Furthermore, only one of the H4IIE^{GFP+/BFP+} clones was identified as a homozygous *Uri1* KO, despite the high prevalence of CRISPR/Cas9-mediated URI1 gene edits among H4IIE^{GFP+/BFP+} cells. These 2 observations suggest *Uri1* gene expression is important for cell survival.

Two compound heterozygous *Uri1* KO H4IIE clones, *Uri1*^{+/-} 1, and *Uri1*^{+/-} 2, were selected for subsequent experiments. Only one homozygous *Uri1* KO H4IIE clone was identified, *Uri1*^{-/-}, and this clone was included for subsequent experiments. Characterization of *Uri1* KO H4IIE clones was carried out by RTCA, which showed that *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells possess

growth characteristics similar to WT H4IIE cells. However, *Uri1*^{+/-} 2 H4IIE cells showed significantly increased growth rates compared to WT H4IIE cells. Further observations using standard light microscopy showed a potential lack of contact inhibition of proliferation in growing *Uri1*^{+/-} 2 H4IIE cells.

Next, BODIPY staining in OA-treated *Uri1* KO and WT H4IIE cells was undertaken to assess the role of *Uri1* in neutral lipid accumulation. BODIPY staining in *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells showed reduced neutral lipid accumulation in response to vehicle and OA treatments compared to WT controls. In contrast, *Uri1*^{+/-} 2 H4IIE cells showed increased neutral lipid accumulation. It should be noted that these cells exhibit characteristics which were unique compared to WT and other *Uri1* KO H4IIE cells. In summary, the evidence indicates *Uri1* has a role in promoting hepatic lipid accumulation. Conditions which normally cause the upregulation of neutral lipid accumulation for H4IIE cells did not occur in *Uri1* KO H4IIE cells, indicating *Uri1* is required for this process.

In conclusion, t10,c12 CLA promotes hepatic steatosis in C57BL/6 mice by upregulating hepatic *de novo* lipogenesis and FA uptake which may have been achieved by t10,c12 CLA-mediated activation of mTOR. Obesity- and t10,c12 CLA-induced hepatic steatosis also resulted in increased levels of URI1. In parallel, URI1 protein levels and neutral lipid accumulation *in vitro* both peaked after 24 hours of OA treatment. Lastly, genetic ablation of URI1 blunted OA-induced neutral lipid accumulation *in vitro*, suggesting a role for URI1 in the processes leading to hepatic lipid accumulation.

Liver Weight and Triacylglyceride Concentration

NAFLD is the most common chronic liver disease in the world (21). Due to the increasing rate of obesity, the major risk factor of NAFLD, the prevalence of NAFLD is expected to rise as well (25,26). Weight loss has been shown to be an effective method for the treatment of NAFLD (60). A previous dietary intervention study performed by our lab showed that experimental lean and *db/db* mice fed t10,c12 CLA diet experienced severe fat mass loss (54% and 75%, respectively) (173). However, rather than improving obesity-related hepatic steatosis in *db/db* mice, the t10,c12 CLA diet promoted hepatic steatosis in lean mice and caused a significant increase of relative liver weight in *db/db* mice. This result highlights that weight loss does not necessarily lead to improvements in NAFLD.

The manner by which weight loss is achieved is important to consider for disease regression. Weight loss through exercise, diet, and bariatric surgery have been shown to be effective for improving NAFLD in humans (60,241). In contrast, t10,c12 CLA-induced weight loss in mice had an opposite effect. Implementation of any of these strategies achieves weight loss through different mechanisms. Our lab has shown that calorie restriction-induced weight loss is achieved through reductions of lean tissue and adiposity, while t10,c12 CLA-induced weight loss is achieved exclusively through reductions of adiposity in mice (173).

Weight loss achieved through t10,c12 CLA supplementation may occur via browning of white adipose tissue (WAT). There are two types of adipose tissue, WAT, whose main role is storage of metabolic energy, and brown adipose tissue (BAT), whose main role is heat production through the dissipation of chemical energy (in the form of proton leakage across the

electrochemical gradient within the inner mitochondrial membrane using uncoupling protein 1 (UCP1)) and increased metabolic activity. WAT may acquire some characteristics of BAT as an adaption to increased thermogenic demand through a reversible process called browning (242). Our lab and others have shown that t10,c12 CLA may induce fat mass loss by increased browning of WAT (173,243). The notion of t10,c12 CLA-induced fat mass loss via t10,c12 CLA-induced adipose browning is supported by evidence showing increased levels of browning markers in the WAT of t10,c12 CLA-fed mice, including nuclear localization of T-box protein 1 (a transcription factor which promotes WAT browning) and UCP1 expression (173,243). Furthermore, t10,c12 CLA feeding in mice results in increased inflammation of WAT and adipocyte apoptosis (185,186). Lastly, our lab has shown that, *in vitro*, t10,c12 CLA inhibits 3T3-L1 preadipocyte adipogenesis, through t10,c12 CLA-induced elevation of β -catenin levels resulting in inhibition of PPAR γ , a key regulator of adipogenesis (244). Thus, t10,c12 CLA may induce fat mass loss by limiting WAT's capacity for energy storage through altering its characteristics, promoting adipocyte apoptosis, and preventing normal adipocyte development.

As observed in t10,c12 CLA-fed mice, t10,c12 CLA is very potent at inducing fat mass loss. However, fat mass loss through impairment of WAT to facilitate its role in normal health has consequences for the entire body. It has been shown that, in response to inflammation, WAT secretes inflammatory cytokines, including TNF- α and interleukin-6, which contribute to the development of MetS and NAFLD (245). Adipose tissue serves a crucial role in energy storage. In the event that adipose tissue is unable to fulfill this role, ectopic lipid accumulation ensues (ie. the accumulation of lipid in non-adipose tissues) (246). Ectopic lipid accumulation

results in a myriad of detrimental consequences for afflicted tissue including insulin resistance and cell death (12,246). Since hepatocytes serve as focal point of lipid metabolism and have limited ability to safely store lipids, the liver may become the preferential location of ectopic lipid accumulation during t10,c12 CLA-induced fat mass loss (3).

In conclusion, t10,c12 CLA induces fat mass loss in an unhealthy manner. Promoting poor adipose tissue health for fat mass loss has negative consequences for the entire body. Organs do not operate in isolation and negative health outcomes for one organ will translate to negative health outcomes for other organs. Adipose tissue contributes to crucial processes required for maintaining normal health, including energy storage and regulating energy homeostasis (247). Therefore, strategies for weight loss should aim to preserve the health of adipose tissue.

Several studies have previously shown that t10,c12 CLA induces hepatic steatosis in mice (248–251). This is the first study to investigate the effect of t10,c12 CLA-feeding on hepatic steatosis in *db/db* mice. CLA supplements are readily available for consumers and it is claimed they have weight loss benefits. However, the results in this study show that t10,c12 CLA-induced weight loss may be accompanied by hepatic lipid accumulation, due to increased hepatic TG concentration observed in t10,c12 CLA-fed lean C57BL/6 mice. Deleterious outcomes of t10,c12 CLA-induced weight loss are also applicable to populations with MetS, since it was observed that t10,c12 CLA feeding in *db/db* mice resulted in a significant increase of relative liver weight. This is a particularly interesting finding since individuals with MetS traditionally would benefit more from fat mass loss compared to healthy individuals. The

hepatic TG concentration was also higher in t10,c12 CLA fed *db/db* mice compared to CTL lean mice, but it was not statistically different from the *db/db* mice fed the CTL diet. The increase of relative liver weight in *db/db* t10,c12 CLA-fed mice likely indicates increased liver injury (e.g., increased macrophage infiltration) compared to *db/db* mice fed the control diet.

Interestingly, the effects of t10,c12 CLA are species specific. A previous study by our lab has shown that feeding obese fa/fa Zucker rats (an alternative animal model of MetS which exhibits less severe MetS compared to *db/db* mice) a CLA mixture which included t10,c12 CLA (0.4% w/w) reduced liver lipid levels and improved liver function (193,252). Similar findings have been shown by other research groups (253,254). Furthermore, t10,c12 CLA feeding does not have a major effect on body weight in rats (5-10% increase), whereas it causes considerable weight loss (30%) in *db/db* mice (173,252–254).

It may be argued that results observed in mice are not transferrable to humans due to interspecies variability of the response to t10, c12 CLA feeding. However, the human response to t10,c12 CLA feeding most closely resembles the effects in mice. Humans and mice have both shown increased indications of inflammation, insulin resistance, and fat mass loss in response to t10,c12 CLA feeding (178,183,184,186,187). This may indicate that t10,c12 CLA activates similar pathways in both humans and mice to promote hepatic steatosis. Therefore, using mouse models is likely better than rat models for predicting the health outcomes of t10,c12 CLA intake in humans. However, studying t10,c12 CLA intake in rat models is also worthwhile since the processes which t10,c12 CLA intake utilizes to improve NAFLD in rats may be deployed through a different manner in humans.

Nevertheless, t10,c12 CLA-induced weight loss in mice is severe compared to humans. This may be due to the difference in t10,c12 CLA dosage between human and animal trials. A 0.5% (w/w) t10,c12 CLA dietary intervention (which is often used in dietary intervention trials in murine models) is roughly equal to 56 g/day/70 kg in humans (255). Furthermore, in a human trial using CLA dosages ranging from 1.7-6.8 g/day, there was no added benefit of using the highest dosage compared to a dosage of 3.4 g/day (256).

For humans, it has been shown that long term intake (at least 12 weeks) of at least 3 g/day of CLA causes significant reductions in body mass index and body weight in overweight/obese cohorts (178,257–259). The largest reduction of body weight and BMI observed in a human clinical trial using CLA supplements was 1.8 kg and 0.6 kg/m², respectively, which was achieved by taking a 3.6 g CLA supplement daily for 1 year (178). In that particular study, the t10,c12 CLA content of the CLA supplement was 38 g per 100 g of fatty acid. However, several studies have shown that CLA supplementation with similar dosage and trial periods does not affect body weight or BMI in overweight/obese populations (183,255,259–263). Importantly, Riserus et al. (183,184) have shown that t10,c12 CLA supplementation causes insulin resistance, oxidative stress and increased circulating C-reactive protein levels in obese men. A main flaw with research in this area is the use of mixtures of CLA isomers which often constitute of equal portions of c9,t11 CLA and t10,c12 CLA isomers. It has been well established in animal model experiments that the weight loss properties of CLA are isomer specific, with t10,c12 CLA being responsible for CLA's weight loss properties (173). It is not known whether CLA purified for t10,c12 CLA would have caused more significant weight loss in previously

mentioned trials. Furthermore, none of the CLA weight loss trials in humans have investigated effects of CLA on the liver lipid levels.

The use of t10,c12 CLA supplementation for weight loss is appealing but caution is warranted due to the negative health effects observed in experiments in humans and mice. However, co-therapies with t10,c12 CLA may allow for t10,c12 CLA-induced fat mass loss, the prevention of t10,c12 CLA-induced hepatic lipid accumulation, and the preservation of insulin sensitivity. Dietary intervention animal trials which include pairing t10,c12 CLA with an insulin sensitizer (flaxseed oil or rosiglitazone) allowed for t10,c12 CLA-induced weight loss and blunted t10,c12 CLA-induced hepatic lipid accumulation and insulin resistance (249,264). Pairing DHA with t10,c12 CLA in a dietary intervention trial in mice also prevented t10,c12 CLA-induced hepatic lipid accumulation and insulin resistance, but prevented t10,c12 CLA-induced weight loss (248). Therefore, investigation into the weight loss properties of t10,c12 CLA may identify effective mechanisms for inducing weight loss and strategies to avoid the associated side effects.

Effect of t10,c12 CLA on Lipid Metabolism Proteins

Hepatic levels of certain lipid metabolism proteins were measured to determine which processes t10,c12 CLA uses to mediate hepatic lipid accumulation. FAS is an enzyme which facilitates *de novo* lipogenesis (78), whereas FAT/CD36 and FABP1 both mediate FA uptake (64,71).

When comparing mouse livers from lean C57BL/6 and *db/db* mice, Western blotting did not show any differences in FAS protein levels between these groups. This is contrary to what other researchers have observed based on reports of increased FAS expression in livers of *db/db* mice, compared to WT controls (265–267). FAT/CD36 expression levels are also often observed to be elevated in *db/db* mice, when compared to WT controls (267–269). The latter finding was repeated in this present study. A previous study which investigated the differences of FABP1 expression between *db/db* and WT C57BL/6 mice showed no significant differences (270). This was also true regarding FABP1 protein levels for this study. These findings suggest that increased hepatic lipid accumulation in *db/db* mice occurs via increased FAT/CD36-mediated hepatic FA uptake.

Regarding mice fed t10,c12 CLA, Western blotting showed t10,c12 CLA feeding in lean C57BL/6 mice resulted in higher hepatic FAS protein levels, but not in *db/db* mice fed t10,c12 CLA. This result mirrors the findings from another study that showed t10,c12 CLA feeding caused increased hepatic expression of FAS compared to control mice (271). Increased hepatic protein levels of FAS in t10,c12 CLA-fed lean C57BL/6 mice coincided with a 5-fold increase in hepatic TG concentration. This suggests that the t10,c12 CLA diet contributed to hepatic lipid accumulation by inducing *de novo* lipogenesis in the livers of lean healthy mice, but not in *db/db* mice which already have hepatic steatosis.

Interestingly, this effect was unique to lean mice. FAS expression is controlled by the transcription factor SREBP-1c (272). Regulation of SREBP-1c is facilitated by various negative feedback loops, which are carried out by downstream products of SREBP-1c-mediated gene

expression (i.e fatty acids, cholesterol, etc.) (273). The *db/db* mice likely already have high levels of these downstream SREBP-1c transcription products since their livers are steatotic. It is possible that negative feedback regulation of SREBP-1c may be preventing SREBP-1c-mediated transcription of FAS in *db/db* mice. This would explain why lean C57BL/6 mice experience t10,c12 CLA-induced elevated hepatic levels of FAS while obese *db/db* mice do not experience this.

Furthermore, t10,c12 CLA feeding upregulated hepatic FA uptake, as demonstrated by increased hepatic levels of both FAT/CD36 and FABP1 in t10,c12 CLA-fed mice. Interestingly, FABP1 levels were solely increased in t10,c12 CLA-fed mice and not in *db/db* mice compared to their respective controls. This may indicate a specific pathway for how t10,c12 CLA facilitates hepatic lipid accumulation which is unique to hepatic lipid accumulation caused by excessive calorie intake.

In vitro experiments using Western blotting to detect protein levels of lipid metabolism proteins in t10,c12 CLA-treated H4IIE cells showed that t10,c12 CLA may directly increase FABP1 levels. The effect of t10,c12 CLA was shown to be FA isomer specific since LA was not able to increase FABP1 protein levels *in vitro*. OA, a prominent inducer of neutral lipid accumulation *in vitro*, was used as a positive control. T10,c12 CLA was able to upregulate FABP1 protein levels as effectively as OA in H4IIE cells. Since combined treatments did not further upregulate FABP1 levels, it may indicate that maximum FABP1 expression was achieved by single FFA treatments and that both function through the same mechanism.

Similar *in vitro* experiments were used to investigate the effect of t10,c12 CLA treatment on FAT/CD36 protein levels in H4IIE cells. Upregulation of FAT/CD36 was observed *in vitro* when the total FFA concentration in the media was 200 μ M (100 μ M of OA and 100 μ M of t10,c12 CLA or LA). This may indicate FAT/CD36 expression is regulated by total FFA concentration and not by the specific FFA which is present. There was uniform augmentation of FAT/CD36 protein levels in OA- and LA-treated H4IIE cells compared to vehicle. Also, 3 of 4 replicates of H4IIE cells treated with OA and t10,c12 CLA showed an increase in FAT/CD36 protein levels, compared to vehicle. Thus, most replicates with 200 μ M of total FFA showed elevated FAT/CD36 protein levels compared to replicates which only contained 100 μ M of total FFA.

Effect of t10,c12 CLA on Cell Signaling Proteins

AMPK Phosphorylation

AMPK detects energy cellular status by interpreting ADP and AMP levels and promotes cellular processes that lead to ATP production. AMPK activation results in the inhibition of anabolic processes and upregulation of catabolic processes (123).

AMPK activation is often observed to be lower in *db/db* mice, compared to WT mice (274–276). However, in this study, hepatic AMPK phosphorylation was not shown to be different between *db/db* and lean C57BL/6 mice.

Hepatic AMPK activation was shown to be increased as a result of t10,c12 CLA feeding in this study. A similar result was observed by another group feeding C57BL/6 mice a 1% (w/w) t10,c12 CLA diet (277). In a previous t10,c12 CLA feeding animal intervention study with rats in our lab, there were no changes in AMPK activation between experimental groups (175). In 3T3-

L1 adipocytes, AMPK phosphorylation was increased in response to 100 μ M t10,c12 CLA (277). Since AMPK promotes catabolic processes, like FA oxidation, it is clear that the effects of AMPK are concealed by other processes.

It is unlikely that the energy status within the liver of t10,c12-CLA fed mice is low given their high hepatic TG concentration. Interestingly, ethanol feeding has also been shown to increase AMPK phosphorylation in mice while also increasing hepatic lipid accumulation (278,279). It is important to note that AMPK is not only regulated by energy status. Calmodulin-dependent protein kinase kinase- β (CAMKK β) is an alternative upstream kinase of AMPK which is activated by high Ca^{2+} levels (280). Obesity has been shown to induce ER stress and disrupt hepatic SERCA activity, leading to increased cytosolic Ca^{2+} levels (86,97,281). Two independent research groups have shown PA-induced ER stress in hepatocyte cell lines results in elevated cytosolic Ca^{2+} levels (282,283). AMPK activation observed in t10,c12 CLA-fed mice may be due to t10,c12 CLA-induced ER stress, which leads to increased cytosolic Ca^{2+} levels, increased CAMKK β activation, and subsequent AMPK phosphorylation. Previous experiments from our lab have shown that t10,c12 CLA induces CAMKK β and AMPK phosphorylation in L6 myotubes (284). Furthermore, CAMKK β is activated in mouse hepatocytes in response to obesity and a selective CAMKK β inhibitor, STO-609, alleviates HFD-induced hepatic steatosis in mice (285,286). Since ethanol is known to cause significant ER stress in hepatocytes, ethanol and t10,c12 CLA may both cause AMPK activation while also causing hepatic lipid accumulation through a similar mechanism (287).

JNK Phosphorylation

JNK activity is increased in response to cellular stress and causes insulin resistance and hepatocyte apoptosis, thus driving NAFLD (114). JNK has been well established as an important component in the pathogenesis of NAFLD through genetic deletion and pharmacological inhibition of JNK in animal models of NAFLD (115,116).

Hepatic JNK activation has been shown to be elevated in animal models of NAFLD (115,288,289). Similarly, in this study, JNK activation was increased when comparing lean and obese *db/db* mice fed the control diet, and in lean t10,c12 CLA-fed mice compared to lean control diet-fed mice. This is the first time t10,c12 CLA feeding in mice has been shown to result in hepatic JNK activation.

JNK activation occurs in response to various stimuli, including cytokines, ROS, pathogens, DAMPs, and environmental stresses (114). JNK activation is an indication that hepatocytes are under metabolic stress. Elevated hepatic TG concentration in *db/db* and t10,c12 CLA-fed lean mice suggests hepatocytes are exposed to high levels of lipotoxicity and increased levels of cytokines, ROS, and DAMPs (12). Furthermore, JNK activation is a component of ER stress UPR (290). Therefore, JNK activation is also indicative that hepatocytes are experiencing ER stress. In conclusion, t10,c12 CLA feeding in lean and *db/db* mice causes hepatocytes to experience high levels of metabolic stress, likely due to elevated lipotoxicity, as indicated by increased JNK activation.

S6 Ribosomal Protein Phosphorylation

p-S6RP was measured to assess the extent of mTOR activation. mTOR activation is indicative of the upregulation of anabolic processes and the downregulation of catabolic processes and has been shown to be an important component of the development of NAFLD in animal models (119,121,122). The effects of mTOR activation directly counteract the effects AMPK activation (123).

The results in this study showed no difference in p-S6RP levels between lean and *db/db* mice. Other research groups have shown increased phosphorylation levels of mTOR associated proteins in *db/db* mice compared WT control mice (274,291,292), which contradicts the results observed in this study.

However, t10,c12 CLA feeding did trigger an increase in p-S6RP levels. This finding is in agreement with *in vitro* experiments by Go et al. (176) who showed that t10,c12 CLA-mediated neutral lipid accumulation in HepG2 cells was achieved through activation of mTOR by t10,c12 CLA, as demonstrated by increased phosphorylation levels of various mTOR associated proteins after t10,c12 CLA treatment. The observed upregulation of p-S6RP levels in t10,c12 CLA fed mice adds evidence to the notion that t10,c12 CLA causes hepatic lipid accumulation through mTOR activation. In addition, this study also found that t10,c12 CLA treatment (100 μ M) for 3 hours caused increased p-S6RP levels in H4IIE cells compared to vehicle and LA treatments. This shows that t10,c12 CLA directly causes mTOR activation *in vitro*.

T10,c12 CLA-induced mTOR activation may be due to upregulation of toll-like receptor 4 (TLR4) expression. TLR4 is an innate immune system receptor which recognizes inflammatory

stimuli (i.e. lipopolysaccharides) and then activates the TLR4-mediated signalling pathway which results in the production of pro-inflammatory cytokines (293). Increased TLR4 expression in hepatocytes promotes a stronger inflammatory in obesity (294). Experiments using muscle of mice and a myoblast cell line have shown that t10,c12 CLA feeding and treatment, respectively, result in increased expression of TLR4 (295). Furthermore, the TLR4 signaling pathway facilitates the activation of inositol-requiring enzyme 1 α (IRE1 α), one of the three major UPR mediating signaling transduction pathways activated during ER stress (11). Lastly, PA-induced IRE1 α activation has been shown to result in mTOR activation in a hepatocyte cell line (296). Therefore, t10,c12 CLA feeding may result in mTOR activation via a TLR4-IRE1 α -mTOR signaling axis. This conjecture is supported by the Western blot in this thesis showing that t10,c12 CLA feeding results in JNK activation, since IRE1 α facilitates JNK activation (97).

In vitro, OA was also shown to increase p-S6RP levels in H4IIE cells. OA is known to be a potent inducer of neutral lipid accumulation. Since both fats are able to increase p-S6RP levels, it is possible that the manner by which they cause neutral lipid accumulation is through activation of mTOR. An mTOR inhibitor, rapamycin, has been shown to reduce OA-mediated neutral lipid accumulation in HepG2 cells (120).

Experiments in this study show that OA is a more potent mTOR activator compared to t10,c12 CLA. OA treatment was able to upregulate p-S6RP levels in H4IIE cells 24-hours post-treatment, while t10,c12 CLA treatment was not. Time course experiments investigating the upregulation of p-S6RP by OA treatment over 24 hours showed that OA-mediated upregulation

of p-S6RP levels is strongest after 2 hours. It is possible that this applies to other FFAs, since t10,c12 CLA treatment in H4IIE cells only causes a transient upregulation of p-S6RP levels.

Future experiments should include comparisons between OA- and t10,c12 CLA-mediated neutral lipid accumulation in H4IIE cells. Also, experiments with rapamycin would help elucidate whether t10,c12 CLA-mediated neutral lipid accumulation is dependent on mTOR activation.

Inspection of p-S6RP levels in liver tissue by Western blotting showed noticeable variability in p-S6RP levels within samples from the same experimental groups. These mice were not fasted prior to being sacrificed. For this reason, certain mice could have eaten more recently before being sacrificed compared to others. As shown in *in vitro* experiments, S6RP phosphorylation increases acutely in response to exposure to t10,c12 CLA or OA. Therefore, the large variability of p-S6RP levels among mice within the same experimental group could be due to certain mice having eaten more recently compared to other mice before sacrifice.

Effect of t10,c12 CLA Feeding and Obesity on Hepatic URI1 Protein Levels

There is inconsistency among the information for the URI1 antibodies and the predicted size of URI1. CST, Thermofisher, and Biorbyt URI1 antibodies predict a single URI1 band with the molecular weight of ~79, ~60, or ~48 kDa, respectively, in a gel after electrophoresis. CST, Thermofisher, and Biorbyt used 263T human embryonic kidney cells, LOVO human colonic epithelial cells, and U87 human glioblastoma cells, respectively, to validate their URI1

antibodies. All three of these bands, including an additional ~35 kDa band, were detected using human URI1 overexpression lysate and the CST URI1 antibody. The most prominent band in this Western blot was the ~79 kDa band which may indicate that this band represents native URI1. URI1 antibody characterization experiments in this thesis suggest all of these bands are different forms of URI1, including the additional ~35 kDa band. Furthermore, there was inter-species variability for the URI1 band(s) detected by the antibodies. Using the CST antibody and the human over-expression lysate, the antibody detected all of 4 of the previously mentioned bands. In rat H4IIE cells, the CST URI1 antibody only detected the ~79 and ~35 kDa bands. Lastly, in murine liver, the CST antibody only detected the ~48 kDa band. Most literature discusses the ~79 kDa band of URI1 (158,163,164). Investigation into the differences between URI1 bands is required for improved understanding of URI1. There should be agreement amongst antibody providers on which URI1 band should be targeted by their antibodies and consistency or differences across species should be confirmed.

The NCBI database indicates that a ~50 kDa isoform of URI1 exists and 2 separate URI1 antibodies detect a band at approximately this molecular weight. This is evidence that the band detected by the CST URI1 antibody in murine liver lysate represents URI1. URI1 was found to be elevated in the livers of *db/db* and *t10,c12* CLA-fed lean mice compared to their respective controls. Furthermore, the hepatic protein levels of URI1 significantly correlated with relative liver weight and hepatic lipid concentration ($R=0.64$, $P<0.0001$). These results indicate that URI1 protein levels are influenced by hepatic lipid content or vice versa or that they are influenced by similar factors. Even though only one isoform of murine URI1 is shown to be correlated with

relative liver weight, it shows that URI1 gene expression is increased when there is increased hepatic lipid accumulation. Similarly, Gomes et al. (169) showed that hepatic protein levels of URI1 were increased in mice with HFD-induced hepatic steatosis. Unfortunately, the molecular weight of this band was not disclosed. The finding in this study shows that, in mice, hepatic URI1 protein levels are increased by obesity- or t10,c12 CLA-induced-hepatic steatosis.

Another instance when hepatic URI1 protein levels were observed to be elevated in mice was when mice were intraperitoneally injected with the hepatotoxin, diethylnitrosamine (25 mg/kg) (162); these authors did not disclose the molecular weight of the URI1 band. Since the effects of t10,c12 CLA on hepatocytes appear to be generally detrimental and URI1 levels are also increased in response to hepatotoxins, this may indicate that t10,c12 CLA behaves like a hepatotoxin. Investigation into the significance of this relationship is warranted due to the high prevalence of NAFLD in the general population and the oncogenic properties of URI1.

Effect of Oleic Acid Treatment on *in vitro* Neutral Lipid Accumulation and URI1 Protein Levels in H4IIE Cells

Complementary *in vitro* experiments using H4IIE cells and OA treatment were used to determine the relevance of URI1 for hepatic lipid accumulation. The progression of neutral lipid accumulation in H4IIE cells in response to OA treatment was first investigated. This experiment showed that neutral lipid accumulation was elevated the most at 24 hours after OA treatment.

A similar experiment was carried out to determine whether URI1 protein levels change in response to OA treatment over time. Western blotting of H4IIE cell lysates revealed two

distinct bands situated near the 75 and 35 kDa molecular weight markers. The ~79 kDa URI1 band was used for analysis of H4IIE cells and the implications of this are discussed below. The results of this experiment showed that URI1 was detectable after 4 hours of OA treatment and that it continued to increase over time. URI1 protein levels were the most elevated after 24 hours of OA treatment.

The results of these experiments show neutral lipid accumulation and URI1 protein levels respond similarly to OA treatment in H4IIE cells. They both reached the greatest elevation after 24 hours of OA treatment. These analogous results suggest that these two variables are influenced in a similar manner. These results mirror what was observed in liver tissue, where hepatic lipid concentration correlated with hepatic URI1 protein levels in *db/db* and t10,c12 CLA-fed C57BL/6 mice and their respective controls. This is the first time it has been reported that URI1 levels are increased in response to OA treatment *in vitro*.

The observation that the ~79 kDa band of URI1 only becomes detectable after exposure to FFA exposes a potential novel mechanism for regulation of URI1 expression. While the ~35 kDa URI1 band remained relatively unchanged over the timepoints examined, the ~79 kDa URI1 band increased with time. Investigation into the band which is responsive to changing growing conditions in a predictable manner would likely disclose how URI1 is regulated by cellular conditions. It is important to note that protein samples were collected after H4IIE cells had become quiescent. The presence of the 79 kDa URI1 band may have been influenced by the growth state of the cell. Furthermore, studies have shown that the ~79 kDa band of URI1

exhibits the most biological activity (binding capacity to PP1 γ , RPB5, and OGT) (160,164,166). Therefore, the ~79 kDa band is likely to have the greatest biological relevance.

Furthermore, it is important to consider whether the conditions which allow for detectable levels of URI1 in H4IIE cell culture are also applicable to hepatocytes *in vivo*. A study measuring serum FFA levels in NAFLD patients reported that individuals with NAFLD and their age matched controls who were fasted overnight, had serum FFA levels of 650 μ M and 450 μ M, respectively (297). Also, OA has been reported to make up 26.3% and 24.4% of the plasma FFA composition in individuals with NASH and their age matched healthy controls, respectively (298). Judging from these 2 separate studies, individuals with NAFLD and healthy individuals may have serum OA levels of ~171 and ~110 μ M, respectively, after an overnight fast. H4IIE cells were incubated with 100 μ M of OA during experiments. Therefore, hepatocytes *in vivo* are likely regularly exposed to FFA concentrations similar to the H4IIE cells in this study. *In vitro* experiments did not investigate the effects of different concentrations of OA on URI1 protein levels, but *in vivo* results did show that hepatic URI1 protein levels were correlated with hepatic TG concentration levels. Lastly, this study did not reveal whether URI1 protein levels are uniquely responsive to OA as they could be responsive to FFAs in general (i.e. PA).

The Development and Characterization of CRISPR/Cas9 *Uri1* KO H4IIE Cells

There were several interesting observations during the process of generating *Uri1* KO H4IIE cells which may suggest the importance of the URI1 gene for H4IIE cell viability. The process of generating *Uri1* KO H4IIE cells was done under the guidance of Dr. Jeusset and the

McManus lab. The first step for creating *Uri1* KO H4IIE cells was the delivery of sgRNA strands, which were targeted for the *Uri1* gene, and the Cas9 endonuclease into H4IIE cells. sgRNA strands were delivered into cells using lenti-viral transduction. FACS showed that the transduction rate of sgRNA into H4IIE cells was 0.03%. The Cas9 endonuclease was delivered into H4IIE cells using nucleofection, an electroporation-based transfection method, and FACS showed that the transfection rate in H4IIE cells was 42%.

Next, the isolation of H4IIE^{BFP+/GFP+} clones was achieved by limiting dilution, and Western blotting was used to screen for *Uri1* KO H4IIE cells. Initially, URI1 Western blots using quiescent H4IIE^{BFP+/GFP+} cells and the CST URI1 antibody only showed the ~35 kDa band of URI1 so this band was used for screening. Several clones with zero or reduced expression of the ~35 kDa URI1 band were selected for further screening experiments. While using this band was effective for finding clones with CRISPR/Cas9-induced gene edits in the *Uri1* gene, it was poor at predicting whether homozygous or heterozygous *Uri1* gene deletions had occurred. Certain clones showed zero expression of the ~35 kDa URI1 band but had no PTCs within the *Uri1* gene. It is possible that the ~35 kDa band represents rat URI1 isoform X2, since the calculated molecular weight of this isoform is 42 kDa and the band is above the 35 kDa molecular weight marker. Rat URI1 isoform AA alignment using BLAST showed that the AA sequences varied only at the N-terminus. If a cell were edited by the *sgUri1-1*, theoretically, this isoform would be expressed since the start codon for this isoform is upstream from the site targeted of *sgUri1-1*. In contrast, *sgUri1-2* targets a site in the *Uri1* gene closer to the C-terminus and would therefore result in genetic ablation of rat URI1 isoform X2. Interestingly, despite, *Uri1*^{+/-} 2 H4IIE

cells being edited by sg*Uri1*-1, there were no indications of the presence of a ~35 kDa URI1 band.

As previous results have shown, the ~79 kDa band of URI1 was present at detectable levels in H4IIE cells after a 4-hour incubation with 100 μ M of OA. When *Uri1*^{+/-}1 and *Uri1*^{+/-}2 H4IIE cells were treated with OA (100 μ M), there was a 50% reduction in the ~79 kDa band of URI1 compared to WT controls, as is expected from heterozygous *Uri1* KO H4IIE cells. The ~79 kDa URI1 band was absent in *Uri1*^{-/-} H4IIE cells in these same conditions which is also expected from homozygous *Uri1* KO H4IIE cells. Therefore, using this band is suitable to determine the nature of the CRISPR/Cas9-induced gene edit. However, certain conditions (ie. OA treatment) must be applied for appropriate detection of this band.

DNA was extracted from H4IIE^{BFP+/GFP+} clones which were suspected to have experienced *Uri1* gene allelic edit(s). PCR was then used to amplify a 600-700 bp DNA sequence which corresponds to the *Uri1* gene exon targeted by the sgRNAs and CRISPR/Cas9 gene editing. PCR products were then sequenced using Sanger sequencing. Chromatograms from Sanger sequencing often showed double peaks, with each peak coming from a different allele, an indication of CRISPR/Cas9 gene edits. The online program, CRISP-ID, was then used to resolve the mixture of the two alleles shown in these chromatograms.

It should be noted that the gold standard of sequencing CRISPR/Cas9-induced indels in diploid cells requires subcloning of these alleles into bacterial vectors. Each allele is subcloned into plasmids that are subjected to DNA sequencing to identify the individual edits to each allele. This method is preferred for sequencing CRISPR/Cas9-induced indels in diploid cells. In

silico methods (including using CRISP-ID) for resolving the differences between alleles in the same chromatogram use algorithms that depend on certain assumptions. Therefore, this method is not guaranteed to accurately determine the exact DNA sequence of these alleles. It is best to separate these alleles using bacterial vectors and to sequence each allele individually. This way, there is certainty of the DNA sequence.

Nevertheless, the pattern of *Uri1* gene edits revealed amongst H4IIE^{GFP+/BFP+} clones were unique and insightful. There are many aspects about the types of gene edits and the number of gene edits found that are worth discussing. Judging from conversations with Dr. Jeusset and Dr. McManus, the process of developing *Uri1* KO H4IIE cells resulted in more *Uri1* edited clones compared to other gene KO endeavours in different cell types and with different gene targets.

Firstly, there were many clones which contained *Uri1* gene edits compared to other gene KO projects. The reason for these differences may be due to the high nucleofection rate of the Cas9 endonuclease compared to the transfection approach that uses lipid-based transfection reagents. As discussed with Dr. Jeusset, a 42% nucleofection rate is very high compared to previous gene KO projects. A previous student from the McManus lab using FT246 cells attained a transfection rate of the Cas9-GFP plasmid of 1.42% (299). A high nucleofection rate may be indicative of high expression levels of the Cas9 endonuclease which could lead to increased gene editing efficiency.

Another possible explanation of the high number of H4IIE^{GFP+/BFP+} clones with *Uri1* gene edits is that the *Uri1* gene is not as essential for cell viability as compared to previous genes investigated. The reason that identifying genetic KO clones is difficult is that the gene which is

knocked out could be important for cell survival. This results in only a few cells with the genetic KO surviving due to them possessing unique characteristics which allow them to survive despite the loss of the targeted gene. Also, if the gene KO negatively affects cell growth, gene KO cells could become diluted amongst the population of WT cells within the cell culture. The process of isolating a clonal population with the gene KO by limiting dilution is based completely on chance. The fact that the CRISPR/Cas9-induced *Uri1* gene edit rate amongst H4IIE^{BFP+/GFP+} cells was very high could indicate that this gene is not essential for cell survival, allowing them to persist in the cell culture as much as WT cells.

On the contrary, there are data that support *Uri1* being an essential gene. In mice, homozygous genetic deletion of *Uri1* resulted in hepatocytes undergoing a massive apoptotic program resulting in liver injury. These mice died 10 days after birth and likely perished due to fulminant liver failure (162). Furthermore, *uri1* deletion in *Caenorhabditis elegans* (*C. elegans*) resulted in sterility, inability to proliferate, and DNA damage (300). Therefore, *Uri1* may have a role in maintaining DNA integrity, proliferation, and fertility, and *URI1* loss may be lethal for the organism.

On this note, some details about the type of edits observed in H4IIE^{BFP+/GFP+} cells supports the previously mentioned notion. Out of all the 26 gene edits observed, 11 of these edits were in-frame indels. These alterations would not introduce PTCs and preserve *Uri1* expression, albeit with an altered AA sequence. This type of edit should constitute no more than one third of all mutations and sgRNA strands are pre-screened to have low in-frame edit rates. Despite this, in-frame mutations constituted 42% of all edits observed. Even though, in

biology, 42% and 33% are similar rates, conversations with other McManus lab students (including Dr. Jeusset) revealed that the prevalence of this many in-frame indels in a gene KO project has not been observed. Due to the novelty of this occurring, it appears that in-frame deletions for NHEJ repair after CRISPR/Cas9-induced DSBs within the *Uri1* gene in H4IIE cells were preferred. A possible reason for this is that despite the abundance of observed *Uri1* gene edits, at least some *Uri1* gene expression is required for cell survival. The AA alterations caused by these indels may not have compromised the URI1 protein's functionality. This notion is further supported by the limited number of homozygous URI1 KO H4IIE cells identified. If the *Uri1* gene was truly non-essential, it is expected that homozygous *Uri1* KO H4IIE cells would be more prevalent. However, out of the 14 clones screened, only one was identified as a homozygous *Uri1* KO.

One homozygous *Uri1* KO clone was generated in this thesis, which is contrary to the notion that *Uri1* is required for cell survival. However, only one homozygous *Uri1* KO H4IIE clone was generated after sequencing 14 H4IIE^{BFP+/GFP+} clones and all but one H4IIE^{BFP+/GFP+} clone possessed a *Uri1* gene edit. Therefore, it is possible that *Uri1*^{-/-} H4IIE cells possess unique characteristics, which are atypical for WT H4IIE cells, yet allow *Uri1*^{-/-} H4IIE cells to survive despite loss of *Uri1*. If *Uri1* was non-essential, there would have been many more homozygous *Uri1* KO H4IIE cells, especially since there was a high *Uri1* gene edit rate among H4IIE^{BFP+/GFP+} clones.

In summary, CRISPR/Cas9-induced *Uri1* gene edits were very prevalent amongst the population of H4IIE^{BFP+/GFP+} cells. The essentiality of *Uri1* is exhibited by the lack of homozygous

Uri1 KO H4IIE cells identified and the skewedness towards *Uri1* gene edits being in-frame deletion indels, which allow for *Uri1* gene expression. The combination of the high prevalence of CRISPR/Cas9-induced DSBs within the *Uri1* gene amongst H4IIE^{BFP+/GFP+} cells and the essentiality of *Uri1* gene expression for H4IIE cell survival, likely led to high number of H4IIE^{BFP+/GFP+} cells with in-frame indels in the *Uri1* gene.

Another interesting observation was the distinct preference of DSBs occurring within exon 10 in the *Uri1* gene. Both exon 4 and exon 10 were targeted by the sgRNAs. However, only 2 of out of the 13 H4IIE^{BFP+/GFP+} clones were shown to have CRISPR/Cas9-induced gene edits within exon 4 of the *Uri1* gene via *sgUri1-1*. Interestingly, the CRISPR/Cas9 edit site targeted by *sgUri1-1* is found within a CLS sequence (shown below in Figure 36). Alterations to this sequence would result in increased localization of URI1 within the nucleus (165). Coincidentally, the only URI1 KO H4IIE clone with a CRISPR/Cas9 gene edit within exon 4 has distorted growth characteristics, including increased growth rate and apparent loss of contact inhibition of proliferation, as evidenced by RTCA analysis and visual monitoring of cell growth using light microscopy.

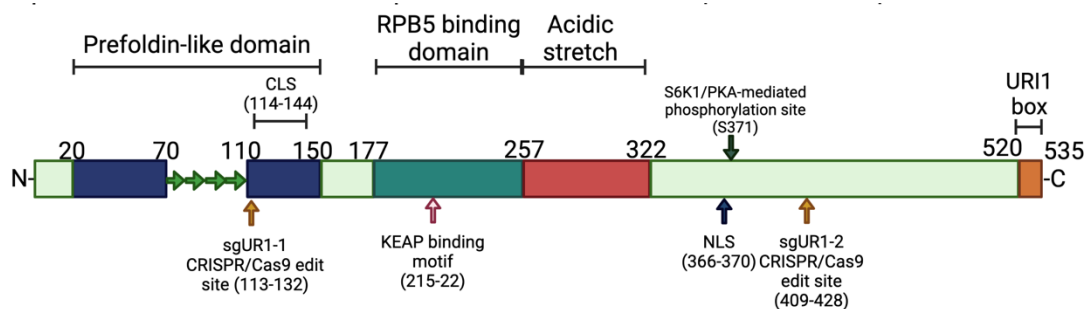


Figure 36: URI1 protein domains in relation CRISPR/Cas9 edit sites.

RTCA was used to characterize *Uri1* KO H4IIE cells. The purpose of this experiment was to assess whether the process of creating *Uri1* KO H4IIE cells had effects on overall H4IIE cell characteristics. These experiments are important since the differences observed between *Uri1* KO and WT H4IIE cells may be due to the extensive process of *Uri1* gene deletion, and not due to the loss of the gene itself. *Uri1* KO H4IIE cells undergo lentiviral transduction, FACS, nucleofection, limiting dilution, and a 2nd round of FACS. Each of these processes apply considerable stress to cells so determining whether their overall characteristics have changed is important. Furthermore, off-target effects from Cas9 endonuclease expression may result in unintended genetic alterations that may adversely impact cell characteristics.

RTCA monitors the growth of cells in real time, by measuring media conductance impedance. As cells populate the bottoms of the well, media conductance is impeded. Media conductance impedance is reported as a unitless measurement called CI. CI can then be used to measure the growth rate of cells in real time.

This assay revealed that 2 of the *Uri1* KO H4IIE clones (*Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells) exhibited growth characteristics similar to WT H4IIE cells. However, *Uri1*^{+/-} 2 H4IIE cells showed significantly elevated growth rate characteristics, including a reduction in the number of hours required to reach confluency, elevated speed of growth (CI/hour) at 50% confluency, and increased CI values between the 30th and 80th hours compared to all other cell types.

These results prompted further investigation into the overall growth characteristics of these cells. This was done by simply photographing cells daily after seeding, using standard light microscopy. These observations showed no apparent differences of growth characteristics

between WT, *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells. Interestingly, *Uri1*^{+/-} 2 H4IIE cells showed apparent distorted growth characteristics compared to WT H4IIE cells. The increased growth rate in *Uri1*^{+/-} 2 H4IIE cells was apparent from photographs. Furthermore, *Uri1*^{+/-} 2 H4IIE cells also appeared to grow much more closely together and disperse less evenly across the bottom of the well which suggests that *Uri1*^{+/-} 2 H4IIE cells have reduced contact inhibition of proliferation. Normally, contact inhibition of proliferation causes cell cultures to slow proliferation as they reach confluency. Contact inhibition of proliferation proposes that the cell proliferation is slowed when there is contact between cell surfaces, thus preventing cells from over-proliferating and forming clumps in a monolayer cell culture (301). The loss of contact inhibition of proliferation, leading to uncontrolled cell growth, is regarded as an important component for the initiation and progression of many forms of cancers (302).

In summary, *Uri1*^{+/-} 2 cells contain CRISPR/Cas9-induced *Uri1* gene edits which lead to the silencing of one *Uri1* allele and alterations to the CLS sequence in the 2nd *Uri1* allele. *Uri1*^{+/-} 2 H4IIE cells exhibit an increased growth rate and grow in dense clusters compared to other H4IIE cell lines. Importantly, these cells exhibit more properties which cancer cells possess (302). There are many possible reasons for this and ample data available which allows for speculation on why these cells behave so uniquely.

Firstly, these differences in characteristics may not be due to *Uri1* genetic deletion, but rather due to CRISPR/Cas9 off-target effects. It has been shown that CRISPR/Cas9 gene editing can result in DSBs at unintended genomic sites (303). Off-target effects can lead to loss of gene function and the creation of cancer cells (209). The high CRISPR/Cas9 editing rate in

H4IIE^{BFP+/GFP+} cells was very beneficial for increasing the feasibility of identifying H4IIE^{BFP+/GFP+} cells with a genetic *Uri1* KO. However, this likely came at a cost. The high nucleofection rate may be indicative of elevated Cas9 expression which lead to increased numbers of off-target effects. These off-target effects may have caused *Uri1*^{+/-} 2 H4IIE cells to gain more qualities of cancer cells. It should be noted that high nucleofection rates do not necessarily correlate with high levels of Cas9 expression. It is only speculated that Cas9 expression was very high in H4IIE^{BFP+/GFP+} cells due to the high nucleofection rate and high *Uri1* gene editing rate.

Another reason for the preference for *sgUri1-2* mediated *Uri1* KO compared *sgUri1-1* mediated *Uri1* KO could be that exon 10 is more accessible to sgRNA. Histones allow for packaging of chromosomal DNA into the microscopic space within the nucleus and the DNA-protein complex is called chromatin (304). According to Horlbeck et al. (305), the organization of the individual units of chromatin, the nucleosomes, can influence sgRNA binding. Therefore, the preference of CRISPR/Cas9-induced DSB occurring within exon 10 rather than exon 4 could be due to the differences of nucleosome organization in their respective regions of DNA.

It is important to consider the different outcomes of *Uri1* deletions depending on where Cas9 was targeted. The preference towards CRISPR/Cas9 edits within exon 10 over exon 4 may be due to exon 4 being important for URI1's functionality. CRISPR/Cas9 gene edits in H4IIE^{BFP+/GFP+} cells always caused some type of alteration in both alleles in this study amongst the CRISPR/Cas9 edited H4IIE^{BFP+/GFP+} clones sequenced in this study. The finding that homozygous *Uri1* KO H4IIE cells are much less prevalent amongst H4IIE^{BFP+/GFP+} cells suggest that this protein is important for cell survival, and, therefore, negative effects on URI1's

functionality would also impair H4IIE cell survival. On this note, *sgUri1-1*-mediated CRISPR/Cas9 edits result in changes to the CLS sequence of *Uri1* (shown above in Figure 34). Changes to the CLS sequence may result in an increase of localization of URI1 within the nucleus which may have deleterious consequences for cell viability (165). URI1 works in conjunction within the prefoldin-like complex to mediate the cytoplasmic assembly of protein complexes, including RNA polymerase (154). Therefore, nuclear localization of URI1 would prevent it from performing this function and impair cell survival. Furthermore, increased localization of URI1 within the nucleus may lead to the altered characteristic observed in *Uri1*^{+/-} 2 H4IIE cells, perhaps via interactions with RPB5 within RNA polymerase (157).

Another possible reason for the preference of *Uri1* gene edits mediated by *sgUri1-2* over *sgUri1-1* is due to their respective truncated versions of *Uri1* supporting or not supporting cell survival. It was not confirmed whether NMD facilitated the complete eradication of *Uri1* mRNAs which harboured CRISPR/Cas9-induced PTCs. If NMD was incomplete, truncated versions of URI1 would persist. Truncated versions of URI1 expressed by *sgUri1-2* edited *Uri1* would contain the prefoldin-like and the RPB5 binding domains. These domains are necessary for cytoplasmic assembly of protein including RNA polymerase II (154,155). In contrast, truncated versions of URI1 expressed by *sgUri1-1* edited *Uri1* would lack a significant portion of the prefoldin-like domain and completely lack the RPB5 binding domain. Therefore, this truncated version of URI1 would less likely support cell survival. Thus, incomplete NMD in *sgUri1-2* edited H4IIE clones may support cell survival but not for *sgUri1-1* edited H4IIE clones. The C-terminal domains which are absent in the truncated version of URI1 expressed by *sgUri1-2* edited *Uri1*

may be important for lipid metabolism which would elucidate the reduced neutral lipid accumulation exhibited by *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells.

Also, from analysis of URI1 isoforms, Uri1 gene expression profile is different in *sgUri1-1*-mediated *Uri1* KO H4IIE cells compared to *sgUri1-2*-mediated *Uri1* KO H4IIE cells. *sgUri1-2* targets a portion of *Uri1* DNA which corresponds closer to the C-terminus of *Uri1* and results in the deletion of all URI1 isoforms. In contrast, since rat URI1 isoforms vary at the N-terminus and *sgUri1-1* targets a portion of *Uri1* DNA which corresponds closer to the N-terminus of URI1, the DNA region targeted by *sgUri1-1* is not present in URI1 isoform X2. Therefore, *sgUri1-1*-mediated *Uri1* KO H4IIE cells would theoretically still express URI1 isoform X2. However, Western blots with *Uri1*^{+/-}2 H4IIE cell samples do not show the ~35 kDa band which may represent URI1 isoform X2. It is possible that isolated expression of URI1 isoform X2 may result in altered growth characteristics. Since URI1 isoform X2 lacks 148 N-terminus AAs, it lacks the prefoldin-like domain which is required for prefoldin-like complex-mediated cytoplasmic protein assembly. Furthermore, it contains the PPIγ binding region which was shown to be important for URI1-induced carcinogenesis in ovarian cancer (161).

Additionally, it is also possible that genetic alterations in the Uri1 gene could have affected cell growth. URI1 is required for the negative feedback inhibition of the mTOR effector kinase, S6K1. URI1 is bound to PP1γ during nutrient/growth factor-deprived conditions, preventing PP1γ-mediated negative regulation of S6K1. Phosphorylation of URI1 by S6K1 results in URI1-PP1γ complex disassociation and PP1γ-mediated negative regulation of S6K1 (160). If a genetic alteration occurs which prevents S6K1-induced phosphorylation or results in a stronger

association between URI1 and PP1 γ , it would result in excessive S6K1 signalling, and consequently increased cell growth and survival. Furthermore, URI1 has been shown to be instrumental for mTOR-mediated suppression of a select set of genes in HeLa cells (159). Since mTOR is associated with cell growth, it is likely that URI1-mediated suppression of these genes results in increased cell growth. Therefore, genetic alterations which potentiate URI1's functionality could result in increased cell growth.

In conclusion, *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells have similar growth characteristics compared to WT H4IIE cells. The strenuous process of generating genetic *Uri1* KO cells did not alter discernable traits in these cells. However, *Uri1*^{+/-} 2 H4IIE cells were heavily altered after this process, and the results observed in *Uri1*^{+/-} 2 H4IIE cells should be evaluated precariously. In contrast, *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells behave similar to WT H4IIE cells and are suitable experimental models for investigating neutral lipid accumulation *in vitro*.

Neutral Lipid Accumulation in *Uri1* KO H4IIE Cells

It was hypothesized that URI1 mediates hepatic lipid accumulation. BODIPY staining in vehicle- and OA-treated *Uri1* KO and WT H4IIE cells revealed that *Uri1*^{+/-} 1 and *Uri1*^{-/-} H4IIE cells have reduced neutral lipid accumulation compared to WT H4IIE cells. This result is evidence that URI1 has a role in facilitating neutral lipid accumulation. These findings complement results shown in WT H4IIE cells and liver tissue where upregulated URI1 protein levels coincide with increased *in vitro* and *in vivo* lipid accumulation, respectively. These results suggest URI1 has a role in facilitating hepatic lipid accumulation.

On the contrary, this claim is refuted by the observation that *Uri1*^{+/-} 2 H4IIE cells showed increased neutral lipid accumulation compared to WT H4IIE cells. However, *Uri1*^{+/-} 2 H4IIE cells have been revealed to share characteristics with cancer cells. It has been shown that HCC tumours exhibit increased expression of key genes involved in *de novo* lipogenesis, including FAS, compared to non-cancerous cells (306). *De novo* lipogenesis in HCC cells promotes HCC cell proliferation whereas interventions which aim to reduce *de novo* lipogenesis in HCC cells have been shown to reduce tumour growth and metastasis in a xenograft mouse model (307). Also, hepatic FFA uptake has been shown to be important for HCC progression since markers of FFA uptake (i.e. CD36 and FABP1) have been shown to be associated with increased HCC progression (144). Therefore, the observation that *Uri1*^{+/-} 2 H4IIE cells, which exhibit properties of cancerous cells, show increased neutral lipid accumulation agrees with previously reported results that suggest HCC cells have increased lipid content compared to non-cancerous cells.

This is the first study to reveal that URI1 may have a role in OA-induced neutral lipid accumulation *in vitro*. This finding mirrors the results shown by Gomes et al. (169) who showed heterozygous genetic deletion of *Uri1* in mice protects against HFD-induced hepatic steatosis. Homozygous *Uri1* deletion could not be investigated using the animal model since homozygous *Uri1* KO mice survived for about 10 days until they died from fulminant liver failure (162). Interestingly, homozygous *Uri1* KO H4IIE clones were generated in this study. This may be due to *Uri1*^{+/-} 2 H4IIE cells possessing unique characteristics which allow them to survive despite the absence of *Uri1*. This may also be due to the context-specific gene essentiality of *Uri1*. As discussed, the responsibilities of the liver for maintaining normal health are extensive. *Uri1*, in

the context of the liver of a mouse, may be essential for the mouse to survive. However, in the context of hepatocyte cell culture, removed from its interactions with other organs and its role in maintaining normal health, *Uri1* may no longer be essential. This may be why homozygous *Uri1* KO H4IIE cells were successfully generated while homozygous *Uri1* KO mice lived for only 10 days. However, it should be noted that heterozygous *Uri1* KO H4IIE cells were much more prevalent compared to homozygous *Uri1* KO H4IIE cells, suggesting that complete loss of *Uri1* expression in H4IIE cells is detrimental for cell survival.

Interestingly, in this study there were no apparent differences between heterozygous and homozygous *Uri1* KO H4IIE cells. They exhibited similar reductions in neutral lipid accumulation compared to WT H4IIE cells. Future experiments should involve direct comparisons between these cell types.

Significance of Findings

CLA is marketed as a weight loss supplement. These claims are strongly supported by experiments using animal models and high amounts of t10,c12 CLA in their diet. CLA-diets have been shown to repeatedly induce severe fat mass loss across different mouse models of MetS (173,187,308,309). However, several studies have shown t10,c12 CLA-induced fat mass loss is often accompanied with hepatic steatosis (248–251). The results in this study add to the current body of evidence that t10,c12 CLA causes hepatic steatosis. These findings support the notion that t10,c12 CLA induces fat mass loss in an unhealthy manner due to resultant hepatic steatosis in mice. As discussed in the literature review, limiting hepatic lipid content below 5%

should be prioritized due to the broad detrimental effects on human health. Furthermore, t10,c12 CLA feeding in lean C57BL/6 mice resulted in JNK activation. JNK activation is associated with MetS and is an indicator that hepatocytes are experiencing metabolic stress (96). Importantly, JNK activation has been shown to be necessary for the development of T2DM (115). These results indicate the use t10,c12 CLA supplementation for promoting weight loss in humans should be discouraged, since t10,c12 CLA feeding may lead to hepatic lipid accumulation and metabolic stress within the liver, thus contributing to the development of T2DM.

This is the first study to show that t10,c12 CLA feeding results in mTOR activation in an animal model. Treating HepG2 cells (a human hepatoma cell line) with t10,c12 CLA has previously been shown to increase mTOR activation and evidence indicates that mTOR has a role in facilitating hepatic lipid accumulation *in vivo* (121,122,176). This result shows that t10,c12 CLA may facilitate hepatic lipid accumulation through the activation of mTOR. This result is further supported by *in vitro* results where t10,c12 CLA was shown to directly increase mTOR activation compared to vehicle and LA.

FAS and FAT/CD36 levels are elevated in response to mTOR activation (121,310). T10,c12 CLA feeding may result in increased FAS and FAT/CD36 protein levels by stimulating mTOR activation. These results may highlight a pathway by which t10,c12 CLA feeding mediates increased hepatic lipid accumulation. Using interventions which deter mTOR activation amid t10,c12 CLA feeding may allow for t10,c12 CLA-induced fat mass loss without concomitant hepatic steatosis.

Interestingly, FABP1 protein levels were found to be elevated in only t10,c12 CLA-fed mice and not in *db/db* mice compared to their respective controls. Furthermore, t10,c12 CLA treatment was able to increase FABP1 protein levels compared to LA or vehicle treatments *in vitro*. This shows that t10,c12 CLA may be increasing FABP1 expression through a unique pathway (i.e interaction with a promoter or a transcription factor). Further research into this result could reveal a unique pathway by which t10,c12 CLA elevates FABP1 expression and increases fatty acid uptake. FABP1 expression is controlled by the transcription factor, hepatocyte nuclear factor 1 alpha (HNF1 α) (311). T10,c12 CLA-induced upregulation of FABP1 may be due to an interaction between the t10,c12 CLA and HNF1 α . Over-expression of HNF1 α in rat livers has been shown to result in HNF1 α -mediated expression of SH2 domain-containing phosphatase -1 (SHP-1) which induces TLR4 activation (312). A potential interaction between t10,c12 CLA and HNF1 α may explain the mechanism by which t10,c12 CLA promotes the development of NAFLD.

Regarding URI1, this study calls attention to the need for additional research into URI1. A total of 4 UR1 bands were detected by Western blotting in a URI1 overexpression HEK 293 cell lysate. Similar sized bands were observed across different samples from mice and rats. These bands were confirmed to be different forms of URI1 which vary in size (possibly due to alternative splicing or post-translational modifications). In tissue samples, the identity of the URI1 bands was confirmed by using 2 antibodies which detect URI1 via separate epitopes. In H4IIE cell samples, the identity of the URI1 bands was confirmed by showing that the URI1 bands detected by the URI1 antibody in WT H4IIE cells were reduced/absent in the

hetero/homozygous *Uri1* KO H4IIE cell lines, respectively. There is a lack of consistency between antibody providers surrounding the molecular weight of URI1. There should be agreement amongst antibody providers regarding the isoform of URI1 targeted by their antibodies. Furthermore, there was inter-species variability on which band was detected by the URI1 antibody. In this study, the CST URI1 antibody was able to detect the ~79 and ~35 kDa URI1 bands in H4IIE cells but could only detect the ~48 kDa URI1 band in mouse liver tissue. Further validation of these antibodies across different species is required for better research on this topic

Nevertheless, the ~48 kDa isoform of URI1 was found to be upregulated in the livers genetically obese *db/db* mice and t10,c12 CLA-fed C57BL/6 mice. There was a close association between URI1 protein levels and relative liver weight ($R=0.64$, $P<0.0001$), indicating that these two variables are regulated in a similar manner. Even though only one URI1 band was shown to be correlated with relative liver weight, it shows that *Uri1* gene expression is increased when there is increased hepatic lipid accumulation. The ~79 kDa URI1 band may only be present in growing cells and transiently expressed in response to certain conditions. The chronic conditions experienced by *db/db* and t10,c12 CLA-fed mice may have led to the increased presence of the lower molecular weight form of URI1.

Interestingly, only H4IIE cells which were treated with OA had the ~79 kDa band of URI1. This observation may be indicative of a novel mechanism of regulation for URI1. Investigation into why this band is exclusively present under these specific conditions may reveal why hepatic URI1 protein levels are elevated by obesity- or t10,c12 CLA-induced hepatic

steatosis. Since the calculated molecular weight of URI1 is only 60 kDa, characterization of the ~79 kDa URI1 band after OA treatment may indicate that these conditions allow for post-translational modification (i.e. glycosylation). Investigation into the consequences of post-translational modification of URI1 is warranted.

While URI1 was observed to be increased in response to OA treatment, it is important to note that all experiments with H4IIE cells were performed after H4IIE cells had become quiescent. A multitude of biological processes are initiated in the post-prandial state. URI1 has already been shown to undergo mTOR- and PKA-induced phosphorylation (160,166). Since OA is an accessible energy source, OA treatment may have shifted starved H4IIE cells from a quiescent state to a growing state. Investigation into how URI1 protein levels respond to changes in glucose, insulin (absent from medium used for serum-starvation), or growth factor concentrations *in vitro* are required to understand URI1's role in NAFLD.

These findings reveal a relationship exists between URI1 expression and hepatic lipid accumulation and this necessitates further investigation due to URI1's association with NAFLD and HCC. It has been reported that hepatic URI1 expression is elevated in HCC patients (162,163,169). This suggests that URI1 protein levels ought to be investigated in NAFLD patients as well. Due to URI1's oncogenic properties, investigation into hepatic URI1 protein levels and the presence of certain URI1 isoforms could be employed to stratify a NAFLD patient's risk for developing of HCC and help determine the best treatment regimen. Also, it may indicate the patient's risk for developing advanced disease forms of NAFLD (i.e. NASH or fibrosis).

The results observed in WT and *Uri1* KO H4IIE cells further support the results observed in tissues. *Uri1* KO H4IIE cells showed that URI1 has a direct role in facilitating neutral lipid accumulation *in vitro*, since *Uri1* KO H4IIE cells showed reduced neutral lipid accumulation compared to WT H4IIE cells. Therefore, URI1 may not only have a role in progression to HCC, it may also have a role in progression of NAFLD severity. These results highlight the relevance of this protein for MetS, since NAFLD is the hepatic manifestation of MetS. Determining how URI1 protein levels are regulated by the diet may help individuals at risk of severe NAFLD avoid further progression. Importantly, the prevalence of NAFLD as the aetiology of HCC has been growing in recent years, but despite this, studies investigating how NAFLD and HCC are connected are lacking (140). This research explores how a protein with oncogenic properties also has a role in advancing NAFLD. Since rates of obesity have been growing, and NAFLD and HCC are projected to increase in parallel over time, research investigating the connection between NAFLD and HCC is crucial (25,26).

Strengths

- T10,c12 CLA was available in a high purity FFA form, allowing investigation of isomer specific effects of t10,c12 CLA. Many studies investigating t10,c12 CLA's effect on adiposity and hepatic lipid accumulation utilize t10,c12 CLA formulations which contain a significant proportion of the c9, t11 CLA isomer.
- Complementary experimental designs using *in vivo* and *in vitro* experimental models for investigating various cellular processes and their roles in hepatic lipid accumulation. The

in vivo approach was used to establish the relevance of several cellular processes, including the relevance of URI1, for hepatic lipid accumulation in an animal model. *In vitro* experiments were used to establish the mechanistic role of these cellular processes in neutral lipid accumulation in rat hepatoma cells.

- The use of both healthy lean C57BL/6 mice and genetically obese *db/db* mice. *Db/db* mice are a widely accepted model for investigating NAFLD and MetS (195). The use of both lean and *db/db* mice allowed for identification of cellular processes which t10,c12 CLA exploits to induce hepatic steatosis that are unique from obesity-induced hepatic steatosis.
- Characterization of URI1 bands in relation to growth/metabolic state.
- Use of both homozygous and heterozygous *Uri1* KO H4IIE cells. These experimental models gave strong evidence of the importance of URI1 for neutral lipid accumulation since *Uri1* KO H4IIE cells have reduced capacity for neutral lipid accumulation. The consequences of *Uri1* genetic ablation provides strong indication of what role the *Uri1* gene normally fulfills.
- Analysis of CRISPR/Cas9-induced gene edits and editing rate to develop hypotheses regarding the importance of *Uri1* for cell survival.
- First study investigating the role of URI1 in NAFLD using homozygous and heterozygous *Uri1* KO H4IIE cells and exhibiting the feasibility of generating *Uri1* KO H4IIE cells.
- Demonstration of 2 *Uri1* KO H4IIE clones possessing similar reduced capacity for neutral lipid accumulation, thus supporting that the observed difference in characteristic

between WT and *Uri1* KO H4IIE cells are related to the *Uri1* KO and not to CRISPR/Cas9 off-target effects.

Limitations

- Amount of t10,c12 CLA provided to mice is not attainable for humans through food or supplements.
- All mice used during the diet intervention study were male. Therefore, results in this study may have differed slightly for female mice.
- Lack of available information surrounding the different molecular weights of URI1 bands detected across different species via Western blotting.
- Sequencing of *Uri1* alleles in *Uri1* KO H4IIE cells was done by using an in-silico sequence analysis tool. The gold standard for characterizing indels resulting from CRISPR/cas9 gene editing is subcloning separate alleles into plasmids and sequencing the 2 alleles individually.
- WT H4IIE cells were used as controls when investigating *Uri1* KO H4IIE cells. H4IIE cells which undergo transduction with a non-targeting sgRNA and nucleofection with a Cas9 plasmid are the ideal control. This way, it is confirmed that differences in characteristics between control and *Uri1* KO H4IIE cells is not related to transduction, nucleofection or FACS.
- All heterozygous *Uri1* KO H4IIE clones were compound heterozygous *Uri1* KO H4IIE cells. *Uri1*^{+/-} 1 H4IIE cells contained an in-frame deletion in the *Uri1* allele which did not

contain a PTC and *Uri1*^{+/-} 2 H4IIE cells contained a SNP in the *Uri1* allele which does not have a PTC. The effect of these mutations on URI1 protein's functionality is not known.

Future Research

- Investigation of *in vitro* t10,c12 CLA-induced neutral lipid accumulation in WT and *Uri1* KO H4IIE cells. This would reveal if t10,c12 CLA also causes increased neutral lipid accumulation in H4IIE cells and whether it is facilitated by URI1.
- Measuring FAS protein levels in H4IIE cells after t10,c12 CLA treatment to determine if t10,c12 CLA directly increases hepatic *de novo* lipogenesis *in vitro*.
- BODIPY staining in liver sections of experimental mice for hepatic lipid droplet visualization and to complement hepatic TG concentration assay results.
- Using a TG assay to measure TG concentration in FFA treated *Uri1* KO and WT H4IIE cells to complement BODIPY staining results.
- Investigation of protein levels of molecular markers of cellular processes which have been revealed to be relevant for hepatic lipid accumulation (i.e. p-S6RP, FABP, p-JNK and etc.) by Western blotting in *Uri1* KO H4IIE cells. These experiments may help elucidate how URI1 contributes to hepatic lipid accumulation.
- Use of immunofluorescence to investigate the localization of lipid droplet associated proteins (LDAP) (perilipin 1 and 2) relative to lipid droplets in WT and *Uri1* KO H4IIE cells. Also, using Western blotting to measure the total amount of LDAP in WT and *Uri1* KO

H4IIE cells to complement the previous results. This would reveal if *Uri1* influences the expression of LDAPs and promotes neutral lipid accumulation through this mechanism.

- Measurement of insulin sensitivity in t10,c12 CLA treated H4IIE cells to investigate whether t10,c12 CLA causes insulin resistance in H4IIE cells. This can be done by using Western blotting to measure levels of insulin-stimulated phosphorylation of insulin signaling pathway proteins. Investigation into the effect of t10,c12 CLA on insulin sensitivity is crucial because insulin resistance is an important component of NAFLD.
- Measurement of insulin sensitivity in *Uri1* KO H4IIE cells to investigate whether URI1 affects insulin sensitivity using the method above.
- Investigate if genetic *Uri1* deletion in H4IIE cells results in increased expression of NKX3. This could reveal a mechanism by which URI1 promotes the development of HCC since URI1 has been shown to interrupt androgen receptor-mediated transcription of NK3 homeobox 1 (NKX3.1) (156), NKX3.1 expression is reduced in human HCC, and its overexpression in HCC cells reduces HCC cell proliferation and tumorigenicity (313).

Overall Conclusion

From reviewing the literature about NAFLD, t10,c12 CLA, and URI1, it was hypothesized that t10,c12 CLA will promote hepatic lipid accumulation through upregulation of URI1 expression and that genetic ablation of URI1 in H4IIE cells will result in reduced fatty acid induced neutral lipid accumulation. Experimentation with t10,c12 CLA-fed lean and *db/db* C57BL/6 mice revealed that t10,c12 CLA feeding results in increased hepatic lipid accumulation

and elevated URI1 protein levels. Furthermore, OA-induced neutral lipid accumulation was blunted in *Uri1* KO H4IIE cells compared to WT H4IIE cells. These results support the possibility that URI1 mediates hepatic lipid accumulation, and that promotion of hepatic steatosis by t10,c12 CLA feeding may involve upregulation of URI1 expression.

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