The role of *de novo* phosphatidylcholine synthesis in the gut-liver axis and intestinal homeostasis

by

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Abstract

The intestine and liver are in constant communication to maintain lipid processes and to prevent disease states such as non-alcoholic fatty liver disease (NAFLD) and colitis. Phosphatidylcholine (PC) can be synthesized in the liver by the CDP-choline pathway or the phosphatidylethanolamine N-methyltransferase pathway, while the small intestine is only capable of synthesizing PC through the CDP-choline pathway. Cytidine triphosphate:phosphocholine cytidylyltransferase-α (CTα) is the rate limiting enzyme in the CDP-choline pathway.

Hepatic CTα deletion in mice from birth causes reduced circulating very low-density lipoprotein levels and NAFLD when fed a high-fat diet (HFD). Our aim was to determine the impact of an acute deletion of hepatic CTα in adult mice on lipid handling in the liver (fasting) and intestine (postprandial). We found that chow- and HFD-fed acute CTα liver knockout ($CT\alpha^{LKO}$) mice quickly lose weight, have reduced fasting TG levels, and develop NAFLD. When analyzing lipid metabolism in the postprandial state, we also found that chow and HFD-fed $CT\alpha^{LKO}$ mice have reduced lipid absorption, leading to a reduction in the appearance of plasma TG. This data suggests that that hepatic CTα-derived PC synthesis is important for regulating lipid metabolism in fasting and postprandial states.

When CTα is deleted from intestinal epithelial cells (IECs) of HFD-fed adult mice ($CT\alpha^{IKO}$ mice), they present with weight loss, lipid malabsorption, and high postprandial plasma glucagon-like peptide 1 (GLP-1) levels. We aimed to characterize the changes that occur in the small intestines of $CT\alpha^{IKO}$ mice. We found that impaired *de novo* PC synthesis in the gut is linked to altered lipid metabolism and induction of endoplasmic reticulum (ER) stress, cell death, and inflammation. Induction of the host defence response in $CT\alpha^{IKO}$ mice was also associated with loss of goblet cells. Additionally, we found that impaired fatty acid uptake occurs in isolated intestinal

sacs from CTα^{IKO} mice. Antibiotic treatment prevented acute weight loss and normalized jejunum TG concentrations after refeeding but did not alter enhanced postprandial GLP-1 secretion, induction of host defence and ER stress transcripts, or loss of goblet cells in CTα^{IKO} mice. Dietary PC supplementation partially prevented loss of goblet cells but was unable to normalize jejunal TG or plasma GLP-1 concentrations after refeeding in CTα^{IKO} mice. Together these data show that there is a specific requirement from *de novo* PC synthesis in maintaining small intestinal homeostasis.

Patients with ulcerative colitis have low concentrations of the major membrane lipid, PC, in gastrointestinal mucus. Therefore, we aimed to determine the role that PC plays in colonic barrier function. Inducible loss of CTα in the intestinal epithelium reduced colonic PC concentrations and resulted in spontaneous colitis. Colitis development in CTα^{IKO} mice was traced to an ER stress response caused by an altered phospholipid composition. This ER stress response was linked to the necroptotic death of IECs leading to excessive loss of goblet cells, formation of a thin mucus barrier, elevated intestinal permeability, and infiltration of the epithelium by microbes. These experiments show that maintaining the PC content of IECs protects against colitis development and maintains colonic homeostasis.

Enterohepatic circulation and biliary homeostasis are critical for the digestion, absorption, and processing of lipids. PC has an important role in this cycle as it is the second most abundant component found in bile, after bile acids, and is heavily involved in lipid metabolism. We discovered that CTα^{IKO} mice have reduced postprandial circulating cholecystokinin (CCK) levels leading to enlarged gallbladders. Our aim was to determine whether improving the presence of bile in CTα^{IKO} mice could improve weight loss or lipid malabsorption. When CTα^{IKO} mice were injected with exogenous CCK they did not acutely lose weight yet still experienced lipid

malabsorption. When CTα^{IKO} mice were fed a diet supplemented with bile acids, there were no improvements in weight loss or lipid malabsorption.

In conclusion, CDP-derived PC is an important regulator of the gut-liver axis and is involved in maintaining cellular, organ and systemic homeostasis. This research demonstrates that maintaining appropriate levels of CDP-derived PC in the liver and intestine is necessary for appropriate fasting and postprandial lipid metabolism. Additionally, CDP-derived PC has an intricate role within the gut-liver axis and altering the pathway of CDP-choline synthesis leads to the development of disease states such as NAFLD and colitis.

Preface

This thesis is original work by Stephanie Carlin. All procedures regarding animal handling, feeding, and surgeries were approved by the University of Alberta's Institutional Animal Care Committee in accordance with guidelines of the Canadian Council on Animal Care. The contributions made by the candidate, Stephanie Carlin, and the co-authors of these studies, are described below.

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List of Abbreviations

AB/PAS	Alcian blue/Period acid-Schiff
ABCB4	ATP binding cassette subfamily B member 4
ABCB11	ATP binding cassette subfamily B member 11
ABCG5/8	ATP-binding cassette sub-family G member 5/8
ACAT2	Acyl-coenzyme A:cholesterol acyltransferase two
ACC	Acetyl-CoA carboxylase
ALT	Alanine transaminase
ApoB48	Apolipoprotein B48
ApoB100	Apolipoprotein B 100
ApoCII	Apolipoprotein C II
ApoE	Apolipoprotein E
ASBT	Apical sodium-dependent bile acid transporter
AST	Aspartate transaminase
ATF6	Activating transcription factor 6
ATGL	Adipose triglyceride lipase
BSEP	Bile salt export pump
CA	Cholic acid
CDCA	Chenodeoxycholic acid
CCK	Cholecystokinin
CDP	Cytidine diphosphate
CE	Cholesterol esters
CEPT	CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase
CES3	Carboxylesterase 3
ChREBP	Carbohydrate response element binding protein
CIDEC	Cell death inducing DFFA-like effector c
CMP	Cytidine monophosphate
CPT	CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CPT-1	Carnitine palmitoyltransferase 1
CSD	Choline supplemented diet
CTα	Cytidine triphosphate:phosphocholine cytidylyltransferase-α
$CT\alpha^{IKO}$	CTP:phosphocholine cytidylyltransferase alpha intestinal knockout
$CT\alpha^{LKO}$	CTP:phosphocholine cytidylyltransferase alpha liver knockout
$CT\alpha^{PLKO}$	CTP:phosphocholine cytidylyltransferase alpha permanent liver knockout
CTβ	CTP:phosphocholine cytidylyltransferase beta
CTP	Cytidine triphosphate
DAG	Diacylglycerol
DGAT1/2	Diacylglycerol acyltransferase 1/2
ER	Endoplasmic reticulum

FABP1/2	Fatty acid binding proteins1/2
FAS	Fatty acid synthase
FATP4	Fatty acid transport protein 4
FITC	Fluorescein isothiocyanate
FGF15/19	Fibroblast growth factor 15/19
FXR	Farnesoid X receptor
GI	Gastrointestinal
GLP-1	Glucagon-like peptide 1
GM-CSF	Granulocyte-macrophage colony-stimulating factor
H&E	Hematoxylin and eosin
HDL	High-density lipoprotein
HFD	High-fat diet
IBD	Inflammatory bowel disease
IEC	Intestinal epithelial cell
IFN-γ	Interferon-γ
IL	Interleukin
INSIG	Insulin induced gene
IPA	Ingenuity pathways analysis
IRE1α	Inositol-requiring enzyme 1-α
KO	Knockout
LFD	Low-fat diet
LDL	Low-density lipoprotein
LPCAT3	Lyso-phosphatidylcholine acyltransferase 3
LPS	Lipopolysaccharide
MAG	Monoacylglycerol
MCP-1	Monocyte chemoattractant protein-1
MDR2	Multidrug resistant gene-2 glycoprotein
MGAT2	Monoacylglycerol acyltransferase 2
MTP	Microsomal transfer protein
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis
NEFA	non-esterified fatty acid
NPC1L1	Niemann-Pick C1-Like 1
NTCP	Na/taurocholate cotransporting polypeptide
OATP1	Organic anion transporting polypeptide 1
OSTα/OSTβ	Organic solute transporter alpha and beta
PBA	4-phenyl butyrate
PC	Phosphatidylcholine
PCSD	Phosphatidylcholine supplemented diet
PE	Phosphatidylethanolamine

PEMT	Phosphatidylethanolamine N-methyltransferase
PERK	Protein kinase R-like ER kinase
PLA2	Phospholipase A2
PPi	Pyrophosphate
PUFA	Polyunsaturated fatty acid
S-AdoHyc	S-adenosyl-L-homocysteine
S-AdoMet	S-adenosyl-L-methionine
SCAP	SREBP cleavage activating protein
SR-B1	Scavenger receptor, class B type 1
SREBP-1c	Sterol response element binding protein one-c
TG	Triacylglycerol
TNF-α	Tumor necrosis factor-α
TUNEL	Terminal deoxynucleotidyl transferase dUTP nick end
UC	Ulcerative colitis
UPR	Unfolded protein response
VLDL	Very low-density lipoprotein
XBP-1	X-Box Binding Protein 1

Chapter 1

Introduction

1.1 Phosphatidylcholine

1.1.1 Phosphatidylcholine summary

Phosphatidylcholine (PC) is an amphipathic molecule containing a glycerol backbone with two hydrophobic fatty acid tails and a hydrophilic phosphocholine headgroup. PC is an ester phospholipid ideal for spontaneously forming lipid bilayers due to its cylindrical shape. As such, PC is the most abundant phospholipid found in the plasma membranes and organelles of mammalian cells (Van Meer, Voelker, & Feigenson, 2008). The importance of PC has not only been established for the structure of cells, but also for many signalling processes that occur within the cell and for regulating systemic lipid metabolism (Alvaro et al., 1986; Exton, 1994; Skipski et al., 1967). As the most abundant phospholipid secreted into bile, PC is important for the digestion of dietary lipids (Alvaro et al., 1986). PC creates the outer layer of mixed micelles facilitating the emulsification of fat globules in the dietary tract. Also, PC is important for lipid transport throughout the body as it is the most abundant phospholipid found in the outer layer of all lipoprotein particles (Skipski et al., 1967). The amphipathic properties and structure make PC uniquely invaluable in these processes. PC can create a monolayer surrounding the hydrophobic molecules in mixed micelles and lipoproteins with the fatty acid tails facing inward to allow for the dissolvement and transportation of bile salts and lipids within. Additionally, the hydrophilic outer surface that the phosphocholine head group supplies allow for the protection of membranes of surrounding tissues which would be easily damaged by the hydrophobic contents within (Karaman, Demirbilek, Sezgin, Gürbüz, & Gürses, 2003; Voshol et al., 2000).

1.1.2 Synthetic pathways

There are two *de novo* pathways for PC synthesis in the body including the CDP-choline pathway and the phosphatidylethanolamine N-methyltransferase (PEMT) pathway (Figure 1.1). The CDP-choline pathway (also referred to as the Kennedy pathway) is the most common pathway for PC synthesis and is performed in all cells that contain a nucleus in mammals (Kennedy and Weiss, 1956). The first step in the pathway involves the conversion of choline, an aqueous molecule obtained by absorption from dietary sources or by breakdown of PC molecules by phospholipases (Zhaoyu Li & Vance, 2008), to phosphocholine by choline kinase. Phosphocholine is then converted to cytidine diphosphate-choline (CDP-choline) by the enzyme cytidine triphosphate:phosphocholine cytidylyltransferase (CT) utilizing cytidine triphosphate as the energy source. This second step is the rate limiting step under physiologic conditions (Choy, Farren, & Vance, 1979; Choy, Paddon, & Vance, 1980; Vance & Choy, 1979). CDP-choline and diacylglycerol (DAG) are then converted to PC by CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) or by CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase (CEPT) (J. van der Veen et al., 2017). The PEMT pathway for PC synthesis occurs primarily in the liver and accounts for 30 % of PC synthesis in hepatocytes – where the remaining 70 % occurs from the CDP-choline pathway. This pathway involves the conversion of phosphatidylethanolamine (PE) to PC by three successive methylation reactions by the PEMT enzyme (Figure 1.1) (Bremer, Figard, & Greenberg, 1960; DeLong, Shen, Thomas, & Cui, 1999; Sundler & Akesson, 1975a, 1975b; Tasseva et al., 2016).

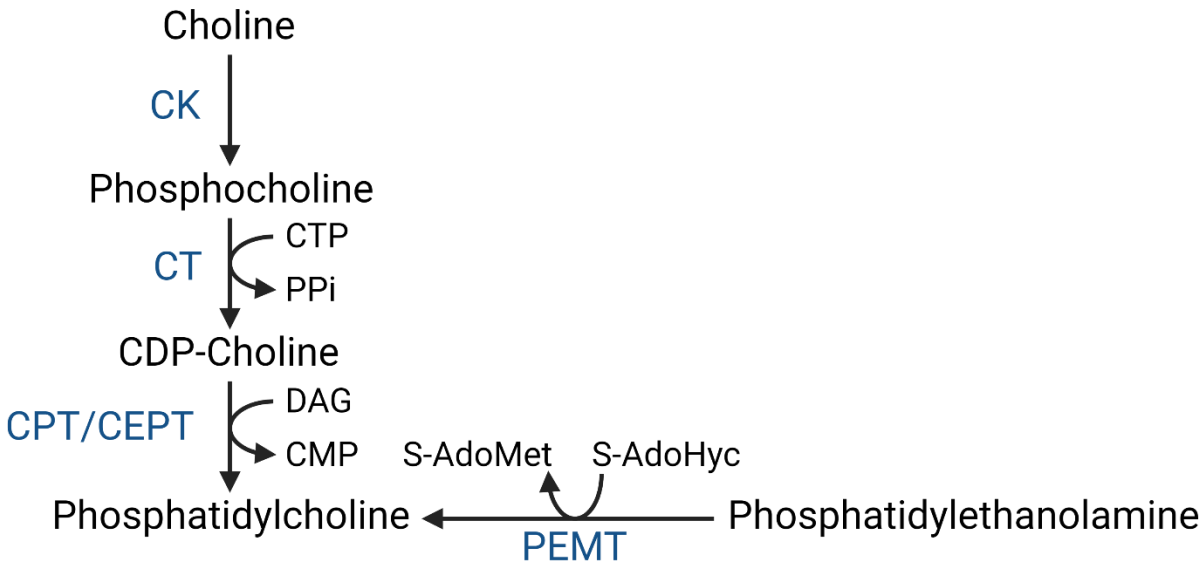

Figure 1.1: Phosphatidylcholine synthesis. Phosphatidylcholine can be synthesized through the CDP-Choline pathway whose precursor is choline. Phosphatidylcholine can also be synthesized through the PEMT pathway whose precursor is phosphatidylethanolamine. Enzymes are in blue. Abbreviations: CDP (cytidine diphosphate), CEPT (CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase), CK (choline kinase), CMP (cytidine monophosphate), CPT (CDP-choline:1,2-diacylglycerol cholinephosphotransferase), CT (cytidine triphosphate:phosphocholine cytidylyltransferase), CTP (cytidine triphosphate), DAG (diacylglycerol), PEMT (phosphatidylethanolamine N-methyltransferase), PPi (pyrophosphate), S-AdoHyc (S-adenosyl-L-homocysteine), S-AdoMet (S-adenosyl-L-methionine).

1.1.3 CTP:phosphocholine cytidylyltransferase

CT is the rate limiting enzyme in the choline pathway of PC synthesis (Choy et al., 1979, 1980; Vance & Choy, 1979). CT is an amphipathic protein that resides in a soluble, inactive state until binding to membranes where the protein becomes activated (Johnson, Xie, Singh, Edge, & Cornell, 2003). CT has increased binding to membranes when enriched with DAG and fatty acids (R. Cornell & Vance, 1987; S. L. Pelech, Pritchard, Brindley, & Vance, 1983). Once membrane bound the enzyme becomes active and is able to synthesize CDP-choline. There are two isoforms, CTα and CTβ. CTα is ubiquitously expressed but significant levels of CTβ are only found in brain and gonadal tissues of adults (Rosemary B. Cornell & Ridgway, 2015; Karim, Jackson, & Jackowski, 2003; Lykidis, Baburina, & Jackowski, 1999). The two main differences between the isoforms are that CTα has increased phosphorylation sites and a nuclear localization signal targeting the enzyme to the nucleus while CTβ resides in the cytosol (Rosemary B. Cornell & Ridgway, 2015).

PC synthesis and degradation are continually occurring within a cell to ensure total PC levels remain in homeostasis (Fagone & Jackowski, 2013; Steven L. Pelech & Vance, 1989). CTα has been shown to be an important regulator of total cellular PC. When CTα activity was stimulated, both PC synthesis and degradation rates were increased in order to maintain homeostatic levels (Tercé, Record, Tronchère, Ribbes, & Hugues, 1991; Walkey, Kalmar, & Cornell, 1994). There is also some evidence to suggest that through alterations in activity, CTα can alter alternative membrane lipid levels including DAG and phosphatidic acid (Steven L. Pelech & Vance, 1989).

CTα is encoded by the *Pcytla* gene and mRNA levels are regulated by transcriptional and post-transcriptional factors. The cell cycle is thought to be one of the most important

transcriptional regulators of *Pcytla* as CTα has an important role in cell growth and division (Hogan, Kuliszewski, Lee, & Post, 1996; Tessner, Rock, Kalmar, Cornell, & Jackowski, 1991). CTα activity is regulated by both the phosphorylation status of the protein and by membrane binding. CTα is fully phosphorylated when in the inactive, soluble state. Dephosphorylation is required for enzyme activity and increases membrane binding, though it has been shown that a phosphorylated CTα can bind to membranes prior to dephosphorylation (Hatch, Jamil, Utal, & Vance, 1992; Houweling, Jamil, Hatch, & Vance, 1994; Y. Wang, MacDonald, & Kent, 1993; Watkins & Kent, 1991). Additionally, CTα has an auto-inhibitory regulatory domain that blocks the catalytic site when in the soluble form (Y. Wang & Kent, 1995). During binding, the regulatory domain is inserted into the membrane which arrests the inhibition of the catalytic site (R. B. Cornell et al., 1995; Friesen, Campbell, & Kent, 1999; W. Yang, Boggs, & Jackowski, 1995).

Binding of the regulatory domain to membranes also leads to increased dephosphorylation of CTα and increased enzyme activity (Houweling et al., 1994). CTα protein and subsequent activity levels are increased during times of membrane enlargement to provide the necessary PC component for growth. CTα expression has been shown to increase during cell division, during times of increased lipid droplet formation, and under times of endoplasmic reticulum (ER) stress such as during the unfolded protein response (Aitchison, Arsenault, & Ridgway, 2015; Fagone et al., 2007; Krahmer et al., 2011; Sriburi et al., 2007). The increase in activity levels can be attributed to an increase in translation of CTα, by reduced turnover of the enzyme and by an increase in DAG levels in membranes (Aitchison et al., 2015; Fagone et al., 2007; Krahmer et al., 2011; Sriburi et al., 2007).

1.2 Role of phosphatidylcholine in liver health

1.2.1 Lipid metabolism of the liver

The liver is an important organ in lipid homeostasis. The liver is involved in synthesizing, storing, secreting, absorbing, and degrading lipids in order to maintain fuel sources and energy levels during fluctuating times of fasting and feeding (Hodson & Gunn, 2019). Hepatocytes are the major cells of the liver where these lipid processes occur. During times of feeding the hepatocytes synthesize *de novo* fatty acids to store as fuel for energy (Figure 1.2). Fatty acid synthesis occurs in times of high metabolic substrates, high glucose levels, and high insulin levels. Acetyl-CoA is the building block of fatty acids. In the cytosol, acetyl-CoA is converted to malonyl-CoA by acetyl-CoA carboxylase (ACC) enzymes which is the rate limiting step in fatty acid synthesis. Also in the cytosol, malonyl-CoA then undergoes a series of elongation reactions by fatty acid synthase (FAS) to create primarily palmitic acid, a saturated 16 carbon fatty acid (Hodson & Gunn, 2019). Palmitic acid is the most common fatty acid in the body and can also be elongated and desaturated on the ER into almost all other fatty acids that the hepatocytes are capable of synthesizing.

The transcription of ACC and FAS is under the regulation of transcription factors including carbohydrate response element binding protein (ChREBP) and sterol response element binding protein one-c (SREBP-1c) which are activated during times of high glucose and high insulin respectively (Ferré & Foufelle, 2010; Linden et al., 2018; Uyeda & Repa, 2006). ChREBP is located in the cytosol in the inactive state and is activated in the presence of glycolysis by-products to enter the nucleus and increase the expression of ACC and FAS genes (Uyeda & Repa, 2006). SREBP-1c is located in the ER in the inactive state complexed to two other proteins – SREBP

cleavage activating protein (SCAP) and insulin induced gene (INSIG). Insulin activation leads to the dissociation of INSIG and the SREBP-1c-SCAP complex is able to travel to the Golgi apparatus where SREBP-1c is cleaved to its active, mature form, which can enter the nucleus and increase expression of ACC and FAS genes (Ferré & Foufelle, 2010). While ChREBP and SREBP-1c are independently activated by glucose and insulin, there is evidence that they are cooperate with each other as both glucose and insulin need to be present for activation of either transcription factor (Figure 1.2) (Linden et al., 2018). Excess of fatty acids are stored as TG molecules whose synthesis primarily occurs on the ER, but smaller amounts are also synthesized on lipid droplets and the nuclear envelope (Hodson & Gunn, 2019).

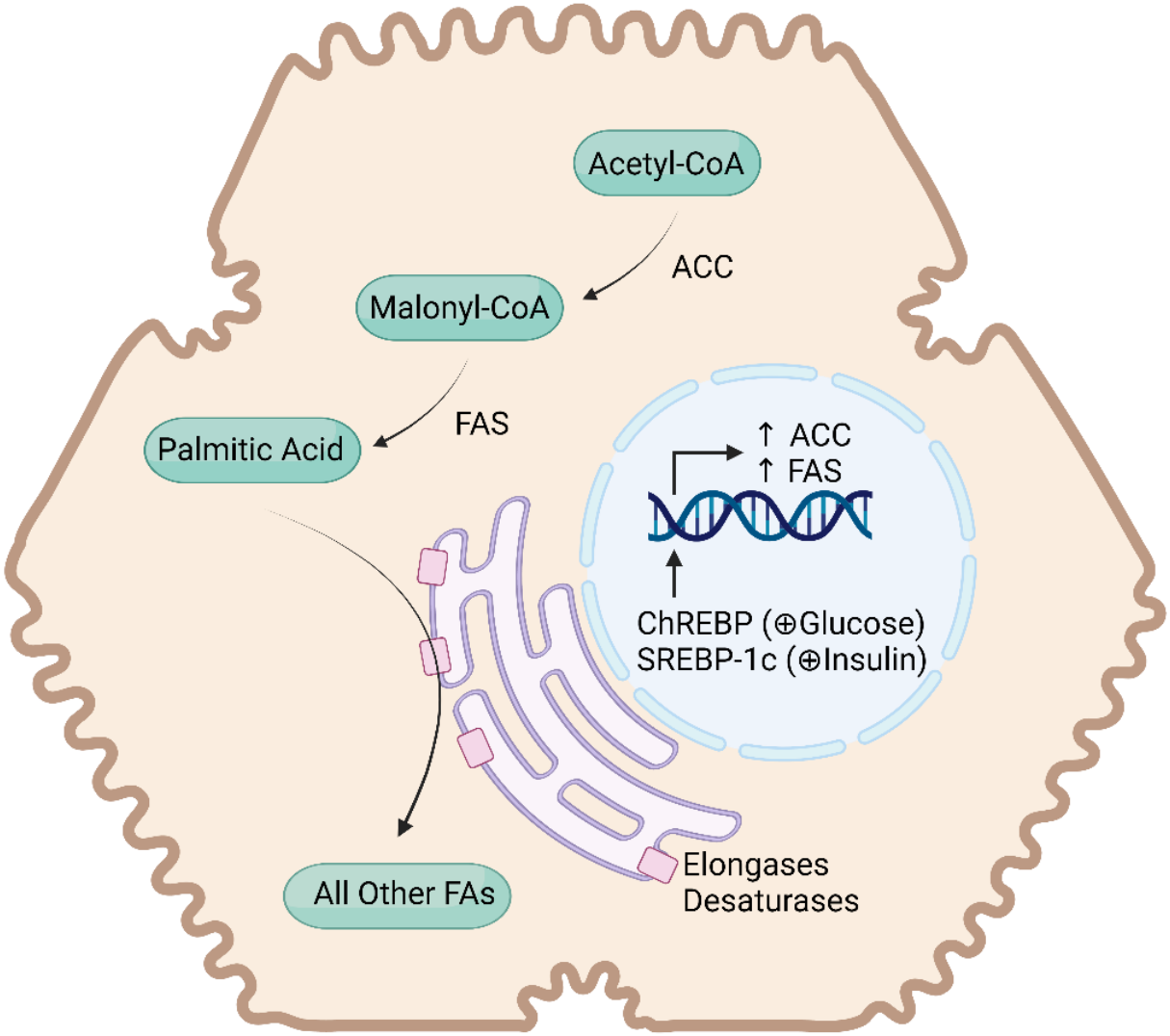

Figure 1.2: *De novo* fatty acid synthesis in hepatocytes. The precursor for fatty acid synthesis is acetyl-CoA. Synthesis begins in the cytosol where acetyl-CoA is converted to malonyl-CoA by ACC. Malonyl-CoA is then synthesized to palmitic acid by FAS. Palmitic acid can then be converted to all other fatty acids (that hepatocytes are capable of synthesizing) on the endoplasmic reticulum by elongases and desaturases. ChREBP and SREBP-1c are transcription factors activated by glucose and insulin respectively that increase the expression of ACC and FAS. Abbreviations: ACC (acetyl-CoA carboxylase), ChREBP (carbohydrate response element binding protein), FAs (fatty acids), FAS (fatty acid synthase), SREBP-1c(sterol response element binding protein one-c).

As fatty acids and TG molecules can be lipotoxic to hepatocyte cells, they are stored as lipid droplets (Figure 1.3) (Egnatchik, Leamy, Jacobson, Shiota, & Young, 2014; Listenberger et al., 2003). Lipid droplets can contain fatty acids and TG from *de novo* lipogenesis, from the uptake of circulating lipoproteins, and from non-esterified fatty acids (NEFAs) released from adipocytes. Lipid droplets are made of a neutral lipid core, primarily TG and cholesterol esters, surrounded by a monolayer of phospholipids (DiAugustine, Schaefer, & Fouts, 1973; Thiam, Farese, & Walther, 2013). Within, or associated with, the phospholipid monolayer is a variety of proteins which are involved in trafficking, signaling, and lipid metabolism (Bersuker et al., 2018; Khan, Wollaston-Hayden, Markowski, Higgins, & Mashek, 2015). The levels of these proteins depend on the nature of the lipid droplets, the nutritional state, and the needs of the cell (Bersuker et al., 2018; Kramer, Quiroga, Lian, Fahlman, & Lehner, 2018). Lipid droplets initially form as an accumulation of neutral lipids between the ER phospholipid bilayer (Joshi et al., 2018; Seebacher, Zeigerer, Kory, & Krahmer, 2020). Eventually, the accumulation of neutral lipids grows and buds off of the ER creating a lipid droplet, though the mechanism of how this occurs has not been elucidated (Seebacher et al., 2020).

The size and stability of lipid droplets are dependent on multiple factors, one being the ratio of phospholipids found in the surrounding monolayer (Aitchison et al., 2015; Krahmer et al., 2011). The most abundant phospholipid in lipid droplet is PC followed by PE. PC stabilizes lipid droplets and if the levels of PC to PE in lipid droplets fall, they become destabilized. Lipid droplets high in PE will coalesce to form fewer, larger lipid droplets. Unsurprisingly, a reduction of PC in lipid droplets leads to the activation of CTα to increase PC synthesis (Aitchison et al., 2015; Krahmer et al., 2011; Sołtysik, Ohsaki, Tatematsu, Cheng, & Fujimoto, 2019). There are also proteins on the surface of lipid droplets which influence their size like cell death inducing DFFA-

like effector c (CIDEC) proteins which are able to promote the exchange of lipids between various lipid droplets (Figure 1.3) (Gong et al., 2011; Langhi & Baldán, 2015).

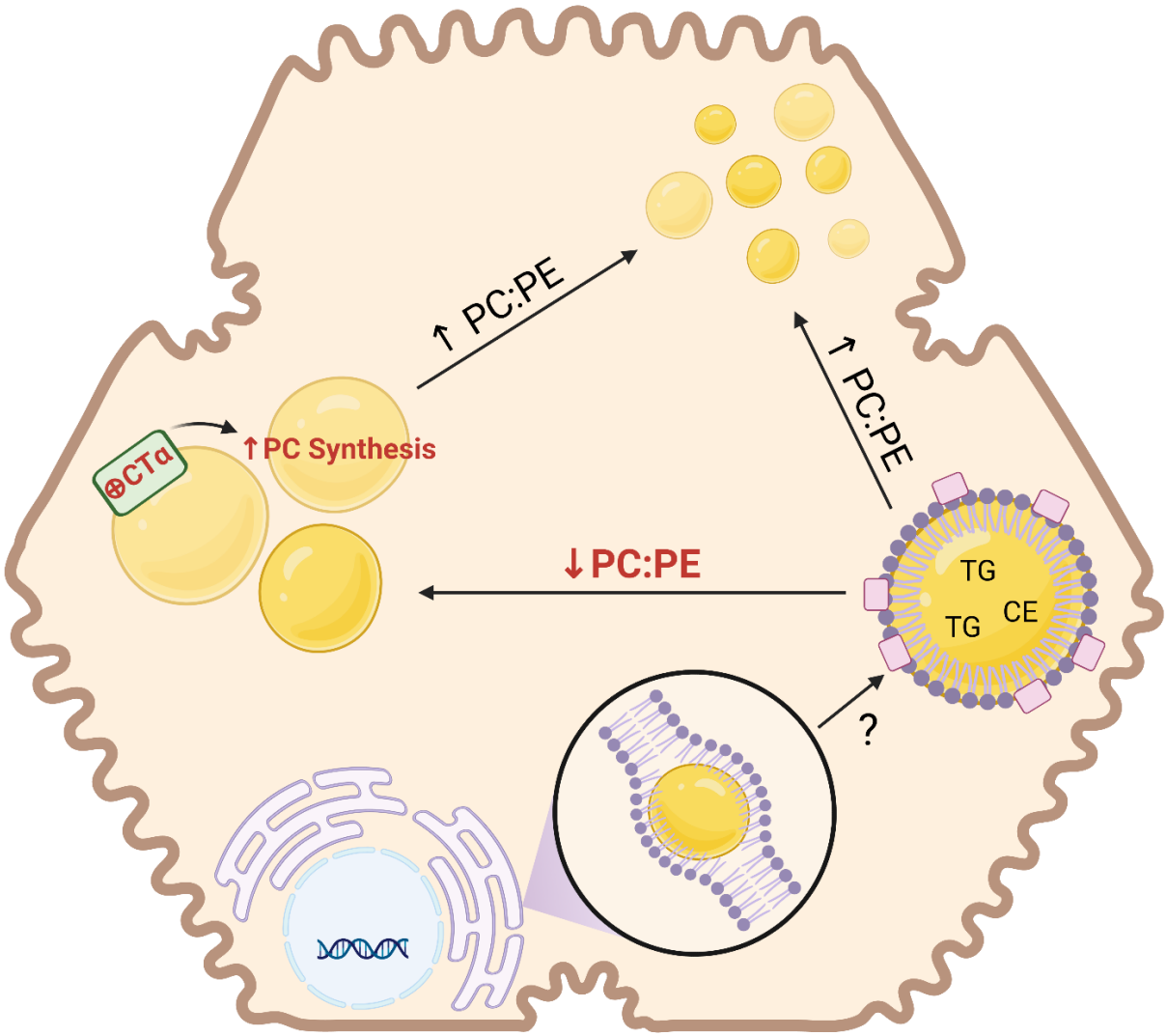

Figure 1.3: Lipid droplet formation in hepatocytes. Lipid droplet formation begins as an accumulation of neutral lipids between the leaflets of the ER membrane. The lipid accumulation eventually buds off through unknown mechanisms to form a lipid droplet, coated in phospholipids and proteins. A stable lipid droplet has an increased PC:PE ratio leading to smaller, more numerous lipid droplets. If the PC:PE ratio is reduced fewer, larger, and unstable lipid droplets form activating CTα to increase the synthesis of PC. The increase in lipid droplet PC restores the PC:PE ratio leading to the formation of stable lipid droplets. Abbreviations: CE (cholesterol ester), PC (phosphatidylcholine), PE (phosphatidylethanolamine), TG (triacylglycerol).

Under physiologic conditions, lipid droplets do not remain in hepatocytes for storage but are targeted for either secretion from the cell in the form of very low-density lipoproteins (VLDLs) or for degradation. Lipid droplets are primarily degraded by lipolysis, though some are targeted for lipophagy (Figure 1.4). Lipid droplets that undergo lipophagy are initially surrounded by a double membrane complex from the ER known as a phagophore to create an autophagosome. The autophagosome is targeted to lysosomes for degradation of the lipid droplet (Singh & Cuervo, 2012; Singh et al., 2009). Lipolysis of lipid droplets involves the release of fatty acids from the neutral lipid pool by lipases. One of the most important lipases involved in the turnover of lipids in hepatocytes is adipose triglyceride lipase (ATGL) (Haemmerle et al., 2006). ATGL has been shown to be important for mobilizing fatty acids for β-oxidation (Ong, Mashek, Bu, Greenberg, & Mashek, 2011; Reid et al., 2008).

Fatty acids are able to enter the mitochondria for β-oxidation via the carnitine palmitoyltransferase 1 (CPT-1) transporter (Houten, Violante, Ventura, & Wanders, 2016). CPT-1 is inhibited by malonyl-CoA, the first product produced in *de novo* lipogenesis, and subsequently inhibits β-oxidation (Houten et al., 2016; McGarry, Mannaerts, & Foster, 1977). Within the mitochondria, fatty acids are degraded to acetyl-CoA molecules which are then targeted for the citric acid cycle or ketogenesis depending on oxaloacetate supply and the nutritional status of cell. During times of feeding, glycolysis produces an excess of oxaloacetate which can combine with acetyl-CoA in the mitochondria and the complex can enter the citric acid cycle for energy production. During times of extreme fasting, the supply of oxaloacetate in hepatocytes is used for gluconeogenesis and acetyl-CoA then enters ketogenesis to provide an alternative fuel source for the body (Figure 1.4) (Bach, 1978; Hodson & Gunn, 2019).

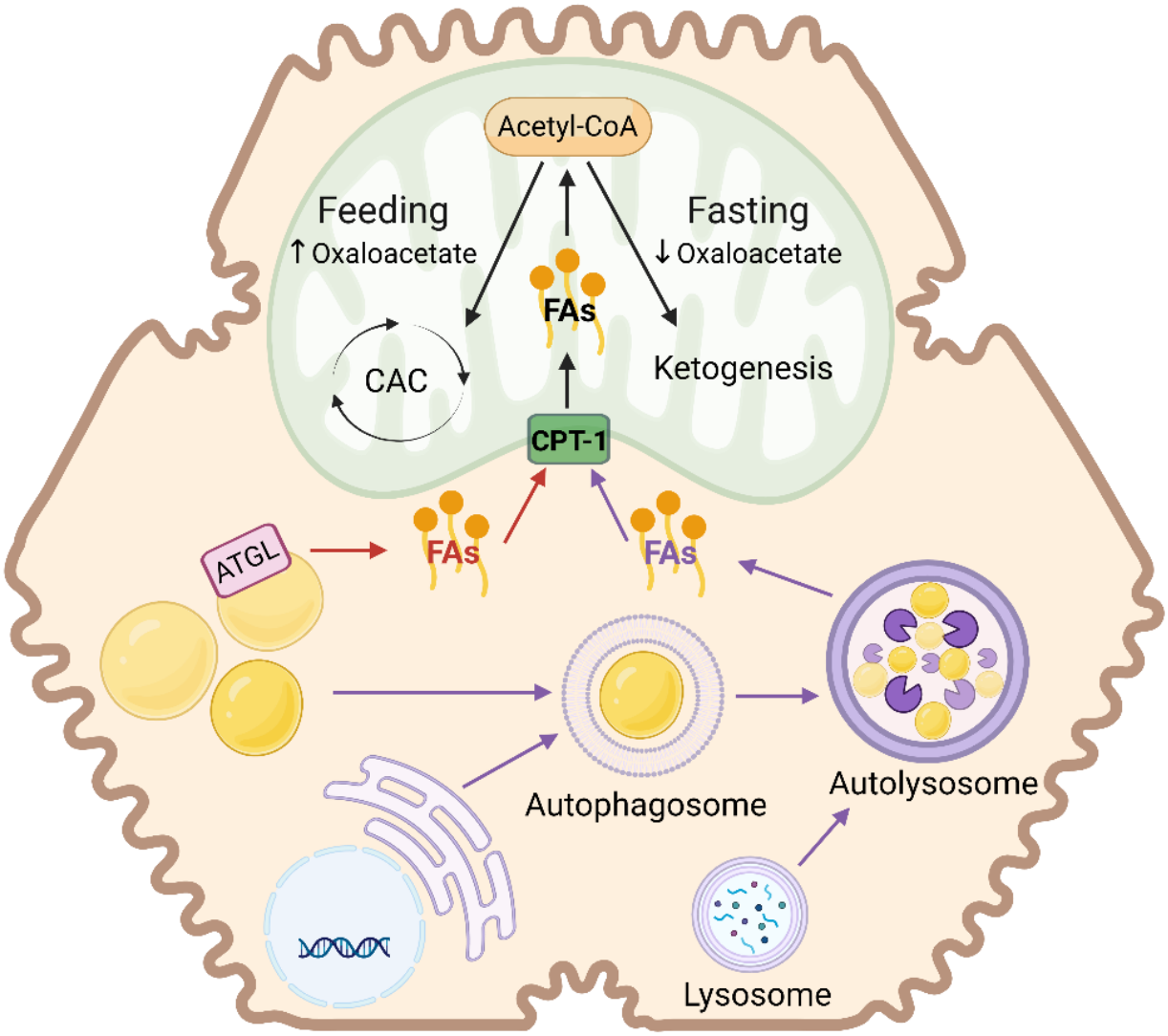

Figure 1.4: Degradation of lipid droplets in hepatocytes. Lipid droplet degradation can occur through lipolysis (red arrows) or lipophagy (purple arrows). Lipolysis involves the release of fatty acids from lipid droplets. ATGL is important in targeting fatty acids for β-oxidation through lipolysis. Lipophagy involves coating lipid droplets with membrane bilayer from the ER creating an autophagosome. The autophagosome fuses with a lysosome to create an autolysosome which breaks down the lipid droplet releasing fatty acids targeted for β-oxidation. Fatty acids enter the mitochondria through the CPT-1 transporter and are broken down to acetyl-CoA molecules. In the fed state, levels of oxaloacetate are high, and acetyl-CoA is targeted to the CAC for energy production. In the fasting state, levels of oxaloacetate are low, and acetyl-CoA is targeted to ketogenesis to create an alternative fuel source. Abbreviations: ATGL (adipose triglyceride lipase), CAC (citric acid cycle), CPT-1 (the carnitine palmitoyltransferase 1), FAs (fatty acids).

The alternate fate of hepatic lipid droplets involves transportation out of the cell via VLDL particles which contain apolipoprotein B 100 (ApoB100) (Figure 1.5) (Lehner, Lian, & Quiroga, 2012). The process of VLDL synthesis begins with the translation of ApoB100. In the smooth ER, ApoB100 undergoes primary lipidation with TG, phospholipid and cholesterol ester molecules by microsomal transfer protein (MTP) (Jiang, Liu, Hussain, Atkinson, & McKnight, 2008; Lehner et al., 2012; Rava, Ojakian, Shelness, & Hussain, 2006). This primordial VLDL particle then undergoes a secondary lipidation through unknown mechanisms to create a mature VLDL particle. There are two main hypotheses surrounding the secondary lipidation of VLDL – the first being that TG in lipid droplets first undergo lipolysis then are reesterified into TG molecules within VLDL particles. The second theory – whose evidence is lacking support – is that the primordial VLDL particle fuses with lipid droplets (Lehner et al., 2012). Carboxylesterase 3 (CES3) is a lipase that has been shown to independently target lipids to VLDL particles. When Ces3 was knocked out in mice, there was a reduction in TG rich VLDL in circulation with an increase in hepatic β-oxidation without a large increase in hepatic lipid droplet formation (Lian et al., 2012).

The levels of mature VLDL produced by hepatocytes is determined by the secondary lipidation step, if this step does not occur the primordial VLDL particle is degraded (Ohsaki, Cheng, Suzuki, Fujita, & Fujimoto, 2008). VLDL undergoes COPII mediated transfer to the Golgi apparatus where other proteins, including apolipoprotein C II (ApoCII) are added to the particle before entering circulation (Figure 1.5) (Gusarova, Brodsky, & Fisher, 2003). In circulation, endothelial lipoprotein lipase (LPL) enzymes are activated by ApoCII and hydrolyze TG molecules from VLDL to mobilize fatty acids (Acta, 1985; LaRosa, Levy, Herbert, Lux, & Fredrickson, 1970; Ramasamy, 2014). As VLDLs lose the neutral lipid core they become intermediate density lipoproteins (IDLs) and pick up apolipoprotein E (ApoE) proteins from

circulating high-density lipoproteins (HDL). IDLs in circulation can either bind to low-density lipoprotein (LDL) receptors via ApoE particles and be taken out of circulation by hepatocytes or continue to be hydrolyzed until they form LDL particles. LDL particles are eventually also taken up in hepatocytes by the binding of ApoB100 to LDL receptors (Ramasamy, 2014).

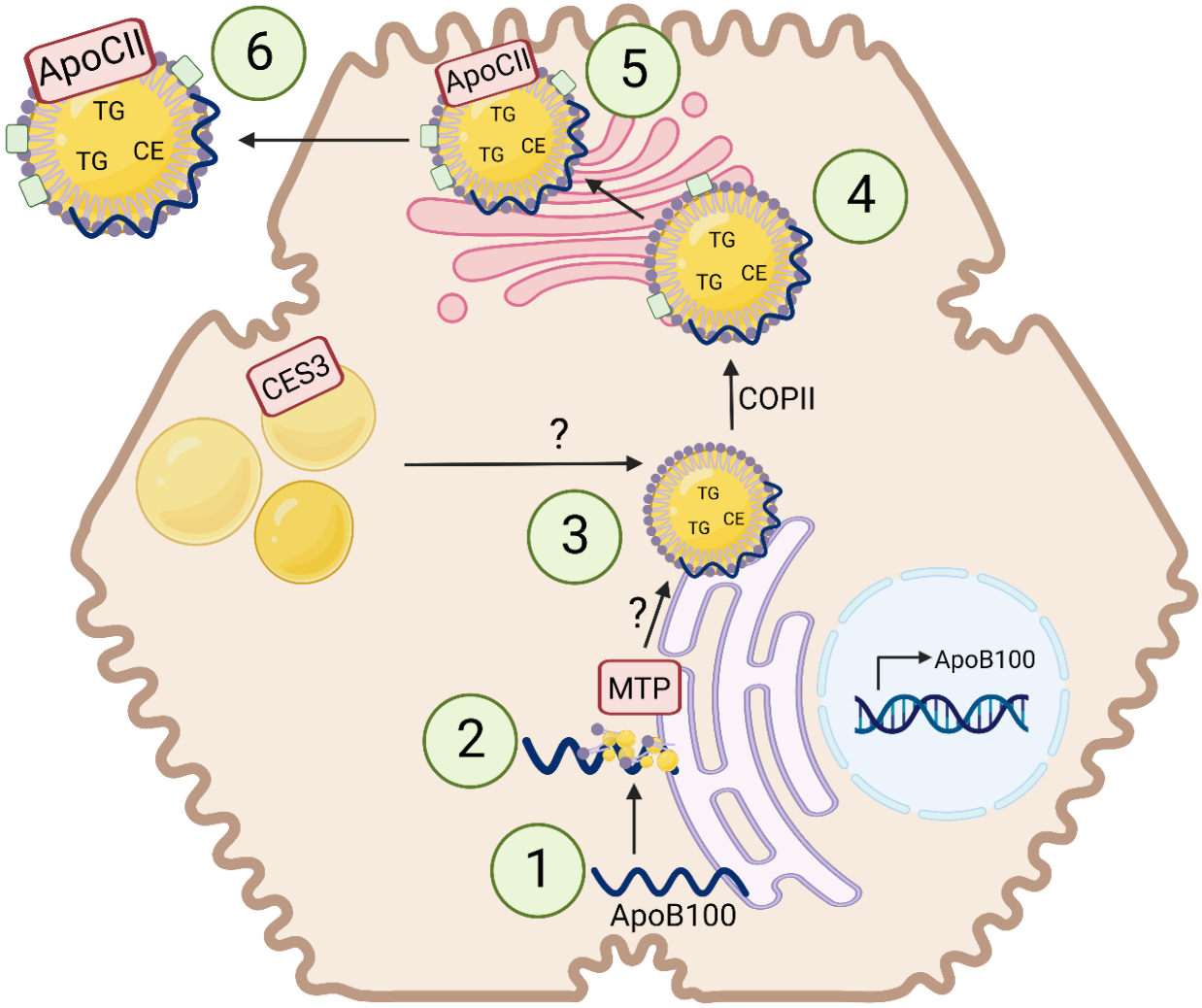

Figure 1.5: VLDL synthesis in hepatocytes. Synthesis of VLDL particles begins with continuous transcription and translation of the ApoB100 protein (1). ApoB100 on the ER is initially lipidated with TG, CE and phospholipids with the help of MTP creating a primordial VLDL particle (2). Through unknown mechanisms, the primordial VLDL particle is lipidated with lipids from lipid droplet to form a mature VLDL particle. CES3 is important for targeting lipids from lipid droplets towards the formation of VLDL particles (3). The mature VLDL particle undergoes COPII mediated transport to the Golgi apparatus (4) where proteins including ApoCII are added to the lipoprotein (5). The VLDL particle is then secreted into circulation for systemic lipid transport (6). Abbreviations: ApoB100 (apolipoprotein B100), ApoCII (apolipoprotein CII), CE (cholesterol esters), CES3 (carboxylesterase 3), MTP (microsomal transfer protein), TG (triacylglycerol).

1.2.2 Non-alcoholic fatty liver disease

The incidence of non-alcoholic fatty liver disease (NAFLD) has been on the rise and is estimated to affect 25 % of the global population (Younossi et al., 2016). NAFLD incidence is also commonly associated with co-morbidities that include hypertension, obesity, hyperlipidemia, type 2 diabetes and the metabolic syndrome (Younossi et al., 2016). NAFLD encompasses a group of diseases ranging from simple steatosis, or fatty liver, to the development of non-alcoholic steatohepatitis (NASH) (Matteoni et al., 1999). NASH involves increased fatty liver deposits, oxidative stress, inflammation, and fibrosis. As the liver can regenerate, even severe forms of NAFLD are reversible, though the progression of NAFLD to cirrhosis or hepatocellular carcinoma is irreversible and has much higher mortality rate (Buzzetti, Pinzani, & Tsochatzis, 2016; Matteoni et al., 1999). Most persons with NAFLD do not show any signs or symptoms and those with simple steatosis alone have the same life-expectancy as the general population (Buzzetti et al., 2016). Unfortunately, the progression of NAFLD to the more severe liver pathologies are not consistent with the severity of NAFLD alone (Ekstedt, Franzén, Mathiesen, & Kechagias, 2012). Despite these findings, an increase in hepatocellular fibrosis has been shown to have poorer outcomes than simple steatosis alone (Angulo et al., 2015). With the current data, it is estimated that the development of hepatocellular carcinoma from NAFLD is low, occurring in 0.44/1000 persons (Matteoni et al., 1999).

There are multiple theories on how NAFLD is developed and progressed. The multiple hit theory is the most recent and is supported by the most evidence (Buzzetti et al., 2016). This theory states that the liver undergoes multiple simultaneous or progressive insults from factors such as genetics, dietary habits, obesity, and insulin resistance – leading to the development of steatosis and fibrosis (Buzzetti et al., 2016). While the development and progression of NAFLD is still

debated, there are commonalities affecting the liver such as increased fatty liver deposits, mitochondrial dysfunction, oxidative stress, ER stress, inflammation, and fibrosis (Buzzetti et al., 2016). The increase in fatty deposits in hepatocytes has been shown to be due to a combination of increased flux of NEFAs from adipocytes, *de novo* lipogenesis, and reduced lipid degradation. In NAFLD, adipocyte regulation of lipolysis is altered leading to an increase in the quantity of NEFAs reaching the liver. Insulin resistance is a common cause of this dysfunction in persons affected by NAFLD (Buzzetti et al., 2016). Insulin resistance is also known to cause an increase in *de novo* lipogenesis in hepatocytes due to a variety of factors including an increased expression of SREBP-1c (Shimomura, Bashmakov, & Horton, 1999). SREBP-1c and ChREBP transcription factors are activated in NAFLD, contributing to the expression and activity of enzymes involved in the *de novo* lipogenesis pathway (Dentin, Girard, & Postic, 2005; Iizuka, Bruick, Liang, Horton, & Uyeda, 2004; Kugimiya, Takagi, & Uesugi, 2007; Shimomura et al., 1999).

While increased NEFA flux and *de novo* lipogenesis contribute the most to lipid accumulation in NAFLD, the contribution of lipid degradation is still debated. There is evidence that autophagy pathways are reduced in steatotic livers, but evidence in humans shows no change in hepatic β-oxidation pathways (Kotronen et al., 2009; Sanyal et al., 2001; L. Yang, Li, Fu, Calay, & Hotamisligil, 2010). Despite the controversy of β-oxidation capacity of hepatocytes, mitochondria are still severely affected in NAFLD leading to structural and functional changes. Mitochondria from NAFLD livers have reduced mitochondrial DNA, respiration, and ATP production (Cortez-Pinto et al., 1999; Pérez-Carreras et al., 2003; Pessayre & Fromenty, 2005). These changes cause an increase in toxic lipid metabolites and reduced flux of electrons through the respiratory chain leading to the development of reactive oxygen species and oxidative stress in hepatocytes (Hensley et al., 2000; S. Yang et al., 2000). Reactive oxygen species can lead to a

further reduction in respiration and increased damage to mitochondrial DNA (D. Gao et al., 2004). Reactive oxygen species are also thought to be an activator of specific liver cells – including stellate and Kupffer cells. These specialized liver cells have an important function in the progression of NAFLD as they are involved in the development of inflammation and fibrosis (Canbay et al., 2003; Rivera et al., 2007; Tomita et al., 2006). The ER is also affected by the changes occurring in hepatocytes including the reduction in ATP produced by mitochondria, by a reduction in PC levels, and by oxidative stress. These processes lead to the activation of ER stress, specifically the unfolded protein response (Kammoun et al., 2009; Ozcan et al., 2004). The unfolded protein response has also been associated with the activation of SREBP-1c, contributing to increased fatty acid synthesis and steatosis in the liver (Zhang et al., 2012). Together, the steatosis, cellular stress, and inflammation and fibrosis characterize NAFLD severity.

1.2.3 Effects of altered phosphatidylcholine levels in the liver

As stated above, liver synthesizes *de novo* PC through two distinct pathways; 70 % via the CDP-choline pathway while the remaining 30 % is from via the PEMT pathway (DeLong et al., 1999). The independent roles of PC derived from these two pathways have been analyzed in the regulation of liver function. As whole-body CTα knockouts are embryonically lethal, a hepatocyte specific CTα knockout ($CT\alpha^{LKO}$) mouse was developed to analyze the role of the PC derived from the choline pathway (Jacobs, Devlin, Tabas, & Vance, 2004; L. Wang, Magdaleno, Tabas, & Jackowski, 2005). When chow-fed $CT\alpha^{LKO}$ mice were fasted, they had reduced circulating levels of plasma TG corresponding to a reduction in HDL and VLDL levels. While the lipoprotein reduction was gender independent, only female $CT\alpha^{LKO}$ mice had an accumulation of hepatic TG without an increase in plasma markers of liver damage, including aspartate transaminase (AST)

and alanine transaminase (ALT). Finally, chow-fed CTα^{LKO} mice had an increase in PEMT and CTβ protein and activity levels to compensate for the reduction in PC synthesis through the CTα pathway (Jacobs et al., 2004). When CTα^{LKO} mice were switched to a HFD, they developed NASH within 1 week (Niebergall, Jacobs, Chaba, & Vance, 2011). HFD-fed CTα^{LKO} mice had increased hepatic TG accumulation, inflammation, and oxidative stress – all markers of NAFLD. Additionally, HFD-fed CTα^{LKO} mice had increased plasma ALT levels. PC levels were manipulated in HFD-fed CTα^{LKO} mice by providing alternative sources of PC substrates, including lysophosphatidylcholine or betaine. These treatments were unable to fully prevent the development of NAFLD, indicating an important role for PC derived from *de novo* PC synthesis through the CTα pathway in hepatocytes functioning (Niebergall et al., 2011).

The alternative route of hepatic *de novo* PC synthesis has been also analyzed through whole-body knockouts of PEMT (PEMT$^{-/-}$) in mice. Initially, chow-fed PEMT$^{-/-}$ mice were thought to not have altered hepatic PC levels, nor significant changes in plasma or liver lipid levels, and normal hepatocyte morphology (Walkey, Donohue, Bronson, Agellon, & Vance, 1997). Recently, chow-fed PEMT$^{-/-}$ mice were found to have a small increase in ER stress associated with a reduced hepatic ER membrane PC level and to develop steatosis on histology without a significant increase in hepatic TG levels. These mild changes are thought to be due to a compensatory increase in hepatic PC levels through the CDP-choline pathway. PEMT-derived PC has been shown to be more important under times of liver stress. When PEMT$^{-/-}$ mice are fed choline-deficient diet for only 3 days, reducing the ability of hepatocytes to compensate with increased PC synthesis through the choline pathway, they accumulate hepatic TG and develop liver failure. Choline-deficient PEMT$^{-/-}$ mice also have huge reductions in hepatic PC and plasma lipid levels indicating PEMT pathway of PC synthesis is important under times of choline

restriction (Walkey, Yu, Agellon, & Vance, 1998). Male PEMT$^{-/-}$ mice fed a high-fat/high-cholesterol diet accumulate hepatic TG and have reduced plasma TG and VLDL levels (Noga & Vance, 2003a, 2003b). When PEMT$^{-/-}$ mice are fed a HFD for 10 weeks, they develop fatty liver and NASH despite not gaining weight and remaining insulin sensitive (Jacobs et al., 2010; J. N. Van Der Veen et al., 2019). At present, there remains controversy surrounding the expression and importance of PEMT in non-hepatic tissues, therefore a hepatic specific PEMT$^{-/-}$ mouse model was created. HFD-fed hepatic PEMT$^{-/-}$ mice maintain a similar phenotype as whole-body knockouts as they do not gain weight and remain insulin sensitive while developing fatty liver and NASH (Wan, van der Veen, et al., 2019). Finally, HFD-fed PEMT$^{-/-}$ mice have a more significant reduction in hepatic ER membrane PC level, leading to ER stress and activation of the unfolded protein response, an important component of NAFLD (X. Gao et al., 2015).

There is a clear link between phospholipid levels, specifically PC, and the development of NAFLD. Interestingly, the ratio between PC and PE in the cell is more important than either level alone (Zhaoyu Li et al., 2006; J. Ling, Chaba, Zhu, Jacobs, & Vance, 2012). While PC is the most abundant phospholipid found in mammalian cell membranes, PE is also enriched in membranes (Van Meer et al., 2008). Biopsies from the livers of patients with NASH show a reduction in hepatic PC:PE ratio (Zhaoyu Li et al., 2006). Both CTα^{LKO} mice and PEMT$^{-/-}$ mice that develop NAFLD also have a reduced PC:PE ratio (Jacobs et al., 2010; Niebergall et al., 2011). The ratio of PC:PE in both these mouse models was also shown to be inversely correlated to the severity of NAFLD development (J. Ling et al., 2012). After a partial hepatectomy, the ratio of PC:PE was also inversely correlated with survival in these mice. Finally, improving the PC:PE ratio in PEMT$^{-/-}$ mice improved NAFLD and improved survival after partial hepatectomy. To prove the PC:PE ratio was a better indicator of liver health than PC levels alone, a PEMT/multi drug-resistant

protein 2 (MDR2) double knockout mouse was created (PEMT/MDR2^{DKO}). MDR2 is the protein responsible for the secretion of PC into bile and MDR2 knockout mice develop liver damage (Smit et al., 1993). When PEMT/MDR2^{DKO} mice were fed a choline deficient diet there was a significant reduction in both hepatic PC and PE levels. Despite the reduced phospholipid levels, the PC:PE ratio was not severely affected and the PEMT/MDR2^{DKO} mice were protected from liver failure and the development of NAFLD (Zhaoyu Li et al., 2006). More recently, it has been discovered that not only a reduction in the PC:PE ratio can lead to an impaired membrane but also an increase in the PC:PE ratio above physiological levels (Fu et al., 2011; Martínez-Uña et al., 2013; J. van der Veen et al., 2017). Together, this research shows the important role of maintaining a physiologic PC:PE ratio to protect the liver from NAFLD development and maintain hepatic lipid homeostasis.

1.3 Role of phosphatidylcholine in small intestinal health

1.3.1 Lipid metabolism of small intestine

Dietary lipid digestion begins in the stomach and is both mechanical and enzymatic. As the stomach contracts the mechanical action allows for an initial emulsification of lipids and allows for the hydrolysis of triacylglycerols (TG) to diacylglycerols (DAG) and fatty acids by gastric lipase. In adults, gastric lipase only has a minimal role in lipid digestion (Bernbäck, Bläckberg, & Hernell, 1989; Ko et al., 2020). When the acidic contents from the stomach reach the duodenum – the proximal segment of the small intestine – they stimulate the release of pancreatic and intestinal secretions containing bicarbonate to neutralize the pH and allow for processing by digestive enzymes. Dietary lipids also stimulate the release of bile and pancreatic secretions for digestion (Ko et al., 2020). In the duodenum, dietary lipids are mixed with lipids secreted in the bile as well

as lipids released from the shedding of enterocytes for digestion and absorption (Figure 1.6) (Shiau, Popper, & Reed, 1985). Bile acids secreted in bile allow for further emulsification of lipids, creating mixed micelles which can be acted upon by lipases (Hofmann, 1999). Mixed micelles have a neutral lipid core containing TG, cholesterol esters (CE) and other neutral lipids, surrounded by a phospholipid monolayer. Mixed micelles can be acted upon by pancreatic lipase and colipase, which hydrolyzes TG and DAG molecules to 2-monoacylglycerol (MAG) molecules, and cholesterol ester hydrolase, which converts cholesterol esters into free cholesterol. Phospholipids in mixed micelles are also digested by hydrolysis to lysophospholipids by phospholipase A2 (Hofmann, 1999; Ko et al., 2020). The small intestine contains an unstirred water layer made up of glycoproteins attached to the apical surface of enterocytes that creates a barrier between luminal contents and the cells of the small intestine. Mixed micelles are able to cross the unstirred water layer by diffusion to facilitate lipid absorption (Figure 1.6) (Ko et al., 2020; Wilson, Sallee, & Dietschy, 1971).

Lipid absorption in the small intestine primarily occurs in the jejunum – the middle segment of the small intestine (Booth, Read, & Jones, 1961; Borgstrom, Dahlqvist, Lundh, & Sjovall, 1957; Turner, 1958). Passive diffusion from mixed micelles into enterocyte membranes is responsible for most fatty acid, MAG, and lysophospholipid absorption as well as having a role in cholesterol and fat-soluble vitamin absorption (Figure 1.6). This passive diffusion only works when the concentration of lipids is higher in the lumen than it is intracellularly. In order to maintain the concentration gradient lipids are rapidly reesterified within enterocytes (Ko et al., 2020). While the majority of fatty acids are absorbed by passive diffusion a saturable, most likely transport protein dependent, mechanism of fatty acid absorption has been identified (Chow & Hollander, 1979a, 1979b; K. Y. Ling, Lee, & Hollander, 1989). Due to these finding a few fatty acid

transporters have been identified including CD36 and fatty acid transport protein 4 (FATP4) (Lobo et al., 2001; Lynes, Narisawa, Millán, & Widmaier, 2011; Stahl et al., 1999).

When CD36 is knocked out in HFD-fed mice, they have alterations in lipid processing leading to lipid accumulation in enterocytes and reduced chylomicron secretion and processing (Drover et al., 2005; Nauli et al., 2006). The importance of CD36 in fatty acid absorption is less known. There is some evidence that CD36 knockout mice have reduced fatty acid absorption in the proximal intestine but that the distal intestine can compensate for the increased lipid flux leading to net normal absorption levels (Nassir, Wilson, Han, Gross, & Abumrad, 2007). Additionally, there is some evidence that CD36 has an important role in the esterification of fatty acids once inside enterocytes leading to an indirect role in fatty acid absorption by maintaining the concentration gradient required for lipid absorption (Xu, Jay, Brunaldi, Huang, & Hamilton, 2013). The role of FATP4 is also contested as *in vitro* studies show a reduction in lipid absorption when FATP4 is knocked out of enterocytes, but *in vivo* studies have not replicated these results. In enterocytes lacking FATP4 the reduced lipid absorption appears to be due to a reduction in the enzymatic function of FATP4 and not the transport function as certain studies have found FATP4 to be localized entirely intracellularly (Milger et al., 2006; Shim et al., 2009). FATP4 is capable of converting fatty acid inside the cell to an fatty acyl-CoA molecule in order to maintain the fatty acid concentration gradient outside the cell and to create less toxic forms of fatty acids inside the cell (Milger et al., 2006).

While passive diffusion of cholesterol occurs in the small intestine, 70 % of cholesterol is absorbed through the Niemann-Pick C1-Like 1 (NPC1L1) transport protein which absorbs both cholesterol and plant sterols (Altmann et al., 2004; Davis et al., 2004). Scavenger receptor, class B type 1 (SR-B1) may also be involved in cholesterol absorption as overexpression of SR-B1 in

enterocytes leads to increased cholesterol absorption though knocking it out does not lead to reduced absorption (Bietrix et al., 2006; Bura et al., 2013; Mardones et al., 2001). SR-B1 is also involved in some fat-soluble vitamin absorption (Figure 1.6) (During, Dawson, & Harrison, 2005; Goncalves et al., 2014; Reboul et al., 2006).

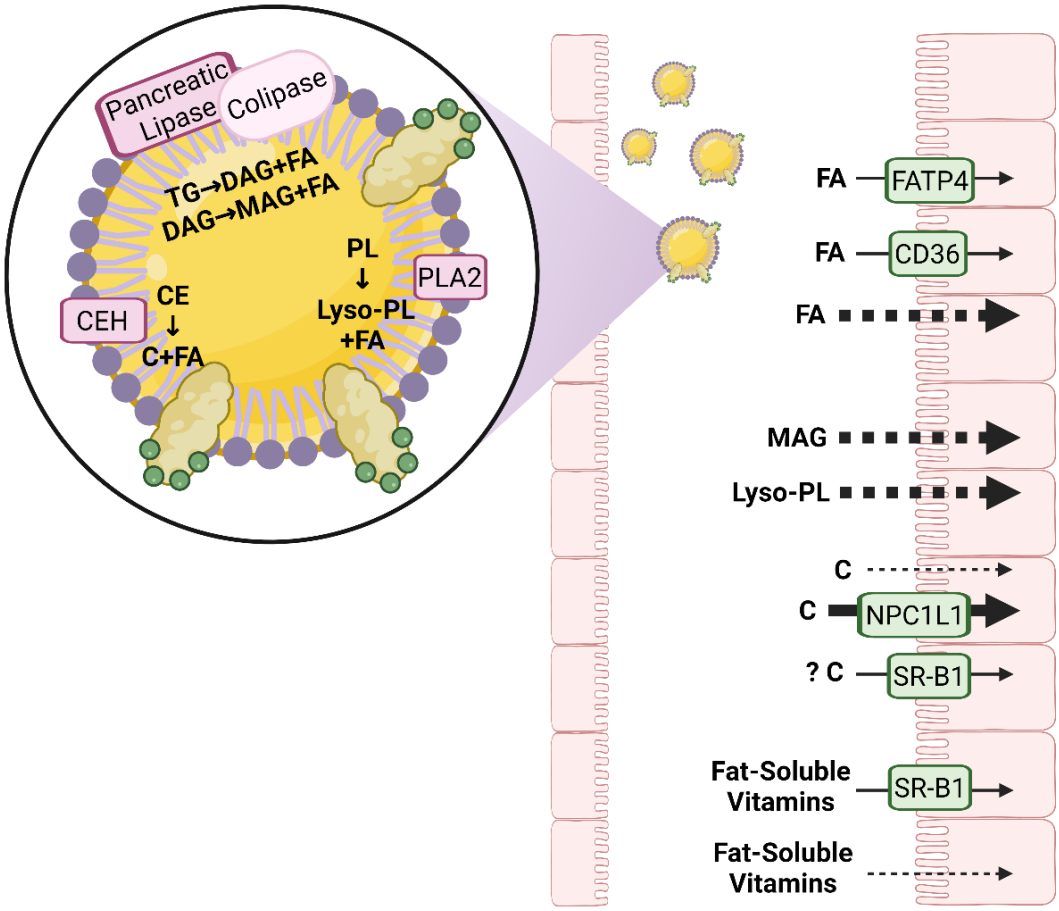

Figure 1.6: Small intestinal lipid digestion and absorption. Mixed micelles aid in the digestion of lipids. They are made of a bile acid, cholesterol, and phospholipid monolayer, with a neutral lipid core. Enzymes bind to the monolayer and facilitate digestion. Pancreatic lipase and colipase digests TG to DAG and FA, and DAG to MAG and FA. CEH digests CE to C and FA. PLA2 digests PL to lyso-PL and FA. MAG, FA, and lyso-PL absorption into enterocytes primarily occurs through passive diffusion. FA absorption is minimally facilitated by FATP4 and CD36 transporters. C is primarily absorbed by the NPC1L1 transporter, though a minimal amount is absorbed through passive diffusion. Currently, there is debate about the importance of SR-B1 C transport. Fat-soluble vitamins are absorbed through passive diffusion and SR-B1 transport. Abbreviations: C (cholesterol), CE (cholesterol ester), CEH (cholesterol ester hydrolase), DAG (diacylglycerol), FA (fatty acid), FATP4 (fatty acid transport protein 4), MAG (monoacylglycerol), NPC1L1 (Niemann-Pick C1-Like 1), PL (phospholipid), PLA2 (phospholipase A2), SR-B1 (scavenger receptor, class B type 1), TG (triacylglycerol).

Once inside the cell fatty acids are transported by fatty acid binding proteins1/2 (FABP1/2) (Figure 1.7) (Alpers, Bass, Engle, & Deschryver-Kecskemeti, 2000; Storch & Corsico, 2008; Alfred E.A. Thumser & Storch, 2000). The role of these proteins has not been fully elucidated though there is evidence that they have a role in reducing fatty acid toxicity within the cell. FABP1 is also capable of binding other lipid metabolites including MAG and lysophospholipids (Burner & Brecher, 1986; Lagakos et al., 2013; A. E.A. Thumser, Voysey, & Wilton, 1994). Most fatty acids can also move to the ER in caveolin-1 containing vesicles associated with lysophospholipids (S. Siddiqi, Sheth, Patel, Barnes, & Mansbach, 2013). Lysophosphatidylcholine actives PKC-ζ targeting the vesicles to ER for lipid conversions into TGs and phospholipids (S. Siddiqi & Mansbach, 2015).

In the small intestinal ER, MAG is esterified with fatty acyl-CoA by monoacylglycerol acyltransferase 2 (MGAT2) to DAG (Kayden, Senior, & Mattson, 1967; Yen & Farese, 2003). DAG is then esterified with another fatty acyl-CoA by diacylglycerol acyltransferase 1/2 (DGAT1/2) to TG (Cases, Smith, et al., 1998; Cases et al., 2001). These enzymes have important roles in the proper absorption and processing of lipids. MGAT2 knockout mice have a delay in lipid absorption leading to an increase in lipids targeted for oxidation and a resistance to diet induced obesity (Nelson, Gao, Yen, & Yen, 2014; Yen et al., 2009). There is some evidence that the esterification of MAG by MGAT2 has an important role in maintaining the concentration gradient required for MAG absorption (Y. Gao, Nelson, Banh, Yen, & Yen, 2013). While DGAT1 does not appear to influence lipid absorption, mice lacking DGAT1 have increased energy expenditure and are resistant to diet induced obesity (H. C. Chen, Ladha, Smith, & Farese, 2003; Smith et al., 2000). DGAT2 knockout mice have a much more severe phenotype as the mice are viable for only a short time after birth and have no fat stores (Stone et al., 2004).

Lysophospholipids are converted into phospholipids by acyltransferases, enzymes that have also been shown to influence lipid absorption in mice (Agarwal, 2012). Lysophosphatidylcholine acyltransferase 3 (LPCAT3) is the most abundant isoform in small intestine and is responsible for adding a polyunsaturated fatty acid (PUFA) to lysophosphatidylcholine molecules synthesizing PC. LPCAT3 knockout mice have lipid malabsorption that was influenced by the altered acyl side chain profile of enterocyte membranes (Zhiqiang Li et al., 2012, 2015; Rong et al., 2015; B. Wang et al., 2016).

Cholesterol is also esterified to a cholesterol ester in the ER of enterocytes by acyl-coenzyme A:cholesterol acyltransferase-2 (ACAT2) (Anderson et al., 1998; Cases, Novak, et al., 1998). ACAT2 has a high affinity for cholesterol but a low affinity for plant sterols (Temel, Gebre, Parks, & Rudel, 2003). Most plant sterols and any non-esterified cholesterol left in enterocytes are effluxed back into the lumen of the small intestine for fecal excretion by ATP-binding cassette sub-family G member 5/ G member 8 (ABCG5/G8) heterodimer (Figure 1.7) (J. Wang et al., 2015). Enterocytes are also involved in the transintestinal cholesterol excretion where they take up circulating lipoproteins and excrete the cholesterol into the lumen for fecal excretion (Jakulj et al., 2016; Temel & Brown, 2015).

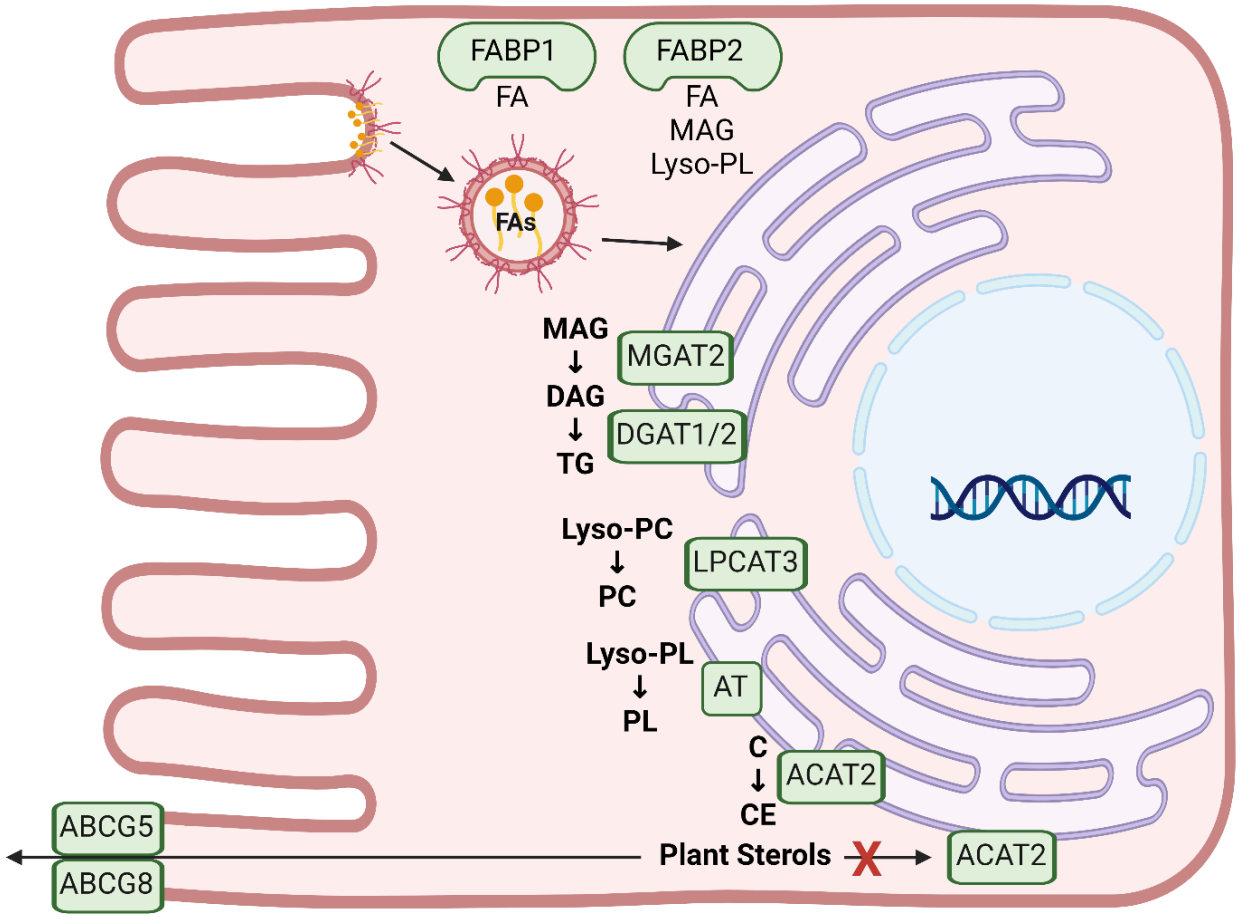

Figure 1.7: Lipid metabolism in enterocytes. Once absorbed, FAs can be transported in bulk to the ER by caveolin-1 mediated endocytosis. Additionally, FAs can be transported to the ER by FABP1 and FABP2 transport proteins. FABP2 is also involved in the transport of MAG and lyso-PLs to the ER. At the ER MAG is converted to DAG by MGAT2 enzymes and DAG is converted into TG by DGAT1 and DGAT2 enzymes. Lyso-PL are converted to PL by ATs and lyso-PC molecules are primarily converted to PC by LPCAT3 enzymes. C is converted to CE by ACAT2 enzymes. ACAT2 is important for the differentiation of C from plant sterols, and plant sterols are then exported from enterocytes by ABCG5/G8 heterodimers. Abbreviations: ABCG5 (ATP-binding cassette sub-family G member 5), ABCG8 (ATP-binding cassette sub-family G member 8), ACAT2 (acyl-coenzyme A:cholesterol acyltransferase 2), AT (acyltransferases), C (cholesterol), CE (cholesterol ester), DAG (diacylglycerol), FA (fatty acid), FABP1 (fatty acid binding protein 1), FABP2 (fatty acid binding protein 2), LPCAT3 (lysophosphatidylcholine acyltransferase 3), MAG (monoacylglycerol), PC (phosphatidylcholine), PL (phospholipids), TG (triacylglycerol).

Lipids absorbed into enterocytes can either diffuse directly into portal circulation or are packaged into chylomicrons. Many short and medium chain fatty acids are able to diffuse directly into portal circulation and are transported via albumin directly to the liver (Guillot, Vaugelade, Lemarchali, & Re Rat, 1993). Chylomicron particles are synthesized primarily in 2 steps revolving around an apolipoprotein B48 (ApoB48) protein. The first step involves ApoB48 proteins being continuously translated and translocated into the ER with the help of microsomal triglyceride transfer protein (MTP) (Ko et al., 2020; Rusin, Jamil, & Vance, 1997). MTP acts as a chaperone to keep nascent ApoB48 particles from being degraded and to help with protein folding to allow for proper transfer of lipids to nascent ApoB48 particle (Atzel & Wetterau, 1993; Hussain, Bakillah, Nayak, & Shelness, 1998; Jiang, Liu, Hussain, Atkinson, & James, 2008). In MTP knockout mice TG absorption and chylomicron secretion is reduced leading to lipid accumulation in enterocytes (Xie et al., 2006). The second step involves lipidation of the ApoB48 particle and this is the step that regulates the degradation of ApoB48 particles under physiological conditions which is independent of fasting or feeding state. The size of chylomicrons produced vary depending on the amount of lipids in a meal, though the number of chylomicrons secreted usually remain the same (Hayashi et al., 1990).

Similar to VLDL lipidation in the liver, it is still unknown how chylomicrons expand with neutral lipids. While there is no current evidence, it is hypothesized that the degradation of ApoB48 occurs when the particle is not lipidated as this is how ApoB100 is regulated in the liver (Ko et al., 2020). PC also has an important role in the synthesis of chylomicrons as it is the most abundant phospholipid added to the surface of chylomicrons and is required for regulation of the lipoprotein (O'Doherty, Kakis, & Kuksis, 1973). Once the nascent chylomicron is lipidated it becomes a pre-chylomicron and gets transported to the Golgi apparatus for maturation. The rate limiting step of

lipid absorption to secretion into lymph is this transfer from the ER to the Golgi apparatus. As pre-chylomicrons are relatively large, they utilize a multi protein pre-chylomicron transport vesicle for transport (Kumarand & Mansbach, 1997; S. Siddiqi et al., 2010). Once at the Golgi apparatus, the pre-chylomicron is matured through the addition of an apolipoprotein AI protein as well as glycosylation by endoglycosidases (Berriot-varoqueaux et al., 2001; S. A. Siddiqi et al., 2006). Once chylomicrons mature, they travel in vesicles from the Golgi apparatus to the basement membrane where they are exocytosed into the lamina propria and are taken up by lacteals (Sabesin & Frase, 1977). Chylomicrons then circulate through the lymphatic system before draining into the cardiovascular circulatory system at the subclavian vein, providing lipids to the body prior to being removed from circulation by the liver (Ko et al., 2020).

1.3.2 Effects of altered phosphatidylcholine levels in the small intestine

Enterocytes can obtain PC through 3 sources – *de novo* synthesis, absorption from the lumen (bile, diet, and shedding) and from absorption of circulating lipoproteins (Ridgway & McLeod, 2008). The small intestine is only capable of synthesizing *de novo* PC through the choline pathway. CTα is the major CT isoform found in the small intestine (Karim et al., 2003; Lykidis et al., 1999). To investigate the role of *de novo* PC synthesis in the small intestine a CTα intestinal knockout ($CT\alpha^{IKO}$) mouse was created (Kennelly et al., 2018). Using the Cre-Lox system under the control of a villin promoter, the CTα knockout is specific to the epithelial cells of the small intestine. By regulating the action of Cre recombinase by fusing it to an estrogen receptor, the CTα knockout is inducible so the action of PC synthesis in the small intestine can be analyzed in adult mice. HFD-fed $CT\alpha^{IKO}$ mice lose around 15 % of body weight by 5 days post induction of the knockout, corresponding to a reduction in visceral fat mass. After 50 days of HFD-feeding

unknown compensatory mechanisms appear to be in place as CTα^{IKO} mice begin gaining weight at a similar rate to controls though they maintain reduced total weight and visceral fat mass (Kennelly et al., 2018).

Lipid metabolism analysis was performed on CTα^{IKO} mice after 5 days of HFD-feeding as it appeared to be the most severe phenotype (Kennelly et al., 2018). HFD-fed CTα^{IKO} mice had reduced plasma TG and NEFA in the postprandial state corresponding to a significant reduction in TG levels of circulating chylomicron and VLDL. Despite the reduced lipid levels, plasma ApoB48 and ApoB100 protein levels were not reduced indicating that lipidation of particles is impaired in intestines of CTα^{IKO} mice but not lipoprotein secretion. Interestingly, total plasma cholesterol levels were similar to controls in the postprandial state despite CTα^{IKO} mice having reduced cholesterol in circulating chylomicron and VLDL. The reduced plasma lipid levels appear to be due to a reduction in lipid absorption, as CTα^{IKO} mice have reduced jejunal TG and increased fecal NEFA excretion in the postprandial state. Surprisingly, CTα^{IKO} mice also have reduced cholesterol absorption. The cause of lipid malabsorption remains unknown, though CTα^{IKO} mice have do have similar acyl-side chain profile to controls as well as an intact brush boarder membrane. The main difference found was a reduction in the mRNA levels of certain membrane lipid transporters including *Cd36* and *Npc1l1* (Kennelly et al., 2018).

Along with *de novo* PC synthesis, the small intestine can obtain a significant amount of dietary PC from luminal absorption. PC is found in the lumen of the small intestine from three sources, the diet, the bile, and secretion from or shedding of intestinal cells (Cotton, 1972). PC absorbed form the lumen is reacylated by LPCATs in enterocytes. There are 4 LPCAT enzymes and LPCAT3 is found in highest forms in metabolic tissues such as the liver and small intestine. LPCAT3 is also involved in remodeling of fatty acyl side chain as it adds a PUFA to PC

(Kazachkov, Chen, Wang, & Zou, 2008). In LPCAT3$^{-/-}$ mice, enterocyte membranes have reduced PUFA profile and lipid malabsorption. High levels of PUFAs in membranes improve lipid absorption and the reduction in fluidity from altered PC species in LPCAT3$^{-/-}$ mice is thought to cause the malabsorption (Hashidate-Yoshida et al., 2015; Zhiqiang Li et al., 2015; Rong et al., 2015). When LPCAT3 was specifically knockout out of the intestine, mice had a significant reduction in plasma TG and cholesterol indicating that intestinal LPCAT3 also has an important role in regulating plasma lipid levels (Kabir et al., 2016).

1.3.3 Gut-liver axis

The intestine and liver are in close communication through the gut-liver axis (Brandl, Kumar, & Eckmann, 2017; Plauth, Raible, Gregor, & Hartmann, 1993). Communication and coordination between these organs are closely regulated and can greatly impact the function of each. The gut-liver axis is involved in the absorption of nutrients, drugs, and toxins in the small intestine that enter portal circulation and go first to the liver for metabolism. The small intestine also secretes hormonal signals that act on the liver in a similar fashion through the portal circulation. The gut-liver axis also encompasses liver secretions, such as bile, into the lumen of the small intestine that can then influence the function of the intestine. When the regulation of the gut-liver axis is altered, by dietary changes or disease states, communication between the gut-liver axis can be altered leading to impaired functions in both organs (Brandl et al., 2017; Plauth et al., 1993; Ridgway & McLeod, 2008).

Exogenous and endogenous contents that reach the lumen of the small intestine must pass through a variety of physical and chemical barriers to enter systemic circulation. The first barrier separates luminal contents from the epithelial cell lining of the small intestine through a mucous

layer primarily containing mucin, a glycosylated peptide (Johansson et al., 2011). These glycoproteins are important for creating the physical barrier separating gastrointestinal (GI) bacteria from intestinal epithelial cells (IECs) (Figure 1.8) (Johansson et al., 2011). GI mucus also contains phospholipids, primarily PC, that create an amphipathic layer at the luminal surface of the mucus layer. This phospholipid layer is important for creating a hydrophobic interface at which detrimental luminal contents cannot pass through (R Ehehalt et al., 2004; Sicard, Bihan, Vogeleer, Jacques, & Harel, 2017). The second barrier is the IEC layer itself that helps create a physical, electrical, and chemical barrier. The IECs create a physical barrier by maintaining tight junctions to minimizing the amount of contents that can enter circulation intercellularly (Roda et al., 2010). The brush boarder surrounding IECs also carries a negative charge, repelling any negatively charged content from interacting with IECs such as bacteria (Van Beers, Büller, Grand, Einerhand, & Dekker, 1995). The IECs include enterocytes, goblet cells, Paneth cells, and enteroendocrine cells, each with a unique function in the small intestine. Enterocytes cells are the absorptive cells of the small intestine and account for 80 % of total IEC content (Cheng & Leblond, 1974). Goblet cells are the cells responsible for secreting mucin to form the mucous layer. Goblet cells, along with Paneth cells, also secrete antimicrobial peptides to create a chemical barrier. Finally, enteroendocrine cells are involved in secreting hormones into circulation that can act on most organs in the body, including the liver as a part of the gut-liver axis (Figure 1.8) (Albillos, de Gottardi, & Rescigno, 2020). The third barrier of the GI tract involves the lamina propria – connective tissue formed on the basolateral side of IECs. The lamina propria is home to many plasma cells that secrete antibodies that help protect form bacterial translocation (Mattioli & Tomasi, 1973).

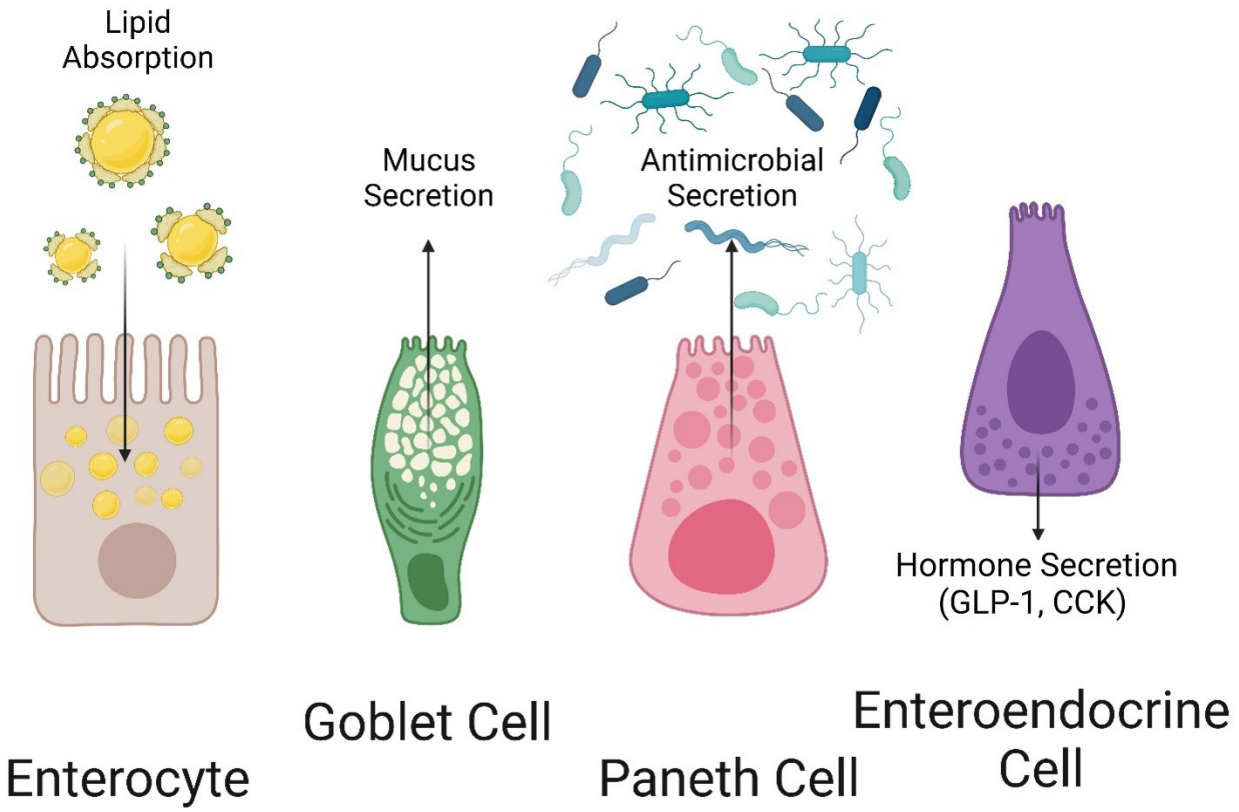

Figure 1.8: Small intestinal epithelial cells. The small intestine has four intestinal epithelial cell types. The enterocytes are the most abundant intestinal epithelial cell and involved in nutrient absorption, including lipid absorption. Goblet cells secrete mucous into the intestinal lumen and are important for creating a physical barrier. Paneth cells secrete antimicrobial peptides into the intestinal lumen and are important for creating a chemical barrier. Enteroendocrine cells are stimulated by luminal contents and secrete hormones into systemic circulation, including GLP-1 and CCK. Abbreviation: GLP-1 (glucagon-like peptide 1), CCK (cholecystokinin).

One well studied pathway of the gut-liver axis involves the enterohepatic circulation of bile acids (Gottlieb & Canbay, 2019; Ridgway & McLeod, 2008). Primary bile acids are synthesized from cholesterol in the liver through two pathways – the classic pathway and the alternative pathway, both of which utilize Cyp27A1 for bile acid synthesis. The classic pathway synthesizes cholic acid (CA) and chenodeoxycholic acid (CDCA), with Cyp7A1 as the rate limiting enzyme, and is responsible for 75 % of bile acid synthesis (Ridgway & McLeod, 2008). While the classic pathway is hepatocyte specific, the alternative pathway of bile acid synthesis is found in extra-hepatic tissues. The alternative pathway in hepatocytes synthesizes only CDCA and utilizes Cyp8B1 enzyme (Russell, 2009). Bile acids in hepatocytes are then conjugated with glycine or taurine to form bile salts that have increased solubility (Hafkenscheid & Hectors, 1975).

The bile salt export pump (BSEP) protein exports bile salts into the canaliculus in the liver where they mix with other components of bile to create mixed micelles (Carey & Small, 1970; Gerloff et al., 1998; O'Máille, Richards, & Short, 1965). In hepatocytes, multidrug resistance-associated protein 2 (MDR2) is a flippase responsible for the addition of PC to bile and ABCG5/G8 is responsible for the addition of cholesterol to bile (Smit et al., 1993; Yu et al., 2002). In the fasted state, bile is stored in the gallbladder (Lanzini, Jazrawi, & Northfield, 1987). With the ingestion food, amino acids and fatty acids stimulate the release of cholecystokinin (CCK) from enteroendocrine I cells which acts on the gallbladder, stimulating contraction and release of bile into the duodenum (Krishnamurthy & Brown, 2002; H. H. Wang et al., 2010).

In the terminal ileum 95 % of bile salts are absorbed by apical sodium-dependent bile acid transporter (ASBT/SLC10A2) where they are released into portal circulation by organic solute transporter alpha and beta (OSTα and OSTβ) (Dawson et al., 2003, 2005; Weinberg, Burckhardt, & Wilson, 1986). Hepatocytes reuptake bile acids with sodium/taurocholate co-transporting

polypeptide (NTCP/SLC10A1) and organic anion transporting polypeptide 1 (OATP1) (Csanaky et al., 2011; Hagenbuch, Jacquemin, & Meier, 1994; Reichen & Paumgartner, 1976; Van De Steeg et al., 2010). The remaining bile acids travel along the intestine to the colon where the microbiota metabolizes them to secondary bile acids. Secondary bile acids are more hydrophobic than primary bile acids and can either be returned into circulation for recycling or excreted in feces. The synthesis of secondary bile acids is what leads to the diversity of bile acids found in bile (Björkhem, Danielsson, Einarsson, & Johansson, 1968; Hofmann, 1984).

Alterations in the gut-liver axis are a consequence of, as well as a cause and amplifier of, disease states – including NAFLD (Han et al., 2021). Damage to the intestinal barrier, alterations in bile acid signaling and alterations in intestinal hormonal signaling all influence the development of NAFLD. The intestinal barrier is required to keep the microbiota from accessing systemic circulation, and disruptions in the intestinal barrier have been linked with bacterial damage to hepatocytes and the development of NAFLD (Mao et al., 2015; Ritze et al., 2013). Many NAFLD patients were found to have disruptions in their intestinal barrier including increased permeability and increased circulating levels of lipopolysaccharide (LPS) – a bacterial toxin (Carpino et al., 2020; Luther et al., 2015; Miele et al., 2009). Increased intestinal permeability allows bacteria and bacterial components, like LPS, to cross into portal circulation and alter hepatic functions leading to the development of NAFLD. Mice fed a HFD or choline deficient diet not only developed NAFLD but also had increased intestinal permeability and circulating levels of bacterial toxins (Luther et al., 2015; Mouries et al., 2019). Furthermore, a reduction in the tight junctions leading to increased intestinal permeability was found in mice after only 48 hours of HFD feeding (Mouries et al., 2019). Further evidence in rats showed that high fat/high sucrose diet feeding leads to reduced tight junctions, increased plasma LPS, and steatosis (Zhou et al., 2014). Finally,

improving the intestinal barrier and reducing intestinal permeability in mice lead to a reduction in the development of NAFLD (Mouries et al., 2019; H. Yang et al., 2022).

Alterations in bile acid homeostasis also contribute to the development of NAFLD. While travelling through the small intestine, bile acids activate farnesoid X receptor (FXR) which leads to increased fibroblast growth factor 15 (FGF15) secretion in rodents or FGF19 secretion in humans (Holt et al., 2003; Inagaki et al., 2005; Kim et al., 2007). FGF15/19 acts on the liver to inhibit the synthesis of bile acids and fatty acids (Holt et al., 2003; Inagaki et al., 2005; Kim et al., 2007; Watanabe et al., 2004). FGF15/19 is important for maintaining hepatic lipid metabolism and HFD-fed FGF15 knockout mice have worsened hepatic lipid accumulation and increased ER stress (Alvarez-Sola et al., 2017). Also, a correlation was found between the severity of NAFLD in patients and plasma bile acid concentration. It was observed that patients with NAFLD had altered composition of bile acids with an increase in bile acids that were weak activators of FXR leading to a reduction in circulating FGF19 (Puri et al., 2018). Interestingly, it was found that lean patients with NAFLD, compared to obese patients with NAFLD, had increased circulating bile acids. This leads to the hypothesis that altered bile acid homeostasis may be one cause of NAFLD in lean patients. When bile acid resorption was inhibited in a lean mouse model of NAFLD, markers of hepatic NASH improved supporting the idea that increased circulating bile acids may influence the development of NAFLD in lean patients (F. Chen et al., 2020).

Finally, hormone secretion from the intestine can directly impact the function of liver. The small intestine secretes a variety of hormonal peptides that influence hepatic metabolic regulation including glucagon-like peptide 1 (GLP-1) (Alvares, Hoffman, Stankovic, & Adeli, 2019). GLP-1 is secreted in the distal intestine during times of feeding and has been shown to be an important molecule for reducing lipid accumulation and inflammation in the liver (Parlevliet et al., 2012;

Taher et al., 2014). Despite a controversy surrounding the presence of GLP-1 receptors on liver cells, GLP-1 receptor agonism in rodent models leads to a reduction in hepatic lipogenesis, fatty liver and VLDL production (Parlevliet et al., 2012; Taher et al., 2014). Additionally, GLP-1 receptor agonism reduced the development of NASH in HFD-fed mice (Trevaskis et al., 2012). In humans with NAFLD, injections of GLP-1 analogues lead to a reduction in hepatic histological markers of NAFLD (Armstrong et al., 2016; Newsome et al., 2021). These data show that altering the gut-liver communication can influence the progression and development of NAFLD.

1.3.4 Effects of altered phosphatidylcholine levels on the gut-liver axis

PC is the second most abundant component of bile after bile acids and the role of *de novo* PC synthesis has been examined in regulating bile homeostasis. The role of both hepatic CTα and PEMT have been analyzed in biliary regulation. Bile stored in gallbladders of fasted, chow-fed $CT\alpha^{LKO}$ mice, had similar concentrations of biliary bile acids, PC, and cholesterol (Jacobs et al., 2004). These results indicate that PEMT and CTβ expression, which were elevated in $CT\alpha^{LKO}$ mice, were sufficient for PC synthesis into bile (Jacobs et al., 2004). Despite these results, when control were mice fed a choline deficient diet – essentially diminishing the role of CT in hepatocytes – there was a 40 % reduction in PC secreted into bile (Agellon, Walkey, Vance, Kuipers, & Verkade, 1999). Together these results may indicate that an acute reduction in the availability of PC through the CT pathway is detrimental, but that hepatocytes can compensate over time and restore biliary homeostasis. In similar experiments, the concentration of PC in bile of chow-fed $PEMT^{-/-}$ mice were analyzed and were not altered compared to control mice (Agellon et al., 1999; Verkade et al., 2007). Further analysis was performed on $PEMT^{-/-}$ mice as it was discovered that PEMT is enriched in portions of the ER membrane that are in contact with

canaliculi – ducts where bile is secreted into from hepatocytes (Sehayek et al., 2003). When PEMT$^{-/-}$ mice were fed a high fat diet they developed cholestasis as seen by an increase in plasma bile acids. Additionally, HFD-fed PEMT$^{-/-}$ mice had a reduction in the secretion of bile acids and PC into bile (Wan, Kuipers, et al., 2019). These results indicate a role or PEMT under times of increased PC need – such as during HFD feeding – to maintain biliary homeostasis.

Interestingly, HFD-fed CTα^{IKO} mice also had alterations in bile regulation despite having minimal changes to the liver (Kennelly et al., 2018). HFD-fed CTα^{IKO} mice had an increase in bile flow which corresponded to an increase in the biliary secretion of bile acids, PC, and cholesterol. Despite the increase in bile acid secretion, CTα^{IKO} mice maintained a similar composition of bile acids and hydrophobicity index in bile, as well as similar plasma and fecal bile acid levels. CTα^{IKO} mice showed an increase in intestinal mRNA expression of genes, including *Slc10A2*, that are involved in the uptake of bile acids from the lumen of the small intestine. These results indicate that a compensatory mechanism for an extra supply of intestinal PC may be occurring in CTα^{IKO} mice by increasing enterohepatic cycling of bile acids (Kennelly et al., 2018). Alterations in biliary PC have also been shown to affect the function of the small intestine. Mdr2 is a flippase in hepatocytes that moves PC from the inner to the outer plasma membrane leaflet. When Mdr2 is knocked out (Mdr2$^{-/-}$), mice no longer secrete PC into bile (Smit et al., 1993). Mdr2$^{-/-}$ mice have a reduction in the appearance of postprandial plasma TG as well as an accumulation of TG in enterocytes. The appearance of postprandial plasma TG were increased in Mdr2$^{-/-}$ mice after a duodenal infusion of whole rat bile. These results indicate that biliary PC is required for the secretion of chylomicrons from the small intestine, but not for TG absorption (Voshol et al., 2000).

1.4 Role of phosphatidylcholine in colonic health

1.4.1 Inflammatory bowel disease

The colon is a dynamic organ regulating absorption and excretion of products passing from the small intestine (Robert Ehehalt, Braun, Karner, Füllekrug, & Stremmel, 2010). The colon maintains an important barrier that allows for the absorption of water and minerals, to maintain systemic electrolyte balance, while excreting waste products such as toxins and unabsorbed nutrients. As in the small intestine, the colonic barrier also protects against the microbiota and regulates inflammation of the colon (Robert Ehehalt et al., 2010). The IEC layer of the colon is populated by similar epithelial cell types to the small intestine. The most abundant IEC in the colon is the colonocyte which is involved in luminal absorption, similar to small intestinal enterocyte (Parikh et al., 2019). The next most abundant cell type in the colon is the goblet cell, the mucous secreting cell type of the intestine. Goblet cells increase in number from the proximal small intestine to the distal colon as the mucous barrier becomes more complex (McCauley & Guasch, 2015; Shamsuddin, Phelps, & Trump, 1982). The next most abundant cell type is enteroendocrine cells, hormone secreting cells of the intestine. Enteroendocrine cells are the most abundant in the proximal small intestine where the numbers decrease through the small and large intestine until they become much more populated again at the rectum (Shamsuddin et al., 1982; Sjölund, Sandén, Håkanson, & Sundler, 1983). Finally, there is controversy surrounding the population of Paneth cells in the colon under normal physiologic conditions. Some studies have shown that the colon only expresses Paneth cells under pathologic conditions, such as inflammatory bowel disease (IBD), while others have shown that the proximal colon does express minimal numbers of Paneth cells under normal conditions (Simmonds, Furman, Karanika, Phillips, & Bates, 2014; Tanaka et al., 2001). These cell types work together to maintain the physiologic functions of the colon.

The mucous barrier of the colon differs from the small intestine in that it exists as 2 layers (G. M.H. Birchenough, Johansson, Gustafsson, Bergström, & Hansson, 2015; Robert Ehehalt et al., 2010). Both mucous layers primarily contain mucins which create the structure of the layers, where muc2 is the most abundant protein. The top layer of the mucous barrier is most similar to the barrier found in the small intestine. This layer is easily removable and has a more open network allowing for the population of the mucous with some bacteria and waste products found in the lumen of the colon. The bottom layer is attached to the IECs and cannot be removed easily (G. M.H. Birchenough et al., 2015; Robert Ehehalt et al., 2010). The mucins in this layer are packed closer together creating a tight barrier that bacteria cannot penetrate (Johansson et al., 2008; Van Der Waaij et al., 2005).

Goblet cells are responsible for creating the mucous barrier and behave in different ways to facilitate the formation of the two layers. There are multiple populations of goblet cells found in the colon with different morphologies, physiologies and regulatory mechanisms (George M.H. Birchenough, Nystrom, Johansson, & Hansson, 2016; Johansson, 2012; Nyström et al., 2021; Specian & Neutra, 1982). The goblet cells found on the surface of the crypts, as well as those found in between crypts, produce the dense bottom layer of the mucous (George M.H. Birchenough et al., 2016; Nyström et al., 2021). The inner layer produced by these goblet cells has a fast, continuous turnover rate that helps protects IEC from bacterial interaction as the old mucous is pushed towards the lumen (George M.H. Birchenough et al., 2016; Johansson, 2012). Goblet cells in the crypt aid in the production colonic mucous, though the mucus produced is detectably different from surface goblet cells. The specific roles of mucous created by the various populations of goblet cells is still not fully understood, though emerging research is showing that each one is

required for maintaining a healthy mucous barrier (George M.H. Birchenough et al., 2016; Nyström et al., 2021).

Over time, the inner mucosal layer is pushed outwards until it becomes the loose outer layer. The outer layer then get pushed into the lumen and excreted in feces – carrying any populated bacteria with it (Johansson, 2012). Finally, as in the small intestine, the outermost portion of the mucous barrier is lined with phospholipids creating a hydrophobic seal (Butler, Lichtenberger, & Hills, 1983). The surface of the mucous layer has the highest hydrophobicity in the stomach and in the colon. It is hypothesized that the hydrophobicity is less in the small intestine to allow for the abundance and variety of nutrient absorption (Mack, Neumann, Policova, & Sherman, 1992; Spychal, Marrero, Saverymuttu, & Northfield, 1989).

Inflammation of the IEC and mucosal layers of the colon can cause common disease states in humans called colitis. Colitis can be caused by a wide range of precipitators including infectious diseases, chemicals, ischemia, and autoimmune diseases. Inflammatory bowel disease (IBD) is a classification of autoimmune diseases including Crohn's Disease and Ulcerative Colitis (Guan, 2019). The clinical presentation of IBD includes systemic and gastrointestinal symptoms caused by the inflammation of the lining of the gut including weight loss, pain, and diarrhea (Hardy, 1949; Warren & Sommers, 1949). The exact pathogenesis of IBD remains unclear though there is increasing evidence that genetics, the environment, the microbiota, and individual susceptibility all have a role (Guan, 2019). Crohn's Disease can occur anywhere in the gastrointestinal tract in a discontinuous fashion and is most commonly found in the ileum. Crohn's Disease involves inflammation of the entire intestinal layer including mucosa, submucosa, muscle, and serosal layers, which can lead to the development of fissures and granulomas (B H Smith, 1967; Hardy, 1949). Ulcerative colitis on the other hand is found only in the colon and begins at the rectum and

moves proximally through the colon in a continuous fashion. Ulcerative colitis involves only inflammation of the mucosa and submucosa which can lead to cryptitis and crypt abscesses (Bargen, 1929; Warren & Sommers, 1949). A common feature of ulcerative colitis is reduced differentiation and damage to goblet cells which leads to altered integrity of the mucosal layer and bacterial interaction with IECs (B H Smith, 1967; Gersemann et al., 2009). In patients with ulcerative colitis, a correlation was found between the thickness of the mucosal layer and the severity of inflammation – showing how important the mucosal layer in the colon (Strugala, Dettmar, & Pearson, 2008). In conclusion, IBD can lead to the disruption of all physiologic functions of the colon, which is why it causes such severe disease patients.

1.4.2 Effects of altered phosphatidylcholine levels in colonic health

The colonic mucous membrane contains phospholipids, the most abundance of which is PC. While there is interindividual variability, PC and lyso-PC account for around 60-80 % of the total colonic mucous phospholipids (R Ehehalt et al., 2004). Lyso-PC accounts for a significant amount of the total colonic mucous phospholipids, ranging between 10-30 % (Braun et al., 2009). The role of both systemic and dietary PC in the gastrointestinal tract has long been studied and has shown to protect against, and improve, inflammation (Eros, Kaszaki, Czobel, & Boros, 2006; Karaman et al., 2003; Voshol et al., 2000). Additionally, in various murine models of colitis, adding different mixtures of PC to the lumen of the colon has shown to cause a direct improvement in the features of colitis (Kovács et al., 2012; Q. Li et al., 2022; Lugea, Salas, Casalot, Guarner, & Malagelada, 2000). Interestingly, phospholipase A2 (PLA2) activity levels are increased in the colons of rodent and human models of IBD (Minami, Tojo, Shinomura, Matsuzawa, & Okamoto, 1994; Murthy & Biondi, 1992). PLA2 is responsible for cleaving fatty acids from the sn-2 position

of phospholipids, creating lyso-phospholipids. When PLA2 activity was inhibited in murine models of colitis, they had an improvement in colitis features (Fabia, Ar'Rajab, Willén, Andersson, & Bengmark, 1993; Krimsky et al., 2003; Zhai et al., 2020). These results indicate that the ratio of lyso-PC to PC in colonic mucous may be very important for its overall function as a barrier.

The role of PC in the mucosal layer of patients with IBD has become a topic of increasing importance. Patients with ulcerative colitis have significantly reduced PC in the mucous collected from their colons (Braun et al., 2009; R Ehehalt et al., 2004). One study found that the mucous of patients with ulcerative colitis had PC species with increased saturated fatty acids and had an increase in the lyso-PC to PC ratio (Braun et al., 2009). The increase in lyso-PC to PC ratio found in these patients supports the hypothesis that this ratio is important for function of the mucous barrier and preventing colonic inflammation. Recently, the use of delayed-release PC has been used in the treatment of ulcerative colitis (Karner et al., 2014; W. Stremmel et al., 2005; Wolfgang Stremmel, Ehehalt, Autschbach, & Karner, 2007). Delayed-release PC is an oral preparation of PC that does not get digested in the small intestine and is released in the colon in its original form. This preparation allows for the selective increase in PC species reaching the colon. The use of delayed-release PC has improved markers of colitis in patients who are not dependent on steroids and in patients who have become refractory to steroids. In these studies, patients with ulcerative colitis given delayed-release PC had significant improvement in disease activity, increased remission, and improved quality of life (Karner et al., 2014; W. Stremmel et al., 2005; Wolfgang Stremmel et al., 2007). A meta-analysis looking at the effectiveness of delayed-release PC also showed that it was able to improve both clinical and histological signs of ulcerative colitis in patients, strengthening the evidence that a reduction in PC in colonic mucous is important in the

development and treatment of ulcerative colitis (Wolfgang Stremmel, Vural, Evliyaoglu, & Weiskirchen, 2021).

1.5 Research plan

1.5.1 Rationale

PC is an amphipathic molecule with a hydrophilic head group and hydrophobic tail. Due to the amphipathic structure, PC has many important cellular and systemic roles (Alvaro et al., 1986; Exton, 1994; Skipski et al., 1967). PC is the most abundant phospholipid found in the membranes of mammalian cells and aids in the formation of membrane bilayers (Van Meer et al., 2008). Additionally, PC can spontaneously create monolayer micelles that aid in the digestion and transport of lipids (Alvaro et al., 1986; Skipski et al., 1967). In all nucleated cells, PC can be synthesized through the choline pathway which uses CTα as the rate limiting enzyme (Choy et al., 1979, 1980; Vance & Choy, 1979). Whole-body knockouts of CTα are embryonically lethal, and so organ specific CTα knockout mice have been previously created to investigate the role of choline-derived PC in whole-body homeostasis (L. Wang et al., 2005).

The role of *de novo* PC synthesis in lipid metabolism of the liver was analyzed through the use of CTα liver knockout mice ($CT\alpha^{LKO}$ mice) (Jacobs et al., 2004; Niebergall et al., 2011). $CT\alpha^{LKO}$ mice have reduced hepatic PC levels leading to lowered circulating HDL and VLDL particles. Additionally, female $CT\alpha^{LKO}$ mice have mild accumulation of hepatic TG (Jacobs et al., 2004). When $CT\alpha^{LKO}$ mice were fed a HFD, they developed NASH after 1 week indicating that CTα-derived PC is important under times of increased stress (Niebergall et al., 2011). The role of *de novo* PC synthesis in lipid metabolism of the small intestine was analyzed through the use of CTα intestinal knockout mice ($CT\alpha^{IKO}$ mice) (Kennelly et al., 2018). When fed a HFD, $CT\alpha^{IKO}$

mice dramatically lose weight and present with lipid malabsorption and reduced plasma TG in the postprandial state. Surprisingly, CTα^{IKO} mice have increased bile flow, despite no other significant changes in the liver (Kennelly et al., 2018). However, the role of PC in interorgan communication and the gut-liver axis has not been fully elucidated.

1.5.2 Objectives and hypotheses

The overall aim of this research was to determine the role of *de novo* PC synthesis in the organs involved in the gut-liver axis and in the communication between them. The following objectives address this overall aim.

1. To determine the role of hepatic *de novo* PC synthesis through the choline pathway on biliary homeostasis and lipid absorption in the small intestine. We hypothesized that a reduction in the availability of hepatic PC would alter hepatic biliary output and reduce lipid absorption in the small intestine.
2. To determine the role of small intestinal *de novo* PC synthesis on IEC stress and maintenance of the mucosal barrier. We hypothesized that a reduction in the availability of small intestinal PC would lead to a damaged mucosal barrier and subsequent IEC stress through increased bacterial interaction.
3. To determine the role of colonic *de novo* PC synthesis in maintenance and development of the mucosal barrier and subsequent prevention of colonic disease states. We hypothesized that a reduction in colonic PC would lead to a damaged mucosal barrier and would lead to the development of colonic inflammation.
4. To determine the role of intestinal *de novo* PC synthesis in maintaining proper gallbladder signaling and functioning. We hypothesized that a reduction in intestinal

PC levels would lead to altered gallbladder signaling and improper functioning of the biliary pathways.

1.5.3 Chapter format

Chapter 2 reports on the effect of reduced *de novo* PC synthesis through the CDP-choline pathway in the livers of mice, addressing the first objective. In summary, within one week of chow- or HFD-feeding, acute CTα liver knockout mice ($CT\alpha^{LKO}$ mice) lose a significant amount of weight, have reduced fasting TG levels, and develop NASH. Additionally, chow- and HFD-fed $CT\alpha^{LKO}$ mice have reduced lipid absorption and reduced circulating TG in the postprandial state. Finally, HFD-fed $CT\alpha^{LKO}$ mice have reduced biliary flow which could account for the reduction in lipid absorption and subsequent weight loss. These results suggest that maintaining proper hepatic PC synthesis is required for fasting and postprandial lipid homeostasis.

Chapter 3 reports on the effect of reduced *de novo* PC synthesis in the small intestines of mice, addressing the second objective. In summary, the previous phenotype of HFD-fed CTα intestinal knockout mice ($CT\alpha^{IKO}$ mice) – including having reduced weight, reduced lipid absorption and increased levels of plasma active GLP-1 – was found to be independent of dietary fat content, occurring during both low-fat diet (LFD) and HFD feeding (Carlin et al., 2022). Additionally, LFD- and HFD-fed $CT\alpha^{IKO}$ mice had increased IEC ER stress, host defense, and cell death leading to reduced goblet cells and altered mucosal barrier. When $CT\alpha^{IKO}$ mice were treated with antibiotics, they had normalized weight gain and improved jejunal TG absorption but maintained increased GLP-1 secretion, ER stress, cell death, and loss of goblet cells. Finally, when $CT\alpha^{IKO}$ mice were fed a diet high in PC, there was a partial prevention of goblet cell loss, but they maintained reduced lipid absorption, increased GLP-1 secretion and induction of ER stress, cell

death and the host defense. These results demonstrate an intricate role of intestinal *de novo* PC synthesis which is necessary for maintaining small intestinal homeostasis (Carlin et al., 2022).

Chapter 4 reports on the effect of reduced *de novo* PC synthesis in the colons of mice, addressing the third objective. In summary, loss of intestinal CTα lead to a significant reduction in colonic IEC total PC levels and induction of spontaneous colitis (Kennelly et al., 2021). Colonic IECs of $CT\alpha^{IKO}$ mice have increased ER stress due to altered PC membrane contents which lead to the induction of necroptosis. Cell death severely impacted goblet cells leading to a reduced mucosal barrier and bacterial infiltration. Dietary PC supplementation and antibiotic treatment were unable to prevent the development of spontaneous colitis in $CT\alpha^{IKO}$ mice. These results indicate that colonic *de novo* PC synthesis is important for preventing disease states of the colon by maintaining IEC function and the mucosal barrier (Kennelly et al., 2021).

Chapter 5 reports on the effect of reduced intestinal *de novo* PC synthesis on gallbladder signaling and function, addressing the fourth objective. In summary, $CT\alpha^{IKO}$ mice have enlarged gallbladders in the postprandial state with a reduction in circulating cholecystokinin (Cck) and jejunal *Cck* mRNA levels. Dietary bile acid supplementation was unable to improve weight loss or lipid malabsorption in $CT\alpha^{IKO}$ mice. CCK injections were able to normalize weight gain in $CT\alpha^{IKO}$ mice, though lipid absorption was not improved. These results indicate that intestinal *de novo* PC synthesis is important for gut-liver axis signalling through the gallbladder.

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Chapter 2

Acute reduction of hepatic phosphatidylcholine synthesis leads to the development of NAFLD and impaired chylomicron secretion

2.1 Introduction

Communication between the liver and intestine is important in regulating metabolic homeostasis (Plauth, Raible, Gregor, & Hartmann, 1993). The gut-liver axis is closely regulated because any nutrient, toxin, or drug absorbed by the intestine and secreted into the circulatory system reaches the liver first through the portal vein. In addition, the liver has an important role in the absorption of nutrients – specifically lipids – through the synthesis and secretion of bile. Bile is composed of bile acids, phospholipids - primarily phosphatidylcholine (PC) – and cholesterol. Bile is secreted in response to hormonal signalling from the small intestine when food reaches the proximal intestine. After their action in facilitating fat absorption in the proximal small intestine, around 95 % of bile acids are reabsorbed in the distal intestine and returned to the liver in a process known as enterohepatic circulation (Gottlieb & Canbay, 2019; Ridgway & McLeod, 2008). The gut-liver axis is also under communication through hormonal signaling from the intestine, which can influence both hepatic lipid metabolism and bile formation (Holt et al., 2003; Inagaki et al., 2005; I. Kim et al., 2007; Parlevliet et al., 2012; Taher et al., 2014; Watanabe et al., 2004). Altering the gut-liver communication may thereby influence disease states of the liver, including the development and progression of non-alcoholic fatty liver disease (NAFLD) (Alvarez-Sola et al., 2017; Smit et al., 1993; Trevaskis et al., 2012).

The global rates of NAFLD have been estimated to be 25 % worldwide and are increasing along with comorbidities such as the metabolic syndrome and diabetes (Younossi et al., 2016). NAFLD encompasses a spectrum of liver pathologies which range from fatty liver to non-alcoholic steatohepatitis (NASH) (Matteoni et al., 1999). The development and progression of NAFLD has not been fully elucidated but the current theory involves a multiple-hit pathogenesis (Buzzetti, Pinzani, & Tsochatzis, 2016). This theory proposes that insults from a variety of factors including

genetics, insulin resistance, and obesity which lead to lipid accumulation and the development of inflammation, oxidative stress, and fibrosis in the liver (Buzzetti et al., 2016). An altered ratio between phosphatidylcholine (PC) and phosphatidylethanolamine (PE) has been implicated in impaired membrane integrity and the progression of NAFLD (Li et al., 2006; Ling, Chaba, Zhu, Jacobs, & Vance, 2012). PC is the most abundant phospholipid in the membranes of mammalian cells and has many important functions in cell signaling and lipid transport (van der Veen et al., 2017). Hepatocytes can synthesize PC through two independent pathways: 70% by the CDP-choline pathway and 30 % from the conversion of PE to PC by 3 methylation reactions performed by phosphatidylethanolamine N-methyltransferase (PEMT) (DeLong, Shen, Thomas, & Cui, 1999; Sundler & Akesson, 1975; van der Veen et al., 2017). The CDP-choline pathway synthesizes PC from choline and has CTP:phosphocholine cytidylyltransferase (CT) as the rate-limiting enzyme.

The independent roles of PC synthesis via the two distinct pathways in the regulation of liver function have been analyzed in our laboratory. CTα is the most abundant isoform of CT in hepatocytes. Chow-fed CTα permanent liver knockout ($CT\alpha^{PLKO}$) mice have reduced hepatic PC levels despite a two-fold increase in hepatic PEMT protein levels (Jacobs, Devlin, Tabas, & Vance, 2004). Additionally, chow-fed $CT\alpha^{PLKO}$ mice have reduced circulating high-density lipoprotein (HDL) and very low-density lipoprotein (VLDL) levels (Jacobs et al., 2004). HFD-fed $CT\alpha^{PLKO}$ mice develop NASH within one week (Niebergall, Jacobs, Chaba, & Vance, 2011). The $CT\alpha^{PLKO}$ mice studied previously (Jacobs et al., 2004; Niebergall et al., 2011) had the CTα gene knocked out from conception onwards which might have led to metabolic adaptations. To define the role of the CDP-choline pathway more directly, we were interested in determining if an acute knockout of liver CTα would have different or similar effects as the permanent knockout. Mice with an acute

CTα knockout are designated CTα^{LKO} mice, differing from the CTα^{PLKO} mice discussed above. We found that an acute knockout of CTα in chow- and HFD-fed CTα^{LKO} mice induced an exacerbation of the permanent phenotype, i.e., induced a dramatic reduction in weight, massive hepatic lipid accumulation and a reduction in fasting plasma TG. To further elucidate the importance of CTα-derived hepatic PC, we investigated postprandial TG metabolism in the intestine. Surprisingly, we found reduced postprandial plasma TG appearance associated with a reduced jejunal lipid content, indicating lipid malabsorption in CTα^{LKO} mice. These results indicate that hepatic CTα-derived PC is important for maintaining proper gut-liver communication to regulate whole-body lipid homeostasis and to prevent the development of NAFLD.

2.2 Methods

2.2.1 Animal handling

C57BL/6J male mice with floxed *Pcyt1a* alleles were housed in a temperature-controlled, 12 h light/dark cycle environment. Mice were given free access to water and a standardized chow diet (5001, Lab Diet, St. Louis, MO) prior to experimentations. Age-matched mice of 14-16 weeks old were given retro-orbital injections of 1 X 10^{11} genome copies of adeno-associated virus (AAV) expressing either Cre recombinase to induce CTα^{LKO} or green fluorescent protein (GFP) as controls. The AAVs were generated at the University of Pennsylvania Penn Vector Core to be liver specific through the use of AAV8 capsid protein and a thyroxine-binding globulin promoter. CTα^{LKO} and control mice received standardized chow diet or switched to a 60 % (fat/calorie) diet (catalog No. F3282, Bio-Serv, Flemington, NJ) for one week ad libitum. Samples were collected after a 16 h fast unless otherwise stated. Mice were euthanized by cardiac puncture and blood was collected in tubes containing EDTA. Plasma was collected for analysis by centrifuging blood

room-temperature at 3000 g for 10 min. The liver was collected and frozen in liquid nitrogen. Small intestines were excised and flushed with a PBS and protease inhibitor cocktail (Sigma) solution. The small intestines were then segmented into equal thirds and frozen in liquid nitrogen. Mice used in the bile cannulation studies were fed the same HFD as above for two weeks and were not fasted prior to gallbladders being cannulated for 30 minutes to analyze bile as described previously (Plösch et al., 2006). The University of Alberta's Institutional Animal Care Committee approved all experiments and follow the guidelines set by Canadian Council on Animal Care.

2.2.2 Lipid analysis

Plasma TG was measured using a commercially available kit (Sekisui Diagnostics). Fasting plasma TG was measured in mice following a 16 h fast and after an intraperitoneal injection of Poloxamer 407 (10 µL/g). Blood was collected at time points 0, 1, 3, and 4 h to measure the appearance of fasting plasma TG. Postprandial plasma TG was measured in mice following a 16 h fast then an intraperitoneal injection of poloxamer 407 (10 µL/g), followed immediately by an olive oil gavage (200 µL). Blood was collected at time points 0, 1, 3, and 4 h to measure the appearance of postprandial plasma TG. Liver and intestinal samples were homogenized and a bicinchoninic acid assay was used to measure protein levels of homogenate. Lipid extractions were performed with liver tissue homogenates (1 mg protein) using the Folch method (Folch, Lees, & Sloane Stanley, 1957). Liver PC and PE levels were isolated using thin layer chromatography in a solvent system containing chloroform, methanol, acetic acid, formic acid, and water (140:60:24:8:2 mL) and visualized with iodine. Intestinal lipid metabolites including TG, free fatty acids, cholesterol, PC, and PE were analyzed by HPLC. Lipids were extracted from 1 mg intestinal protein homogenate in 3.75 mL of chloroform and methanol (2:1) solution containing batyl alcohol (200µg) and PDME

(50µg) as an internal standard; 1.25 mL of mildly acidic 0.9% sodium chloride and 1.25 mL of chloroform were added – vortexing after each. Samples were centrifuged at 3000 rpm for 10 min and the bottom layer was removed and dried down. Samples were redissolved in 100 µL of chloroform and isooctane (1:1) and run on HPLC.

2.2.3 Western blots

50 µg protein from liver and intestinal homogenates were run on sodium dodecyl sulfate polyacrylamide gel (8.5 %) and transferred to a PVDF membrane (0.45 µm). Membranes were probed with CTα (diluted 1:2000, gift from Dr R. K. Mallampalli) and PEMT (raised in our laboratory (Cui, Vance, Chen, Voelker, & Vance, 1993)). Membranes were probed with GAPDH (1:5000, ab8245; Abcam) as a loading control. Enhanced chemiluminescence (WBLUF0500, Millipore) was used for detection of membranes and imaging was performed on Chemi-Doc MP imager (Bio-Rad Laboratories, CA, USA). Protein levels were quantified using Image Lab software from Bio-Rad.

2.2.4 Real-time quantitative PCR analysis

Liver and intestinal samples were homogenized in TRIzol (15596018; Invitrogen) and intestinal total RNA was isolated using RNEasy Mini (74104; Qiagen) kits. Liver and intestinal RNA samples were treated with DNase 1 (18068-015; Invitrogen) and reverse transcribed to cDNA using oligo(dT)12–18 primers (18418-012; Invitrogen), random primers (48190011; Thermo Fisher Scientific), and Superscript II (18064-173 014; Invitrogen). Quantitative PCR was run for 40 cycles using Power SYBR Green PCR Master Mix (4367659; Thermo Fisher Scientific) on StepOne Plus system (Applied Biosystems, MA, USA). Data was normalized to mRNA expression

of Cyclophilin A (*Ppia*) for liver samples and *Rplp0* for intestinal samples. Primer sequences are found in Table 2.1.

Table 2.1: Primers for quantitative PCR.

Gene	Gene Name	Forward Primer	Reverse Primer
Ppia	Peptidylprolyl isomerase A (Cyclophilin A)	TCC AAA GAC AGC AGA AAA CTT TCG	TCT TCT TGC TGG TCT TGC CAT TCC
Rplp0	Ribosomal protein lateral stalk subunit P0	ACT GGT CTA GGA CCC GAG AAG	CTC CCA CCT TGT CTC CAG TC
Pcyt1a	Phosphate cytidylyltransferase 1, choline, α isoform	GCT AAA GTC AAT TCG AGG AA	CAT AGG GCT TAC TAA AGT CAA CT
Cd36	Cluster-determinant 36	TGG CTA AAT GAG ACT GGG ACC	ACA TCA CCA CTC CAA TCC CAA G
Dgat2	Diacylglycerol O-Acyltransferase 2	GGC TAC GTT GGC TGG TAA CTT	TTC AGG GTG ACT GCG TTC TT
Mogat2	Monoacylglycerol O-Acyltransferase 2	TAC AGC TTT GGC CTC ATG C	AGG GCT GTG GTG TCA TCT G
Cidec	Cell Death Inducing DFFA Like Effector C	CAC TGC TAC AAG GCC AAG C	GGT GGC ATC CAG GAA CTG
Fabp1	Fatty acid binding protein 1	CAG AAA GGG AAG GAC ATC AAG	TGG TCT CCA GTT CGC ACT C
Fatp4	Fatty acid transport protein 4	GAA GGG GGA CCA AGC CTA	AGT TCC TGG CAC CTC AAC AC
Npc1l1	NPC1 like intracellular cholesterol transporter 1	TGG ACT GGA AGG ACC ATT TCC	GTG CCC CGT AGT CAG CTA T
Scarb1	Scavenger receptor class B, member 1	GCC CAT CAT CTG CCA ACT	GCC CAT CAT CTG CCA ACT
Mttp	Microsomal triglyceride transfer protein	TGA GCG GTC TGG ATT TAC AAC	CAA GCA CAG CGG TGA CA
Cyp7a1	Cytochrome P450, family 7, subfamily a, polypeptide 1	ACA CCA TTC CTG CAA CCT TC	TCT TGG CCA GCA CTC TGT AA
Cyp8b1	Cytochrome P450, family 8, subfamily b, polypeptide 1	GCA GCA CTG AAT ACC CAT CC	TCT GAG AGC TGG GGA GAG
Cyp27a1	Cytochrome P450, family 27, subfamily a, polypeptide 1	CTT TCC TGA GCT GCT TTT GG	CAC CAG TCA CTT CCT TGT GC
Ntcp	Solute carrier family 10 (sodium/bile acid cotransporter family), member 1	GCT TCC TGA TGG GCT ACA TT	ATG CTG ATG GTG CGT CTG
Oatp1	Solute carrier organic anion transporter family, member 1a1	GAA GTC TGT TGG AAC TGG AAC C	GTC ACA CTC AGG GCC TTT CT
Atp8b1	ATPase, class I, type 8B, member 1	ATG GTG GAC AGA ATT GAT GGT	CTG ACG GTG ATG GTG TTC TG
Bsep	ATP-binding cassette, sub-family B (bile salt export pump), member 11	CAG TGG GTG TGG TAA AAG CA	TGC TGT CGT GAC CAT CTA TCA
Mdr2	ATP-binding cassette, sub-family B (Multidrug resistance 2), member 4	ACA TGT TCT CCC TGG TCT TCT T	CAG CTT TCC CAA ACG TGA AG
Nr1h4	Nuclear receptor subfamily 1, group H, member 4	GGG ATG AGC TGT GTG TTG TC	GGC GTT CTT GGT AAT GCT TC
Nr0b2	Nuclear receptor subfamily 0, group B, member 2	CGA TCC TCT TCA ACC CAG AT	AGC CTC CTG TTG CAG GTG T
Col1a1	Collagen, type I, alpha 1	AGA CAT GTT CAG CTT TGT GGA C	GCA GCT GAC TTC AGG GAT G
Tnfa	Tumor necrosis factor, transcript variant 2	GTC TAC TGA ACT TCG GGG TGA	CAC CAC TTG GTG GTT TGC TAC GAC
Nox2	Cytochrome b-245, beta polypeptide	GAC TGG ACG GAG GGG CTA T	ACT TGA GAA TGG AGG CAA AGG
Pemt	Phosphatidylethanolamine N-methyltransferase	CCG CTC GAG CGT TAT GAG CTG GCT G	CCT GTC AGC TTC TTT TGT GCA

2.2.5 Statistical analysis

Data was statistically analyzed using GraphPad Prism 9 and is expressed at mean ± SEM. Statistical significance ($p<0.05$) was determined either by student's T-test for comparison of two independent groups or two-way ANOVA with uncorrected Fisher's Least Significant Difference test for more than two independent groups.

2.3 Results

2.3.1 Chow-fed CTα^{LKO} mice have reduced fasting plasma TG levels and display features of NAFLD

To determine the effects of an acute reduction in hepatic PC synthesis on liver function, mice were injected with AAV-Cre or AAV-GFP respectively on day 10 of the experiment. From days 10-17 CTα^{LKO} mice lost weight compared to control mice, who remained relatively weight stable (Figure 2.1 A). Surprisingly, CTα^{LKO} mice had increased food intake from day 10-17 compared to control mice that ate similar amounts throughout days 1-17 (Figure 2.1 B). CTα^{LKO} mice showed a reduction in fasting plasma TG compared to control mice (Figure 2.1 C). Additionally, CTα^{LKO} mice had increased hepatic TG levels (Figure 2.1 D). CTα^{LKO} mice also presented with reduced hepatic PC levels, similar hepatic PE levels, and a reduced hepatic PC:PE ratio compared to control mice (Figure 2.1 E-G). Hepatic PC can be synthesized not only through the CTα pathway, but also through the PEMT and CTβ pathway. Despite reduced hepatic PC and PC:PE ratio, mRNA levels of *Pemt* and *Pcyt1b* were also significantly reduced (Figure 2.1 H). The accumulation of lipids in hepatocytes is known to induce cellular stress, therefore mRNA levels of genes involved in cellular stress were measured. mRNA levels of *Col1a1* and *Nox2* were significantly increased in CTα^{LKO} mice compared to control mice (Figure 2.1 H). *Col1a1* and *Nox2* are involved in the development of fibrosis and oxidative stress development respectively (S. Y. Kim et al., 2017; Qi, Wang, Li, Wang, & Liu, 2017). Together, this data indicates that an acute reduction in hepatic PC synthesis via the CDP-choline pathway results in dramatic weight loss, reduced fasting plasma TG, and the induction of NAFLD.

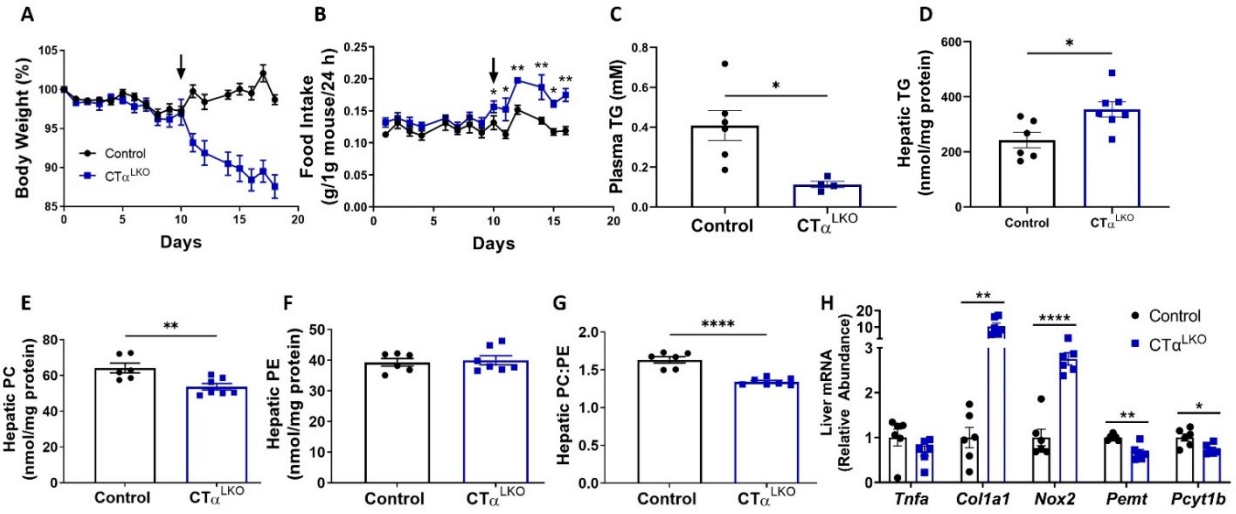

Figure 2.1: One-week chow-fed CTα^{LKO} mice have reduced plasma TG and increased markers of NAFLD. (A) Body weight and (B) food intake of control and CTα^{LKO} mice (arrow indicates date of AAV injection). (C) Plasma and (D) hepatic TG in control and CTα^{LKO} mice. Hepatic (E) PC, (F) PE, and (G) PC:PE ratio in control and CTα^{LKO} mice. (H) Liver mRNA levels of *Tnfa*, *Col1a1*, *Nox2*, *Pemt*, and *Pcyt1b* in control and CTα^{LKO} mice. Values are reported as ±SEM, n = 6–7/group. *P < 0.05, **P < 0.01, ****P < 0.0001.

2.3.2 HFD-fed CTα^{LKO} mice lose weight and have reduced VLDL secretion

Next, we sought to determine if short-term HFD feeding in adult mice with an acute CTα^{LKO} would lead to a more severe phenotype in hepatic lipid processes than observed in chow-fed CTα^{LKO} mice. Chow-fed mice were injected with AAV-GFP or AAV-Cre to generate control and CTα^{LKO} mice, respectively, on day 7 of the experiment. Control and knockout mice were fed the same chow diet for 3 days post injection (until day 9) to ensure the knockout had time to take effect. From days 10 until 19, CTα^{LKO} mice lost around 20 % of their total body weight while control mice gained weight, a greater reduction then was seen in chow-fed CTα^{LKO} mice (Figure 2.2 A). Food intake of HFD-fed CTα^{LKO} mice remained similar to that of HFD-fed controls (Figure 2.2 B). To ensure AAV-Cre was effective, hepatic CTα protein levels were measured. Western blot analysis showed a significant reduction in hepatic CTα in CTα^{LKO} mice compared to control mice (Figure 2.2 C). The reduction in CTα levels also corresponded to a reduction in fasting plasma TG and a reduction in the appearance of fasting plasma TG following a Poloxamer 407 injection in CTα^{LKO} mice compared to control mice Figure 2.2 D-E). Together, this data indicates that HFD-feeding increases the severity of the acute reduction in CTα-derived hepatic PC synthesis in lipid processing.

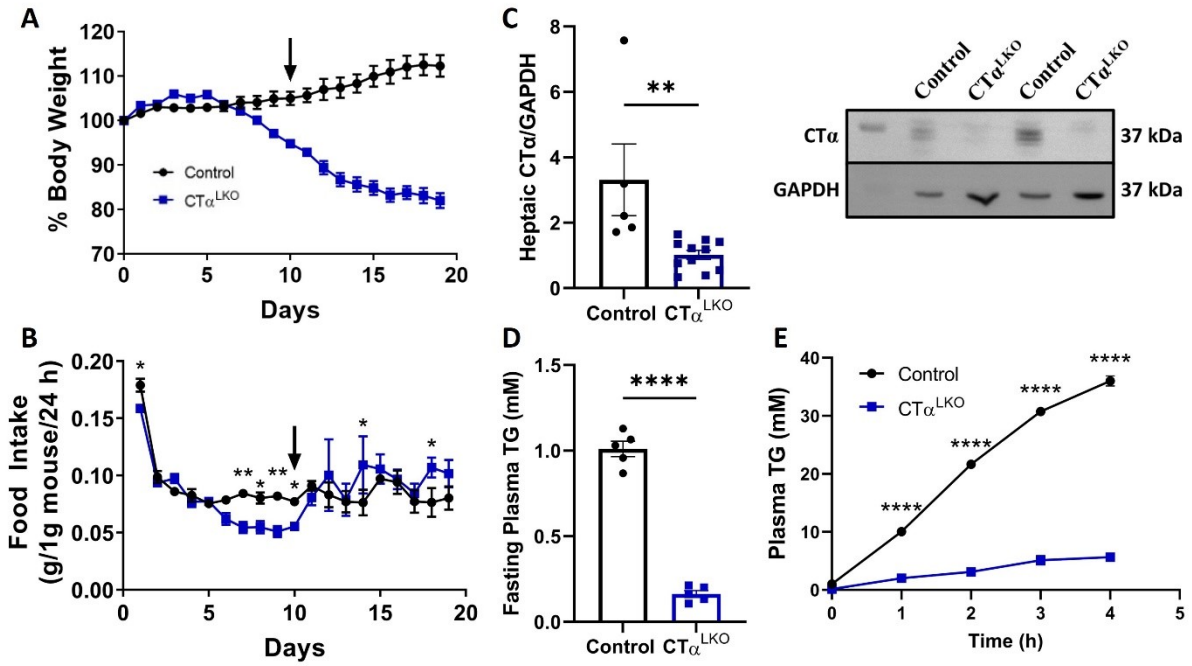

Figure 2.2: One-week HFD-fed CTα^{LKO} mice lose weight and have reduced fasting plasma TG. (A) Body weight and (B) food intake of control and CTα^{LKO} mice (arrow indicates date of AAV injection). (C) Hepatic CTtα/GAPDH protein levels in control and CTα^{LKO} mice. (D) Fasting plasma TG and (E) fasting plasma TG following poloxamer injection (0-4 h) in control and CTα^{LKO} mice. Values are reported as ±SEM, n = 5–11/group. *P < 0.05, **P < 0.01, ****P < 0.0001.

2.3.3 HFD-fed CTα^{LKO} mice have hepatic lipid accumulation and develop NASH

The livers of HFD-fed CTα^{LKO} mice were analyzed to determine how the acute deletion of the gene affects hepatic lipid and stress levels compared to those in the CTα^{PLKO} mice (Jacobs et al., 2004; Niebergall et al., 2011). The hepatic weight to body weight ratio was significantly higher in CTα^{LKO} mice (Figure 2.3 A). This was corroborated by a significant increase in hepatic TG in CTα^{LKO} mice compared to controls (Figure 2.3 B). In addition, we observed reduced PC levels, similar PE levels, and a reduced hepatic PC:PE ratio (Figure 2.3 C-E). We were surprised to see that, despite similar protein levels, mRNA levels of *Pemt* and *Pcyt1b* were significantly reduced (Figure 2.3 F). Western blots analysis of PEMT protein in the livers of CTα^{LKO} mice showed similar levels compared to control mice (Figure 2.3 G). As seen above, HFD-fed CTα^{LKO} mice had increased mRNA levels of *Col1a1* and *Nox2* compared to control mice indicating induction of NASH (Figure 2.3 F). These results indicate that while HFD-fed CTα^{LKO} mice lose body weight and have reduced circulating lipids, they begin to develop NAFLD. Finally, these results were more severe than the HFD-fed CTα^{PLKO} mice, indicating that an acute reduction in hepatic PC synthesis is more challenging on lipid homeostasis than a permanent reduction.

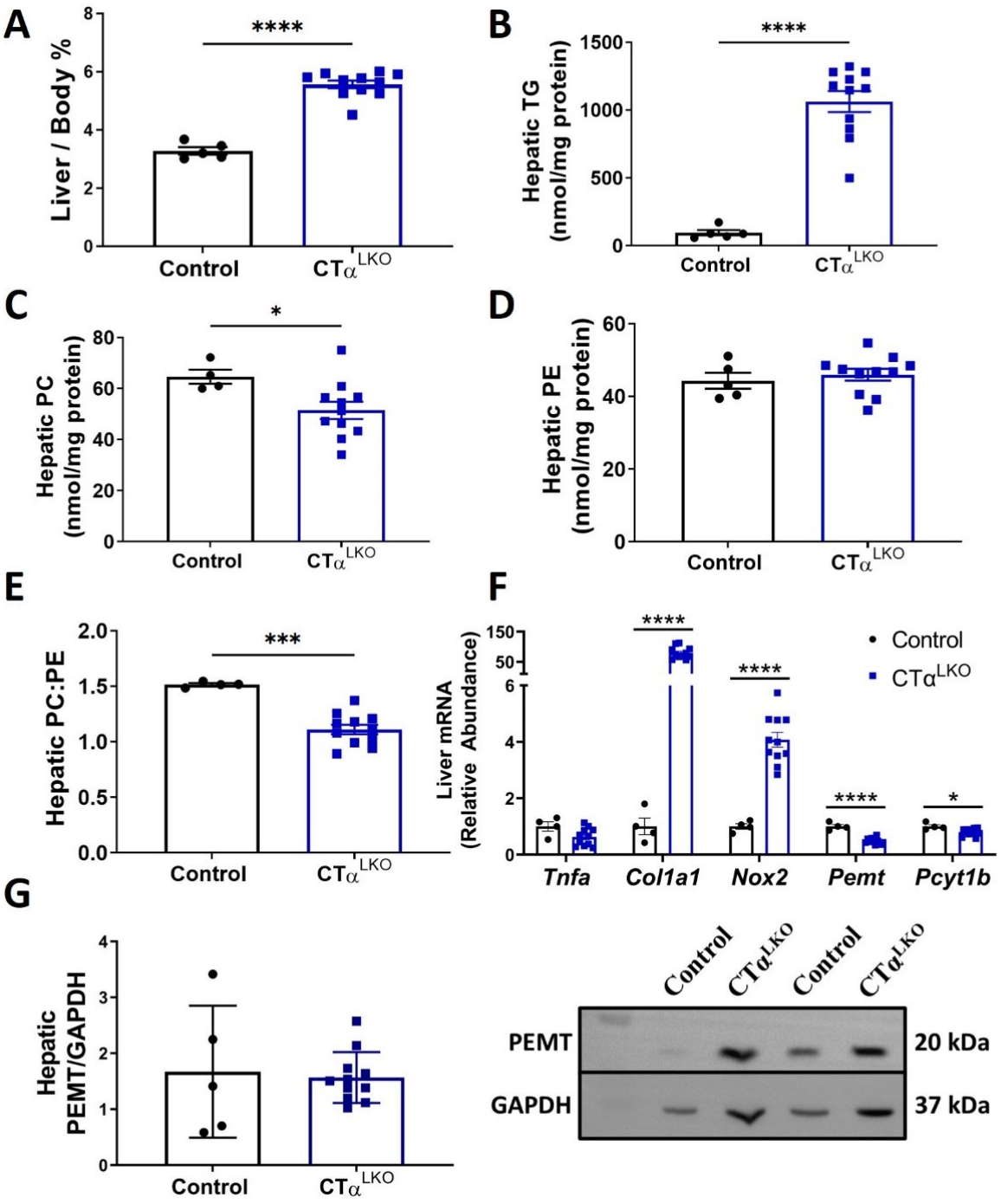

Figure 2.3: One-week HFD-fed CTα^{LKO} mice have increased markers of NAFLD. (A) Liver weight/body weight in control and CTα^{LKO} mice. Hepatic (B) TG, (C) PC, (D) PE, and (E) PC:PE ratio in control and CTα^{LKO} mice. (F) Liver mRNA levels of *Tnfa*, *Col1a1*, *Nox2*, *Pemt*, and *Pcyt1b* in control and CTα^{LKO} mice. (G) Hepatic PEMT/GAPDH protein levels in control and CTα^{LKO} mice. Values are reported as ±SEM, n = 6–7/group. *$P < 0.05$, **$P < 0.01$, *** $P < 0.001$ ****P < 0.0001.

2.3.4 Chow-fed CTα^{LKO} mice show alterations in postprandial lipid handling

To determine if alterations in hepatic PC synthesis leads to changes in intestinal function, we measured postprandial lipid handling in the jejunums of chow-fed CTα^{LKO} mice. As shown previously, CTα^{LKO} mice had reduced fasting plasma TG compared to controls (Figure 2.4 A). To measure intestinal dietary lipid handling, control and knockout mice were injected with Poloxamer 407 and gavaged with olive oil. CTα^{LKO} mice had reduced appearance of postprandial plasma TG compared to controls (Figure 2.4 B). These results indicate that there is a reduction of postprandial lipids entering circulation in chow-fed CTα^{LKO} mice. Despite the reduced circulating TG, CTα^{LKO} mice had similar jejunal TG and FFA levels, and a reduced jejunal cholesterol level compared to control mice (Figure 2.4 C-E). Therefore, CTα^{LKO} mice do not appear to be accumulating lipids in the intestine, indicating a reduction in lipid uptake by the enterocytes. Surprisingly, chow-fed CTα^{LKO} mice had a reduction in jejunal PC levels and a tendency to reduced PE levels and, hence, a similar PC:PE ratio compared to control mice Figure 2.4 F-H). A variety of mRNA levels of lipid absorption and processing genes were measured to elucidate the cause of the suspected lipid malabsorption, yet there were no differences between control and CTα^{LKO} mice (Figure 2.4 I). In summary, CTα-derived hepatic PC synthesis appears to be important for proper communication between the liver and intestine and for the regulation of postprandial lipid absorption and handling.

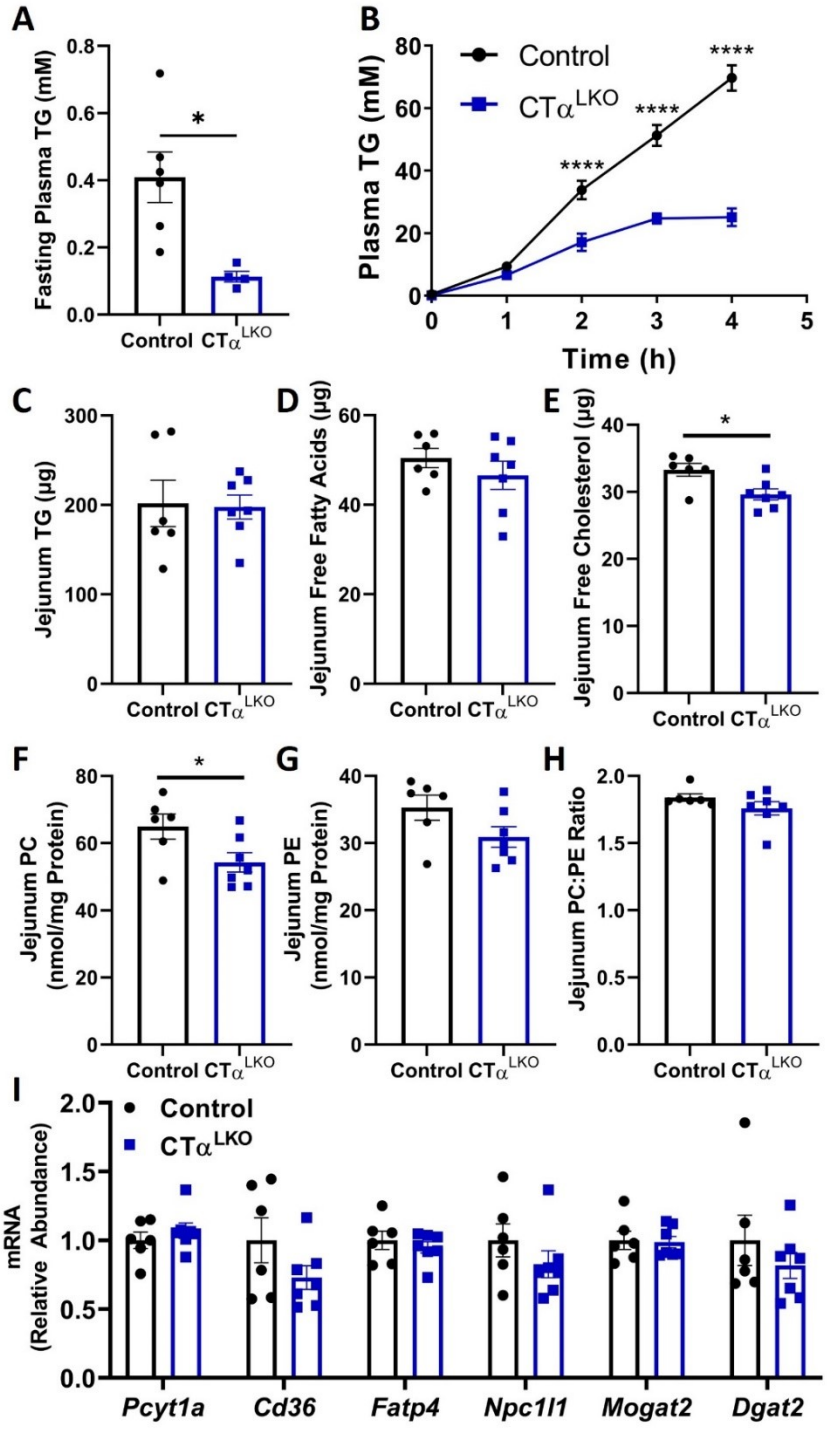

Figure 2.4: One-week chow-fed CTα^{LKO} mice have reduced postprandial plasma TG. (A) Fasting and (B) postprandial plasma TG following poloxamer injection (1-4 h) in control and CTα^{LKO} mice. Jejunum (C) TG, (D) free fatty acids, (E) cholesterol, (F) PC, (G) PE, and (H) PC:PE ratio in control and CTα^{LKO} mice. (I) Intestinal mRNA levels of *Pcyt1a*, *Cd36*, *Fatp4*, *Npc1l1*, *Mogat2* and *Dgat2* in control and CTα^{LKO} mice. Values are reported as ±SEM, n = 6–7/group. *P < 0.05, ****P < 0.0001.

2.3.5 HFD-fed CTα^{LKO} mice have alterations in postprandial lipid handling

Next, we wanted to determine if HFD-feeding would lead to a more severe intestinal phenotype in CTα^{LKO} mice than in chow-fed mice. As shown previously, HFD-fed CTα^{LKO} mice had reduced fasting plasma TG compared to control mice (Figure 2.5 A). HFD-fed CTα^{LKO} mice also had delayed plasma appearance of TG compared to controls after a Poloxamer 407 injection and bolus of olive oil (Figure 2.5 B). HFD-fed CTα^{LKO} mice trended towards a reduction in jejunal TG and free fatty acids (FFAs) and had a significant reduction in jejunal free cholesterol compared to controls (Figure 2.5 C-E). Total jejunal phospholipid analysis did not reveal differences in jejunal PC, PE, or PC to PE ratio (Figure 2.5 F-H). Finally, mRNA analysis of genes involved in lipid absorption and processing were also analyzed in HFD-fed mice. HFD-fed CTα^{LKO} mice had a reduction in *Fatp4* and *Npc1l1* mRNA levels compared to controls (Figure 2.5 I): *Fatp4* is involved in fatty acid uptake and *Npc1l1* is the primary cholesterol transporter protein. These results underscore the importance of hepatic PC synthesis on postprandial intestinal function.

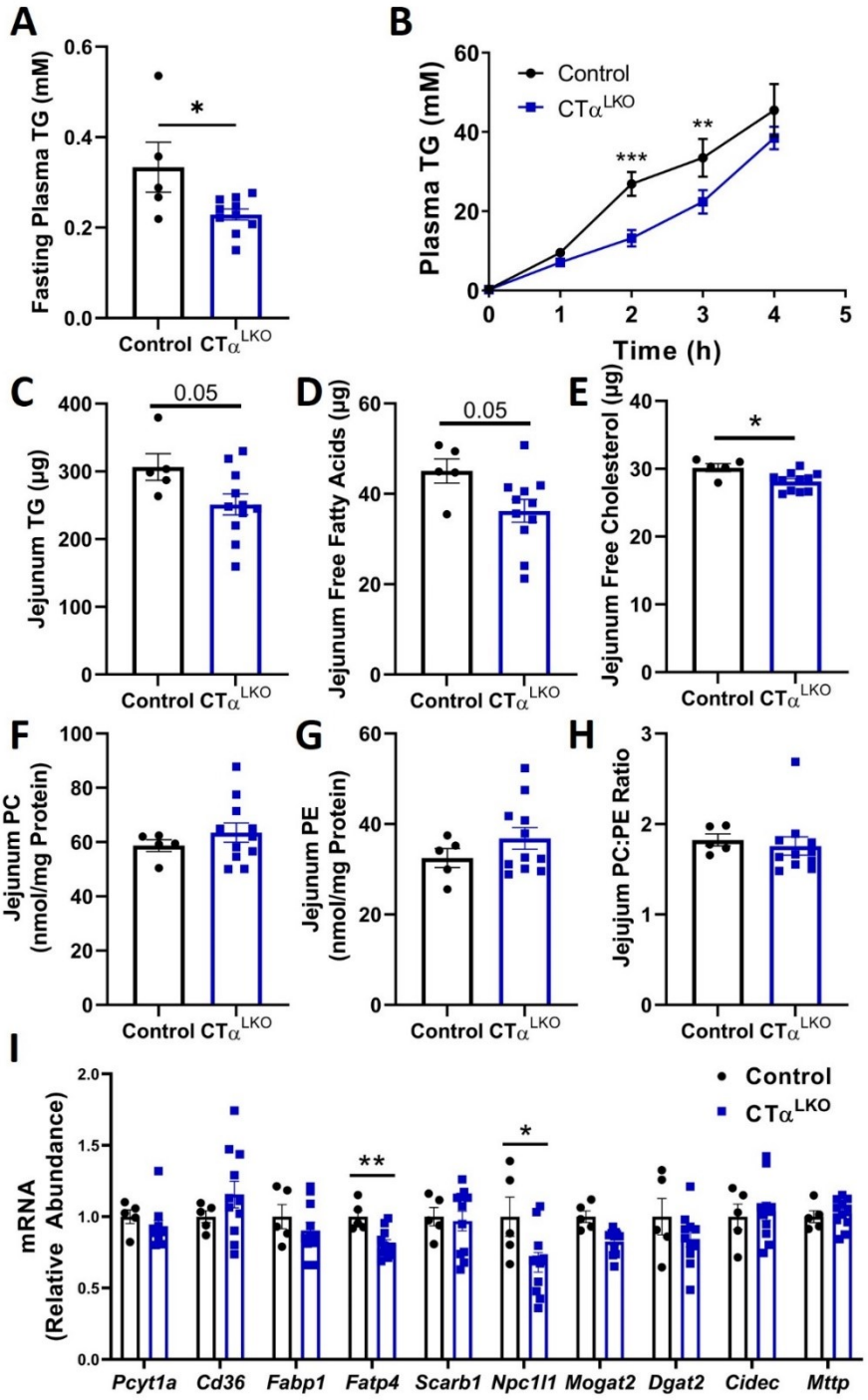

Figure 2.5: One-week HFD-fed CTα^{LKO} mice have reduced postprandial plasma TG. (A) Fasting and (B) postprandial plasma TG following poloxamer injection (1-4 h) in control and CTα^{LKO} mice. Jejunum (C) TG, (D) free fatty acids, (E) cholesterol, (F) PC, (G) PE, and (H) PC:PE ratio in control and CTα^{LKO} mice. (I) Intestinal mRNA levels of *Pcyt1a*, *Cd36*, *Fabp1*, *Fatp4*, *Scarb1*, *Npc1l1*, *Mogat2*, *Dgat2*, *Cidec, Mttp* in control and CTα^{LKO} mice. Values are reported as ±SEM, n = 6–7/group. *$P < 0.05$, **$P < 0.01$.

2.3.6 HFD-fed CTα^{LKO} mice have altered bile formation

To address altered bile production as a potential cause of lipid malabsorption in CTα^{LKO} mice, we analyzed bile flow and bile composition. To this end, gallbladders of CTα^{LKO} and control mice were cannulated. HFD-fed CTα^{LKO} mice showed a reduced bile flow compared to control mice (Figure 2.6 A). Despite the reduced flow, CTα^{LKO} mice had a similar bile acid secretion and a surprising increase in phospholipid secretion (Figure 2.6 B-C). CTα^{LKO} mice had similar levels of plasma bile acids as control mice (Figure 2.6 D). Hepatic mRNA levels of genes involved in bile acid synthesis and secretion were analyzed and no significant effects on expression of major genes involved in bile acid synthesis or transport were observed. Only a significant reduction in *Oatp1* mRNA levels (Figure 2.6 E) was found. *Oatp1* is a gene encoding a bile acid uptake protein that removes circulating plasma bile acids reabsorbed from the intestine yet contributes much less to this process than NTCP. Consequently, plasma bile acid levels were not elevated in CTα^{LKO} mice. *Abcb4*, also referred to as *Mdr2*, encoding the canalicular phospholipid translocator, tended to be induced. Together, these results indicate that biliary homeostasis in CTα^{LKO} mice minimally affected and is unlikely to be contributing to the observed lipid malabsorption or weight loss.

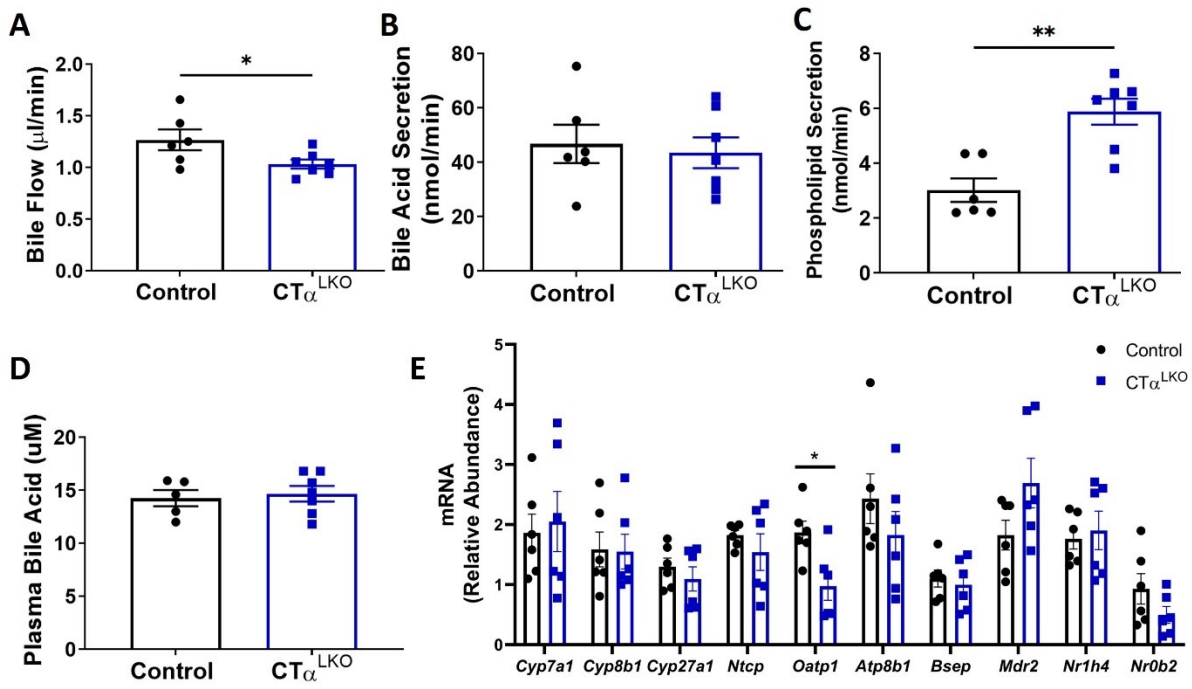

Figure 2.6: Two-week HFD-fed CTα^{LKO} mice have reduced bile flow. (A) Bile flow, (B) bile acid secretion, and (C) phospholipid secretion in control and CTα^{LKO} mice following 30-minute gallbladder cannulation. (D) Plasma bile acid concentration in control and CTα^{LKO} mice. (E) Liver mRNA levels of *Cyp7a1*, *Cyp8b1*, *Cyp27a1*, *Ntcp*, *Oatp1*, *Atp8b1*, *Bsep*, *Mdr2*, *Nr1h4*, and *Nr0b2* in control and CTα^{LKO} mice. Values are reported as ±SEM, n = 6–7/group. *$P < 0.05$, **$P < 0.01$.

2.4 Discussion

Hepatic CTα-derived PC synthesis is an important regulator of hepatic lipid metabolism and necessary to prevent the development and progression of NAFLD. Chow-fed $CT\alpha^{PLKO}$ mice have reduced circulating HDL and VLDL levels, and when switched to a HFD, quickly develop NASH (Jacobs et al., 2004; Niebergall et al., 2011). The aim of the current study was first to determine whether an acute knockout of CTα in adult mice would yield a similar phenotype as $CT\alpha^{PLKO}$ mice. Second, we aimed to determine whether hepatic CTα-derived PC synthesis plays an important role in postprandial lipid handling. Chow- and HFD-fed $CT\alpha^{LKO}$ mice experienced significant weight loss in the week following knockout induction. Chow- and HFD-fed $CT\alpha^{LKO}$ mice also had reduced fasting plasma TG levels, and developed hepatic TG accumulation, and subsequently, NASH. When postprandial lipid handling was investigated in chow- and HFD-fed $CT\alpha^{LKO}$ mice, it was determined that they had reduced appearance of postprandial TG in the plasma and intestine indicating lipid malabsorption. To try and elucidate the cause of lipid malabsorption, bile flow and contents were analyzed. We found that while HFD-fed $CT\alpha^{LKO}$ mice had reduced bile flow, they had the same bile acid secretion and increased phospholipid secretion into bile indicating bile is unlikely to be affecting lipid absorption. Together, these results indicate that hepatic CTα-derived PC synthesis is necessary for proper gut-liver communication and has an important role in fasting and postprandial lipid homeostasis.

PC synthesis in the liver is known to be important for maintaining fasting lipid levels, reducing hepatic TG accumulation, and resisting the development of hepatic ER stress, inflammation, and fibrosis leading to NASH (Gao et al., 2015; Jacobs et al., 2004; Li et al., 2006; Niebergall et al., 2011; Noga & Vance, 2003; Wan, van der Veen, et al., 2019). A previous mouse model was developed to look at the role of CTα-derived hepatic PC using a Cre-lox system fused

to an albumin promoter for a liver specific CTα knockout mouse ($CT\alpha^{PLKO}$ mice) (Jacobs et al., 2004). Initial studies into these mice concluded that CTα-derived hepatic PC was necessary for proper hepatic lipid handling. Chow-fed $CT\alpha^{PLKO}$ mice had a reduction in hepatic PC, with an increase in hepatic PEMT and CTβ protein levels to accommodate the reduction in PC synthesis. As well, chow-fed $CT\alpha^{PLKO}$ mice maintained normal weight despite having a reduction in fasting plasma TG, PC, and cholesterol, that corresponded to a significant decrease in circulating HDL and VLDL particles. The livers of male chow-fed $CT\alpha^{PLKO}$ mice were similar to controls in weight and TG levels and had no increase in circulating AST or ALT indicating a lack of liver injury (Jacobs et al., 2004). Within one-week of HFD feeding the $CT\alpha^{LKO}$ mice maintained the lower fasting plasma TG levels, but had developed fatty liver and NASH, consistent with a significant increase in ALT despite maintaining weight compared to controls (Niebergall et al., 2011). Our first aim was to determine if knocking out hepatic CTα in adult mice would lead to same phenotype seen above. Chow-fed $CT\alpha^{LKO}$ mice not only had reduced fasting plasma TG but had also lost weight, had increased hepatic TG accumulation, and had increased mRNA levels of genes associated with cellular stress, including *Col1a1* and *Nox2*. *Col1a1* upregulation occurs during the development and progression of fibrosis as it encodes the protein type 1 collagen (Qi et al., 2017). On the other hand, *Nox2* upregulation participates in oxidative stress observed in NAFLD as it encodes nicotinamide adenine dinucleotide phosphate oxidase 2, responsible for producing reactive oxygen species (S. Y. Kim et al., 2017). These results indicate that chow-fed acute $CT\alpha^{LKO}$ mice quickly develop NASH. Additionally, chow-fed $CT\alpha^{LKO}$ mice had a significant reduction in the mRNA levels of *Pemt* and *Pcyt1b* – the gene encoding CTβ – despite having reduced hepatic PC levels. The reduction in *Pemt* and *Pcyt1b* mRNA levels is in opposition with the previous results found in $CT\alpha^{PLKO}$ mice (Jacobs et al., 2004). Together, these results indicate that an acute

CTα knockout leads to a more severe phenotype compared to the permanent knockout which could be due to a lack of compensation for the reduction in hepatic PC through alternative PC synthetic pathways (Jacobs et al., 2004).

Next, we wanted to determine if HFD-feeding increases the severity of the acute CTα liver knockout on hepatic lipid regulation. The CTα knockout was determined to be effective, and only around 33 % of CTα levels remained. The remaining CTα levels could be accounted for by non-hepatocyte cells found in the liver. Around 20 % of the liver is made up of non-hepatocytes including stellate and Kupffer cells that also utilize CTα to synthesize PC. HFD-fed acute $CT\alpha^{LKO}$ mice had an even more exacerbated phenotype as observed in chow-fed acute $CT\alpha^{LKO}$ mice. HFD-fed acute $CT\alpha^{LKO}$ mice lost around 20 % of their body weight in only one week compared to controls, as well as a 2.5-fold further reduction in fasting plasma TG than chow-fed knockout mice. Also, HFD-fed acute $CT\alpha^{LKO}$ mice had reduced appearance of fasting plasma TG, indicating reduced hepatic VLDL secretion, and hepatomegaly. Hepatomegaly was not observed in HFD-fed $CT\alpha^{PLKO}$ mice, another finding of increased severity of the acute knockout (Ling et al., 2012). HFD-fed acute $CT\alpha^{LKO}$ mice also had a significant reduction in *Pemt* and *Pcyt1b* mRNA levels, and similar PEMT protein levels compared to controls. Interestingly, HFD-fed PEMT knockout mice lose weight due to a reduction in choline availability, and the reduced PEMT levels could also be accounting for some of the noted weight loss in HFD-fed acute $CT\alpha^{LKO}$ mice (Jacobs et al., 2010; Wan, van der Veen, et al., 2019). Together, these results support the idea that there is a lack of PC synthesis compensation in acute $CT\alpha^{LKO}$ mice, and this may be contributing to their weight loss, increased liver weight, and worsened NAFLD. Future work will involve investigating the compensatory mechanisms occurring in $CT\alpha^{LKO}$ mice over time. In conclusion, HFD-fed acute $CT\alpha^{LKO}$ mice lose a significant amount of body weight, most likely due to a lack of compensation

for reduced hepatic PC synthesis, leading to the development of reduced fasting plasma lipids and NASH. Together, these results provide further evidence that maintaining proper PC levels is important in hepatic health.

Communication between the intestine and the liver has been known to be an important part of regulating whole-body homeostasis (Plauth et al., 1993). They are able to communicate through enterohepatic circulation where substances secreted from the liver in bile are reabsorbed into the small intestine, and can be sent back to the liver through the circulatory system (Ridgway & McLeod, 2008). The intestine can communicate with the liver through hormonal and neuronal signaling, which influences hepatic functioning, including the development and progression of NAFLD (Alvarez-Sola et al., 2017; Taher et al., 2014). Our lab has previously developed an inducible, intestinal specific, CTα knockout ($CT\alpha^{IKO}$) mouse that has lost the ability to synthesize *de novo* phosphatidylcholine in the intestine (Kennelly et al., 2018). While $CT\alpha^{IKO}$ mice do not have any changes to hepatic weight, TG, or PC levels, the loss of intestinal *de novo* PC synthesis lead to changes in biliary secretion. Bile secretion from the liver of $CT\alpha^{IKO}$ mice were analyzed using gallbladder cannulations, and it was determined that they had increased bile flow with an increase in the major bile constituents, including bile acids, PC, and cholesterol. These experiments lead to our second aim, which was to determine if CTα-derived hepatic PC synthesis influenced intestinal homeostasis. Surprisingly, chow-fed $CT\alpha^{LKO}$ mice had a significant reduction in the appearance of postprandial plasma TG, indicating reduced chylomicron secretion, as well as reduced jejunal cholesterol and no difference in jejunal TG or free fatty acids. With reduced chylomicron secretion, we had expected an accumulation of lipids in the intestine, indicating that $CT\alpha^{LKO}$ mice have lipid malabsorption. Finally, we were surprised to see a significant reduction in jejunal PC levels despite the mRNA levels of *Pcyt1a* – the gene encoding CTα – remaining

similar to control mice. The cause of the reduced jejunal PC needs to be further elucidated, though it may be due to an inability of the intestine to handle the one-time efflux of lipids given during the olive oil gavage, as there is an increased demand for PC synthesis during the formation of chylomicron particles (Lee & Ridgway, 2018).

HFD-fed CTα^{LKO} mice were also found to have a minor reduction in the postprandial appearance of plasma TG, as well as reduced jejunal cholesterol with a trend towards reduced jejunal TG and free fatty acids. Intestinal mRNA analysis was performed on certain genes involved in lipid absorption, processing, and secretion to determine which area of lipid metabolism was most affected in HFD-fed CTα^{LKO} mice. The only notable changes were a significant reduction in *Fatp4* and *Npc1l1*. The *Fatp4* gene encodes fatty-acid transport protein 4, one of the redundant fatty acid transport proteins located on the apical membrane of the small intestine (Shim et al., 2009; Stahl et al., 1999). *Npc1l1* gene encodes the protein Niemann-Pick C1 Like 1 protein responsible for the majority of cholesterol absorption in the jejunum (Davis et al., 2004; Duan, Wang, & Wang, 2004). The reduction in the mRNA expression of these genes coincides with the reduced free fatty acid and cholesterol levels in HFD-fed CTα^{LKO} mice. The lack of fat absorption and reduced systemic circulation of postprandial lipids could be contributing the immediate weight loss observed in CTα^{LKO} mice. These results indicate that HFD-fed CTα^{LKO} mice have lipid malabsorption, and that CTα-derived hepatic PC is important in maintaining fasting and postprandial lipid homeostasis.

Bile and the enterohepatic circulation are important for the proper digestion and absorption of lipids. Previous work investigating the role of biliary transport proteins in the liver have shown how altering bile composition leads to disruption in lipid absorption and processing. Bile salt export pump (BSEP) allows for the secretion of bile acids into bile and is produced by the ATP

binding cassette subfamily B member 11 (*Abcb11*) gene. In *Abcb11* knockout mice, bile salt composition is altered leading to lipid malabsorption (Fuchs et al., 2020). PC is the second most abundant constituent of bile, after bile acids, and the amount of PC in bile is regulated by the multidrug resistant 2 (MDR2) protein produced by the ATP binding cassette subfamily B member 4 (*Abcb4*) gene. In *Abcb4* knockout mice, PC secretion into bile is eliminated and it lead to intestinal lipid accumulation and reduced postprandial TG appearance (Voshol et al., 2000). Previous research has investigated the role of PC synthesis in the liver and its impact on bile homeostasis. $PEMT^{-/-}$ mice were found to have a 40 % reduction in the concentration of gallbladder PC (Agellon, Walkey, Vance, Kuipers, & Verkade, 1999). When $PEMT^{-/-}$ mice were fed a HFD, they were found to develop cholestasis with an increase in plasma bile acids, and a reduction in biliary secretion of bile acids and PC (Wan, Kuipers, et al., 2019). The role of hepatic CTα in biliary homeostasis was also investigated by analyzing the gallbladder concentration of biliary constituents in $CT\alpha^{PLKO}$ mice. Chow-fed $CT\alpha^{PLKO}$ mice had no change in the gallbladder concentration of biliary bile acids, PC and cholesterol (Jacobs et al., 2004). These results lead to our hypothesis that alterations in biliary homeostasis may be affecting lipid absorption in our acute $CT\alpha^{LKO}$ mice. While HFD-fed $CT\alpha^{LKO}$ mice were found to have a reduced bile flow they had normal bile acid secretion and a surprising increase in phospholipid secretion. Due to the minimal changes in the amount of biliary bile acids or PC reaching the small intestine, it is unlikely that the lipid malabsorption is caused by changes to biliary homeostasis. Finally, genes associated with bile synthesis, secretion, and reabsorption were measured to attempt to determine the cause of the reduced bile flow. The only gene that was reduced was *Oatp1* which encodes a protein responsible for the reabsorption of bile acids from circulation back into the liver. As there is no increase in

plasma bile acids, a reduction in the expression of *Oatp1* would not be expected to affect plasma bile flow.

In summary, an acute knockout of CTα in the livers of HFD-diet fed mice leads to more severe changes in lipid handling and development of NASH than in $CT\alpha^{PLKO}$ mice. Additionally, we determined that reducing CTα-derived hepatic PC synthesis leads to alterations in the gut-liver axis, including reduced lipid absorption and chylomicron secretion. Our next steps will involve investigating the compensatory mechanisms that lead a reduced NAFLD severity in $CT\alpha^{PLKO}$ mice. Additionally, future research is needed to determine the cause of lipid malabsorption and reduced postprandial chylomicron secretion in $CT\alpha^{LKO}$ mice.

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Chapter 3

De novo phosphatidylcholine synthesis in the small intestinal epithelium is required for normal dietary lipid handling and maintenance of the mucosal barrier

3.1 Introduction

Intestinal homeostasis is maintained by dynamic and complex physiological processes that must operate under stressful environmental conditions (Bischoff, 2011). Intestinal epithelial cells (IEC) are comprised of enterocytes (absorptive cells of the small intestine), goblet cells (mucus-secreting cells), enteroendocrine cells (hormone secreting cells), and Paneth cells (antimicrobial-secreting cells) (Roda et al., 2010). Enterocytes account for 80% of small intestinal epithelial cells and are specialized in nutrient absorption (Cheng & Leblond, 1974). Additionally, goblet cells create a physical barrier to luminal contents by producing the mucus layer, while both goblet cells and Paneth cells create a chemical barrier by producing antimicrobial peptides. The primary constituent of GI mucus is Mucin-2, a glycosylated protein secreted from goblet cells (Sicard, Bihan, Vogeleer, Jacques, & Harel, 2017). Enteroendocrine cells secrete various hormones including glucagon-like peptide-1 (GLP-1) that influence the rate of nutrient uptake, appetite, and intestinal motility.

IECs obtain phosphatidylcholine (PC) by *de novo* synthesis, uptake of PC from the lumen of the GI tract, or uptake of lipoproteins from circulation (Mansbach II & Arnold, 1986). PC in the intestinal lumen comes from both dietary and biliary sources. PC is abundant in many foods including meats, eggs, fish oils and soy (Grandois et al., 2009). Increasing PC supply to the lumen of the small intestine promotes lipid absorption and chylomicron mobilization (Mansbach II, Arnold, & Cox, 1985). The most abundant biliary phospholipid is PC which is involved in the formation of micelles during the digestion of dietary lipids (Barrios & Lichtenberger, 2000). Biliary PC is synthesized in the liver, and its presence in bile is regulated by multidrug resistant gene-2 glycoprotein (MDR2), which is encoded by the Abcb4 gene. *Abcb4*$^{-/-}$ mice, which do not secrete phospholipids into bile, have normal intestinal fatty acid uptake but accumulate lipids in

enterocytes due to impaired chylomicron secretion (Voshol et al., 2000). This observation suggests that biliary PC plays a role in chylomicron assembly or secretion. Once in enterocytes, lyso-PC is re-acylated back to PC by lyso-PC acyltransferase (LPCAT) enzymes. LPCAT3, the most abundant LPCAT enzyme in the small intestinal epithelium, preferentially adds polyunsaturated fatty acids to lyso-PC molecules. Intestine-specific LPCAT3 knockout mice have impaired movement of dietary fatty acids into enterocytes and impaired chylomicron secretion. This phenotype has been linked to impaired incorporation of polyunsaturated fatty acids into PC and altered phospholipid composition of the brush border membrane, leading to impaired passive diffusion of fatty acids into enterocytes (Zhiqiang Li et al., 2015; Rong et al., 2015).

Intestinal *de novo* synthesis of PC occurs through the Kennedy pathway using free choline as a precursor. Cytidine triphosphate:phosphocholine cytidylyltransferase-α (CTα) regulates flux through this pathway. *Pcyt1a*, the gene encoding CTα, is the primary isoform of CT in the intestinal epithelium. Our lab has developed an inducible, intestine specific, CTα knockout ($CT\alpha^{IKO}$) mouse to determine the role that *de novo* PC synthesis plays in intestinal function. The only source of PC in IECs of $CT\alpha^{IKO}$ mice is uptake from the lumen or from circulation. It has previously been determined that $CT\alpha^{IKO}$ mice on a high-fat diet (HFD) rapidly lose body weight and have dietary lipid malabsorption (Kennelly et al., 2018). The aim of the current study is to better understand the factors that contribute to body weight loss and altered intestinal function in $CT\alpha^{IKO}$ mice. We found that impaired *de novo* PC synthesis in the gut is associated with lower abundance of transcripts linked to lipid metabolism and higher abundance of transcripts linked to ER stress, cell death, and inflammation. Impaired movement of fatty acids from the intestinal lumen into enterocytes in $CT\alpha^{IKO}$ mice occurs at high levels of dietary fat intake and occurs in an isolated intestinal sac model. The sac model excludes factors extrinsic to the intestinal epithelium including

bile, gastric emptying, the nervous system, and circulating hormones. Additionally, we found that IECs in CTα^{IKO} mice have high levels of ER stress, induction of transcripts related to host defence, and loss of goblet cells from the small intestinal epithelium. Attempts to modulate these changes through PC supplementation and antibiotic treatment were unable to fully restore intestinal function. Our data suggests that *de novo* PC synthesis is required to maintain intestinal homeostasis and that maintaining PC concentrations in IEC membranes maintains enterocyte metabolic function and prevents the induction of ER stress, cell death, and bacterial infiltration.

3.2 Methods

3.2.1 Animal handling

Female mice were housed in a temperature-controlled environment with 12 h light/dark cycle with free access to water and fed standardized chow diet (5001, Lab Diet, St. Louis, MO) before experiments. Generation of mice by breeding *Pcyt1a*$^{loxP/loxP}$ and *Pcyt1a*$^{loxP/loxP}$;villin-Cre-ER^{T2} mice has been described previously (Kennelly et al., 2018). Female *Pcyt1a*$^{loxP/loxP}$;villin-Cre-ER^{T2} mice were injected intraperitoneally with tamoxifen (1 mg/day) dissolved in sunflower oil for 5 days to induce CTα knockout specifically in intestinal epithelial cells (CTα^{IKO}) which include enterocytes, goblet cells, enteroendocrine cells and Paneth cells (Figure 3.1). Aged matched 8-21 weeks old, tamoxifen treated *Pcyt1a*$^{flox/flox}$ mice were used as controls. After tamoxifen injections, control and CTα^{IKO} mice were given free access to a specialized diet for 4 days, followed by a 16 h fast and 2 h refeed. Unless otherwise stated, mice were euthanized on day 10 of the experiment by cardiac puncture and blood was collected in tubes containing EDTA, dipeptidyl peptidase 4 inhibitor (EMD Millipore, MA) and complete general protease inhibitor (Sigma). Plasma was collected by centrifuging blood at room-temperature at 3000 g for 10 min. After excision, the small

intestines were flushed with solution containing PBS and protease inhibitor cocktail (Sigma). The small intestines were then segmented into duodenum, jejunum, and ileum portions by dividing the intestine by a length ratio of 1:3:2 respectively. A portion of jejunum was collected and stored in 10 % neutral buffered formalin. The rest of the intestinal segments were then opened longitudinally, and the IECs were scraped and frozen in liquid nitrogen. All experiments were approved by the University of Alberta's Institutional Animal Care Committee following guidelines set by Canadian Council on Animal Care.

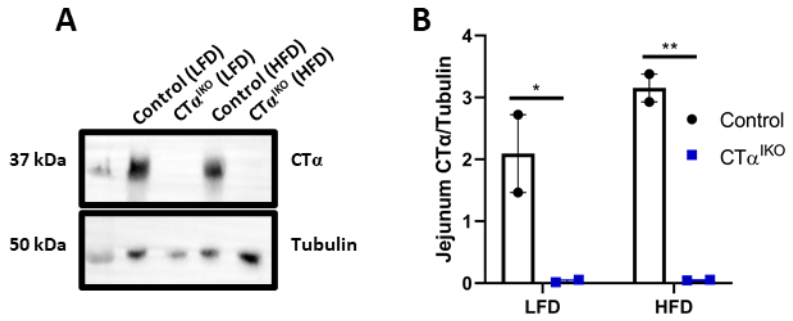

Figure 3.1: CTα knockout in jejunal IECs. Jejunum CTα (A) western blot (B) western blot quantification in control and $CT\alpha^{IKO}$ mice. Mice were 18-22 weeks fed LFD or HFD for 5 days. Values are reported as ±SEM, n=2/group. *P<0.05, **P<0.01.

3.2.2 Diets and feeding trials

In feeding trial 1, control and CTα^{IKO} mice were randomly assigned to a 40 % fat/calorie high-fat diet (HFD), or a compositionally matched 4 % fat/calorie low-fat diet (LFD) for 4 days (8-9 mice/group) (Table 3.1). In feeding trial 2, control and CTα^{IKO} mice were randomly assigned to a 40 % fat/calorie diet with 0.4 % choline wt/wt (choline supplemented diet [CSD]) or a calorie-matched 40 % fat/calorie diet with 0.1 % choline and 0.3 % choline from PC wt/wt (PC supplemented diet [PCSD]) diet for 4 days (6-7 mice/group) (Table 3.1). In feeding trial 3, control and CTα^{IKO} mice were randomly assigned to an antibiotic treatment group or a control group (4-5 mice/group). On the first day of tamoxifen injections, half of the control and CTα^{IKO} mice were given free access to water containing an antibiotic cocktail (bacitracin: 500 mg/L, neomycin: 1g/L and vancomycin: 500 mg/L), while the other half were given free access to antibiotic-free water. On day 6 of feeding trial 3, all experimental mice were switched to HFD for 4 days. Body weights from feeding trials 2 and 3 have been previously reported (Kennelly et al., 2021).

Table 3.1: Composition of experimental diets.

Ingredients	LFD (g)	HFD (g)	CSD (g)	PCSD (g)
Casein	270.0	270.0	270	270
Corn Starch	256.6	170.7	170.65	170.65
Sucrose	293.4	195.4	195.35	195.35
Cellulose	80.0	80.0	80	80
Vitamin Mix	19.0	19.0	19.0	19.0
Mineral Mix	50.0	50.0	50.0	50.0
Calcium Phosphate Dibasic	3.4	3.4	3.4	3.4
Inositol	6.3	6.3	6.3	6.3
L-cysteine	1.8	1.8	1.8	1.8
Choline Bitartrate	4.2	4.2	10	2.5
PC (soy lecithin)	0	0	0	90
Crisco Vegetal Oil	2.4	32.0	32.0	23.0
Mazola Corn Oil	0.8	10.0	10.0	10.0
Lard	11.8	155.0	155.0	127.0
DHAsco	0.1	1.5	1.5	1.5
ARAsco	0.1	1.5	1.5	1.5

3.2.3 Microscopy

Formalin-fixed jejunum samples were paraffin-embedded and sliced (5um thick). The slides were then stained with hematoxylin and eosin (H&E) or Alcian blue/Period acid-Schiff (AB/PAS). Slides were imaged using light-microscopy. AB/PAS slides were scored for goblet cell depletion by an independent pathologist blinded to the experiment. Goblet cell depletion was scored on scale of 0-2 where 0 represented no goblet cell depletion, 1 represented moderate goblet cell depletion, and 2 represented substantial goblet cell depletion.

3.2.4 Gastric emptying

Solid phase gastric emptying was measured in control and CTα^{IKO} mice using a previously reported protocol (Barrachina, Martínez, Wang, Wei, & Taché, 1997; Maida, Lovshin, Baggio, & Drucker, 2008). Briefly, individually housed mice (n=4-5/group) were fasted overnight before being fed a pre-weighed pellet for 2 hr. The stomach was then excised, and the gastric contents were weighed. The rate of gastric emptying was calculated using the following formula: [1-(stomach content wet weight/food intake)] × 100.

3.2.5 Lipid analysis

Plasma triacylglycerol (TG) was measured using a commercially available kit (Sekisui Diagnostics) and active plasma glucagon-like peptide-1 (GLP-1) using an enzyme-linked immunosorbent assay (EMD Millipore). Jejunum IECs were homogenized, and protein levels of homogenate were analyzed using a bicinchoninic acid assay. Lipid extractions were performed on jejunum IEC homogenate (1 mg protein) using the Folch method (Folch, Lees, & Sloane Stanley, 1957). PC and PE (phosphatidylethanolamine) levels were isolated using thin layer chromatography in solvent system containing chloroform, methanol, acetic acid, formic acid, and water (140:60:24:8:2 mL) and visualized with iodine. Total IEC PC and PE levels were determined by a phosphorus assay as described previously (Rouser, Siakotos, & Fleischer, 1966).

3.2.6 Western blots

Jejunum IEC homogenates (50 µg protein) were run on 8.5 % sodium dodecyl sulfate polyacrylamide gel and transferred to 0.45 µm PVDF membrane. Membranes were probed with CTα antibody (diluted 1:2000, gift from Dr R. K. Mallampalli) and α-tubulin antibody (diluted

1:5000, T6199; Sigma-Aldrich) as a loading control. Membranes were detected using ECL (WBLUF0500, Millipore) and imaged on Chemi-Doc MP imager (Bio-Rad Laboratories, CA, USA). Quantification of protein levels was analyzed using Image Lab software from Bio-Rad.

3.2.7 Microarray data acquisition and analysis

Small intestines (n=5 per group) were collected, flushed with ice-cold phosphate-buffered saline containing protease inhibitor cocktail (Sigma, MO, USA), and kept on ice. The small intestine was opened longitudinally and 3 cm of scrapings from the geometric middle of the intestine (jejunum) were collected and immediately frozen in liquid nitrogen. Intestinal epithelial cells were disrupted in Trizol (Invitrogen, CA, USA), and RNA was isolated and purified using RNeasy Mini Kit (Qiagen, UK), according to manufacturer's instructions. RNA concentration and purity were assessed on a NanoDrop 2000 (Thermo Fisher Scientific, MA, USA), and RNA integrity was confirmed using the Agilent 2100 Bioanalyzer system (Agilent Technologies, CA, USA). Microarray analysis was performed by The Centre for Applied Genomics, The Hospital for Sick Children, Toronto, Canada. Whole genome expression profiles were obtained using the Affymetrix mouse Gene 2.0 ST chips (Affymetix, Thermo Fischer Scientific, CA, USA). After confirmation of RNA quality by Agilent Bioanalyzer analysis, 400 ng total RNA was used to produce biotinylated cDNA by the Affymetrix WT Plus kit. cDNA (5.5 μg) was hybridized to the microarray chips. Chips were washed, stained, and scanned on an Affymetrix GeneChip Scanner 3000. Run files were opened and RMA normalized in Expression Console software. Differentially expressed genes were identified with Affymetix Transcriptome Analysis Console (TAC 4.0.1) software using an ANOVA p- value cut-off of 0.05 and fold-change of 1.5. Pathways analysis was conducted with Ingenuity Pathways Analysis (IPA) software (Qiagen Bioinformatics). Gene

Ontology enrichment analysis (Biological process) was performed using DAVID version 6.8 (Huang, Sherman, & Lempicki, 2009). Raw data is available through National Centre for Biotechnology Information with accession number GSE110474.

3.2.8 Real-time quantitative PCR analysis

Jejunum IECs were homogenized in TRIzol (15596018; Invitrogen) and total RNA was isolated using RNEasy Mini (74104; Qiagen) kits. RNA samples were treated with DNase 1 (18068-015; Invitrogen). RNA was then reverse transcribed to cDNA using oligo(dT)12–18 primers (18418-012; Invitrogen), random primers (48190011; Thermo Fisher Scientific), and Superscript II (18064-173 014; Invitrogen). Quantitative PCR was run on StepOne Plus system (Applied Biosystems, MA, USA) for 40 cycles using Power SYBR Green PCR Master Mix (4367659; Thermo Fisher Scientific). Data was normalized to mRNA expression of *Rplp0*. Primer sequences used to determine mRNA levels are in Table 3.2.

Table 3.2: Primers for quantitative PCR.

Gene	Gene Name	Forward Primer	Reverse Primer
Rplp0	Ribosomal protein lateral stalk subunit P0	ACT GGT CTA GGA CCC GAG AAG	CTC CCA CCT TGT CTC CAG TC
Pcyt1a	Phosphate cytidylyltransferase 1, choline, α isoform	GCT AAA GTC AAT TCG AGG AA	CAT AGG GCT TAC TAA AGT CAA CT
Cd36	Cluster-determinant 36	TGG CTA AAT GAG ACT GGG ACC	ACA TCA CCA CTC CAA TCC CAA G
Dgat2	Diacylglycerol O-Acyltransferase 2	GGC TAC GTT GGC TGG TAA CTT	TTC AGG GTG ACT GCG TTC TT
Mogat2	Monoacylglycerol O-Acyltransferase 2	TAC AGC TTT GGC CTC ATG C	AGG GCT GTG GTG TCA TCT G
Cidec	Cell Death Inducing DFFA Like Effector C	CAC TGC TAC AAG GCC AAG C	GGT GGC ATC CAG GAA CTG
HspA5	Heat shock protein family A (Hsp70) member 5	GAG GAT GTG GGC ACG GTG GT	CCC TGA TCG TTG GCT ATG AT
Atf6	Activating transcription factor 6	GGA CGA GGT GGT GTC AGA G	GAC AGC TCT TCG CTT TGG AC
Atf5	Activating transcription factor 5	GCA GCA CCT AGG GTA CAG GT	CGC TGG AGA CAG ACG TAC AC
Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	GAT GAG CCT CCC ATG CAA	AAT GTC CAT CTC AAA TTG TGA CTC
Zbp1	Z-DNA binding protein 1	CAG GAA GGC CAA GAC ATA GC	GAC AAA TAA TCG CAG GGG ACT
Ripk3	Receptor-interacting serine/threonine-protein kinase	GAA CCA TGA TGT AGC AGT CAA GA	CGA AGT CCC ACT GGA GGT C
Birc5	Baculoviral IAP repeat-containing 5	TGA TTT GGC CCA GTG TTT TTT	CAG GGG AGT GCT TTC TAT GC
Muc2	Mucin 2	CCA TTG AGT TTG GGA ACA TGC	TTC GGC TCG GTG TTC AGA G
Tff3	Trefoil factor 3	CTG GGA TAG CTG CAG ATT ACG	CAT TTG CCG GCA CCA TAC
Gfi1	Growth factor independent 1 transcriptional repressor	ATG TGC GGC AAG ACC TTC	ACA GTC AAA GCT GCG TTC CT
Spdef	SAM pointed domain containing ETS transcription factor	GAT GTA CTG CAT GCC CAC CT	GGA GGC GCA GTA GTG AAG G
Klf4	Kruppel-like factor 4	CCG TCC TTC TCC ACG TTC	GAG TTC CTC ACG CCA ACG
Reg3b	Regenerating islet-derived protein 3 beta	GAC AAG ATG CTG CCT CCA A	CGT GCG GAG GGT ATA TTC TT
Reg3g	regenerating islet-derived protein 3 gamma	GCT TCC CCG TAT AAC CAT CA	GCA TCT TTC TTG GCA ACT TCA
Duoxa2	Dual Oxidase Maturation Factor 2	CCT GTT CAT CTT GCC TGG A	CAC GAA CCA GTC TCC ACT GA
Oas1g	2'-5'-Oligoadenylate Synthetase 1G	ATC CGC CTG GTC AAA CAC T	TAC ATC CAT TCC CCT GTT CC

3.2.9 Everted intestinal sac

Everted intestinal sac experiments were performed using a modified version of fat absorption by Strauss (Figure 3.2) (E. W. Strauss, 1966). First, a 10 X stock micelle buffer (1.4 mM sodium cholate, 1.5 mM sodium deoxycholate, 1.7 mM 1,2-dioleoyl-sn-glycero-3-phosphocholine, 2.2 mM oleic acid, 1.9 mM palmitoylglycerol and 0.5 mM choline chloride) was dissolved in chloroform:methanol (1:1). 50 µCi ^{3}H-Oleic Acid and 50 µCi ^{14}C-Choline Chloride were added to the buffer and dried down under nitrogen. 10 X stock micelle buffer was then dissolved in 5 mL of everted intestinal sac buffer (4.8 mM KCl, 118 mM NaCl, 1 mM sodium phosphate monobasic, 24 mM sodium bicarbonate, 1.2 mM magnesium sulfate – anhydrous, 1.6 mM magnesium chloride

hexahydrate and 40 mM glucose – bubbled for 5 min in 95 % oxygen/ 5 % carbon dioxide and adjusted to pH 7.4). 10 X stock micelle buffer was sonicated at medium power in 1 min intervals until solution was clear. Intestines from mice were then excised and flushed with warmed (37 ºC) saline solution. The ileal end of intestine was tied with surgical thread and the entire intestine was everted using a gel loading pipette tip. The serous compartment was filled with warmed (37 ºC) everted intestinal sac buffer and the intestine was tied into individual sacs of 3 cm in length, ensuring sacs representing the jejunum and ileum were used. Links between sacs were cut and each sac was incubated in 5 mL of 1 X micelle buffer (diluted with everted intestinal sac buffer) in shaking water bath at 37 ºC for 2 h. The serous fluid was drained and collected, then intestinal sacs and serous fluid were frozen in liquid nitrogen. Intestinal sacs were homogenized, and protein levels of homogenate were analyzed using a bicinchoninic acid assay. Lipid extractions were performed on tissue (1 mg protein) using the Folch method (Folch et al., 1957). PC, PE, and TG levels were isolated using thin layer chromatography. PC and PE were isolated using solvent system described above and TG was isolated in solvent system containing hexane, di-isopropyl ether, and acetic acid (130:70:4 mL). PC, PE, and TG were visualized using iodine and radioactive levels were quantified with scintillation counting using CytoScint (01882453-CF, MP Biomedical).

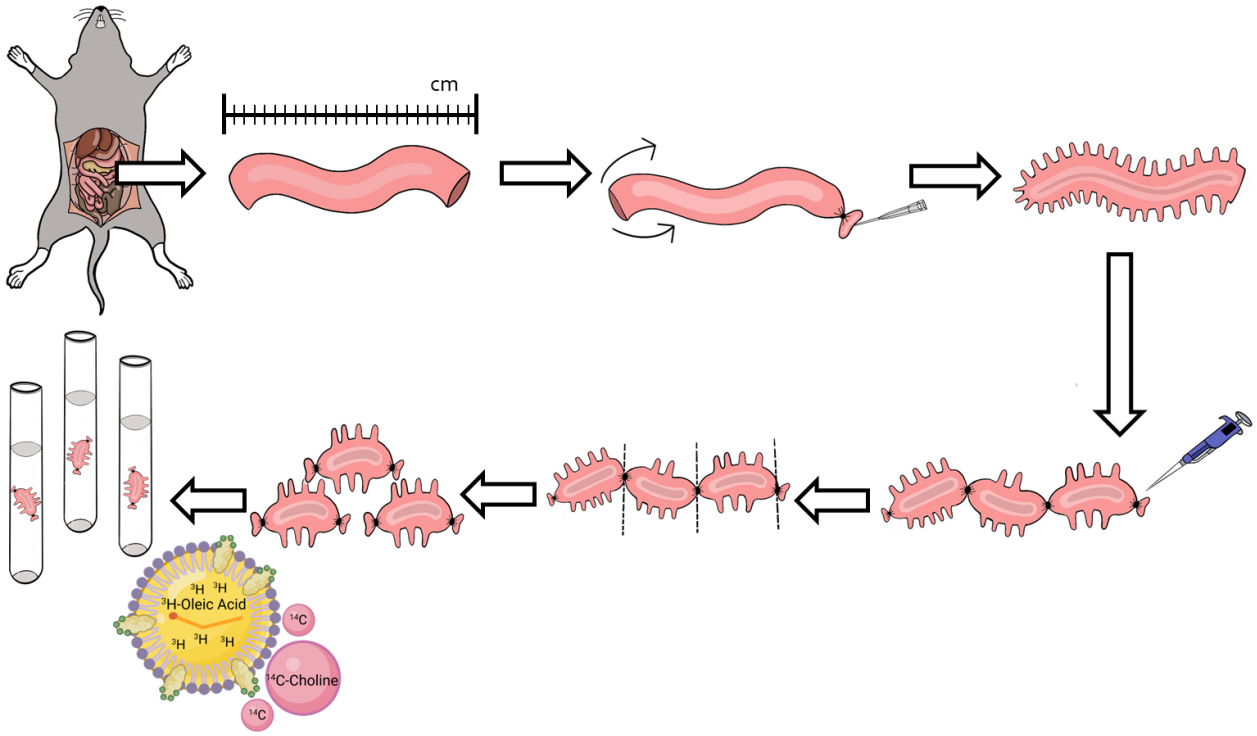

Figure 3.2: Everted intestinal sac methods. The small intestines were collected from 5-day LFD- and HFD-fed CTα^{IKO} mice and tied closed at the ileal end using surgical thread. Using a gel loading pipette tip, the intestines were everted, so the intestinal epithelial cells were on the outside and the intestines were then filled with an oxygenated buffer. The intestine was then tied into individual sacs and then the links between the sacs were cut. The individual sacs were incubated in a buffer containing ^{3}H-oleic acid filled micelles and ^{14}C-choline. The intestinal sacs were then collected for analysis.

3.2.10 Statistical analysis

Statistical analysis of data was performed using Graph Pad Prism 8 and is expressed at mean ± SEM. Data that was not normally distributed were log-transformed. Statistical significance (p<0.05) was determined by two-way ANOVA with uncorrected Fisher's Least Significant Difference test.

3.3 Results

3.3.1 Lower abundance of transcripts related to lipid metabolism and higher abundance of transcripts related to inflammation in the intestines of CTα^{IKO} mice

To gain insight into the mechanisms controlling lipid malabsorption in CTα^{IKO} mice, we conducted transcriptomic analysis of jejunums from HFD-fed control and CTα^{IKO} mice. Pathway analysis showed that ~20% of all down-regulated genes in CTα^{IKO} mice intestines were involved in lipid metabolism. Among the most significantly down-regulated transcripts (Figure 3.3 A) were those encoding proteins involved in fatty acid uptake (*Cd36*, *Slc27a2*), lipid droplet formation (*Plin2*, *Dgat2*, *Cidec*), lipid hydrolysis and oxidation (*Ces1d*, *Lipa*, *Abhd5*, *Acot4*, *Acox2*, *Bco1*), and chylomicron secretion (*Apoc3*, *Sec16b*). Gene Ontology enrichment analysis confirmed that several metabolic processes were repressed in the intestines of CTα^{IKO} mice (Figure 3.3 B). Ingenuity Pathways Analysis (IPA) software indicated that many of the down-regulated genes were controlled by peroxisome proliferator-activated receptor alpha-α (PPARα) (Figure 3.3 D). In contrast to the repression of genes linked to intestinal fatty acid metabolism, the abundance of mRNAs linked to cholesterol biosynthesis was significantly higher in CTα^{IKO} mice (Figure 3.3 C).

Pathway analysis showed that ~70% of up-regulated genes were linked to organismal injury and abnormality. Gene Ontology enrichment analysis further confirmed that immune-

related processes were activated by loss of CTα in the jejunum (Figure 3.3 C). For example, the antibacterial lectins *Reg3b* and *Reg3g*, as well as the hydrogen peroxide generators *Noxo1*, *Duox2* and *Duoxa2*, and many interferon-inducible transcripts were robustly higher in the intestines of CTα^{IKO} mice (Figure 3.3 A). Upstream analysis with IPA suggested that *Irf3* and *Irf7* are involved in the transcriptional control of many of the up-regulated genes in CTα^{IKO} intestines (Figure 3.3 D). This pattern of gene expression is consistent with increased microbial interaction with the small intestinal epithelium due to loss of intestinal CTα (Atarashi et al., 2015).

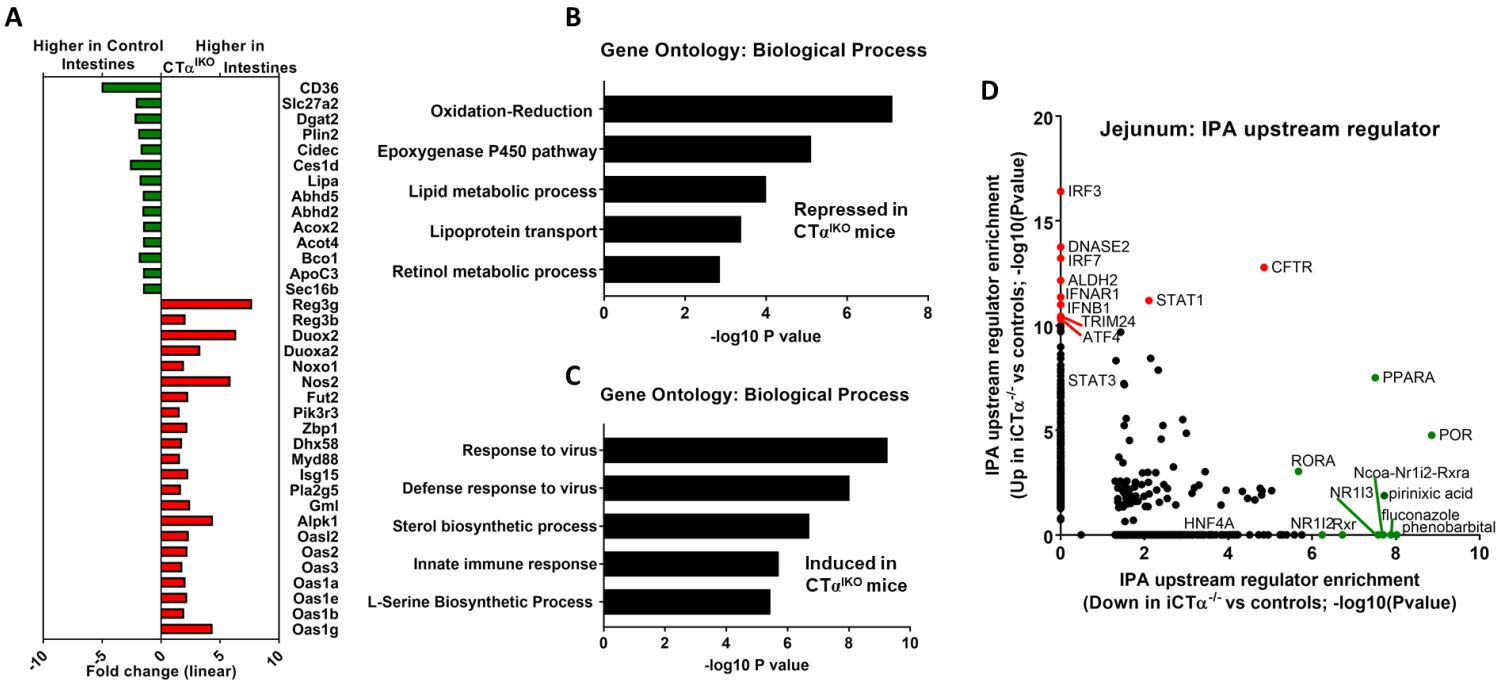

Figure 3.3: Microarray analysis of transcript changes in HFD-fed control and CTα^{IKO} mice. (A) Most significantly up- and down-regulated transcripts in CTα^{IKO} mice. Gene Ontology enrichment analysis showing (B) repressed and (C) induced metabolic processes in CTα^{IKO} mice. (D) Ingenuity pathway analysis showing upstream regulators of up- and down-regulated transcripts in CTα^{IKO} mice.

3.3.2 Factors other than malabsorption of dietary fat contribute to acute weight loss in CTα^{IKO} mice

We previously showed that HFD-fed CTα^{IKO} mice had intestinal lipid malabsorption and higher secretion of the fat-induced satiety hormone GLP-1 compared to control mice (Kennelly et al., 2018). Furthermore, our microarray data show an inverse relationship between transcripts related to lipid metabolism and inflammation in the intestines of CTα^{IKO} mice. Therefore, to determine whether dietary fat is specifically driving acute weight loss in CTα^{IKO} mice, we fed mice either a HFD or a calorically matched LFD. Both LFD- and HFD-fed CTα^{IKO} mice had lower jejunal PC levels compared to control mice (Figure 3.4 A). Interestingly, LFD-fed CTα^{IKO} mice also had lower jejunal PE levels compared to LFD-fed control mice, leading to no change in the jejunal PC:PE ratio between groups (Figure 3.4 B and C). HFD-fed CTα^{IKO} mice had lower jejunal PC, no change in jejunal PE and lower jejunal PC:PE ratio compared to HFD-fed control mice (Figure 3.4 A-C). LFD-fed CTα^{IKO} mice had comparable levels of lipid droplets in H&E stained jejunal segments (Figure 3.4 D) and comparable jejunal TG levels (Figure 3.4 F) compared to LFD-fed control mice. HFD-fed CTα^{IKO} mice had fewer lipid droplets (Figure 3.4 E) and lower jejunal TG (Figure 3.4 F) compared to HFD-fed control mice. The mRNA levels of *Cd36*, an intestinal fatty acid transporter, was lower in both LFD- and HFD-fed CTα^{IKO} mice compared to their respective control groups (Figure 3.4 G). The mRNA levels of other genes associated with lipid metabolism (*Dgat2* and *Cidec*) were only lower in HFD-fed CTα^{IKO} mice, consistent with the impaired movement of fatty acids from the intestinal lumen into enterocytes of HFD-fed CTα^{IKO} mice (Figure 3.4 G). Both LFD- and HFD-fed CTα^{IKO} mice acutely lost body weight (Figure 3.4 H). Additionally, 2 hours after refeeding, CTα^{IKO} mice had lower plasma TG independent of dietary fat intake (Figure 3.4 I). Surprisingly, plasma active GLP-1 levels were ~20-fold higher in

both LFD- and HFD-fed CTα^{IKO} mice compared to control (Figure 3.4 J). Together these data suggest that dietary fat content alone is not driving body weight loss and postprandial GLP-1 secretion after induction of loss of intestinal CTα.

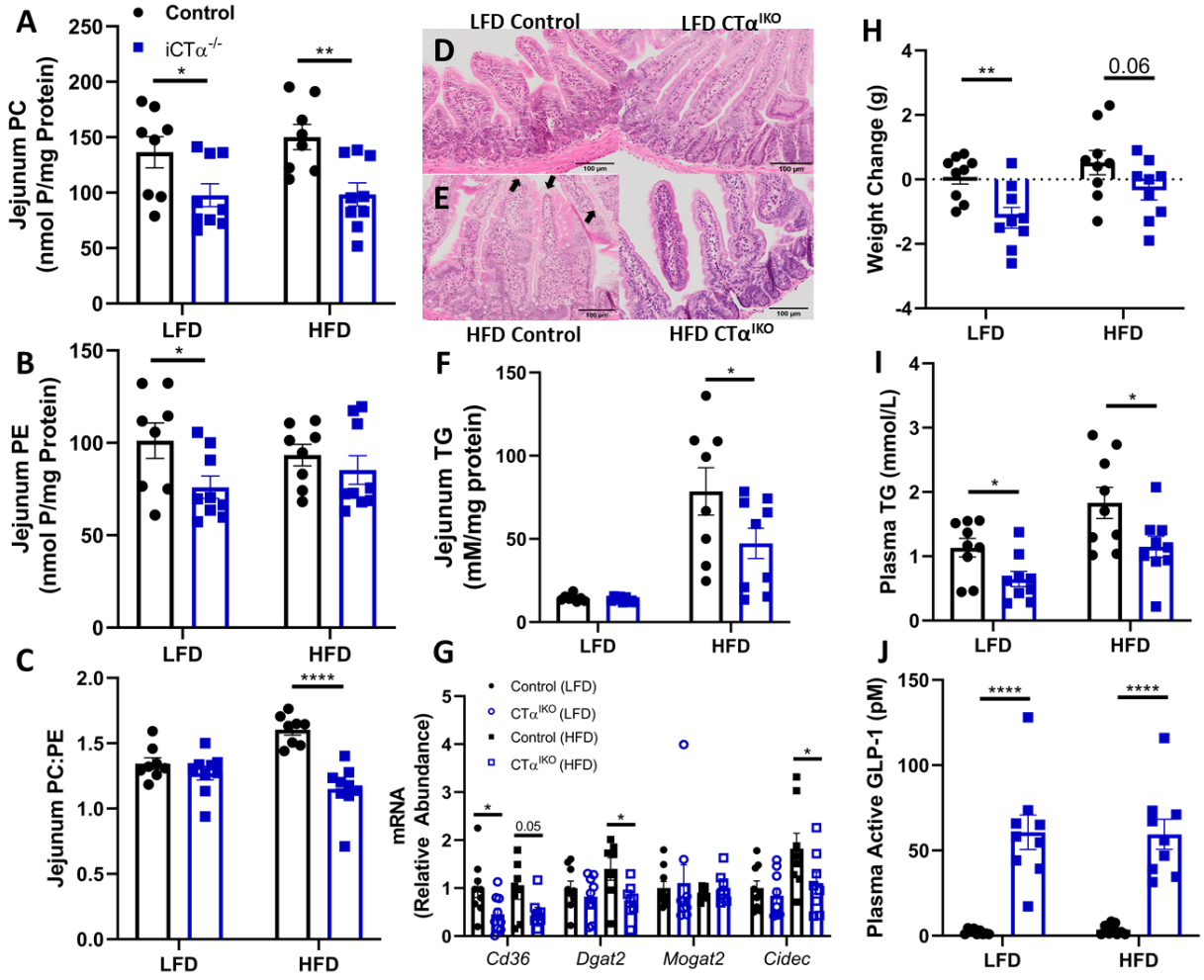

Figure 3.4: LFD- and HFD-fed control and CTα^{IKO} mice. Jejunum (A) PC, (B) PE, and (C) PC:PE ratio in control and CTα^{IKO} mice. Representative jejunum H&E staining in (D) LFD- and (E) HFD-fed control and CTα^{IKO} mice (arrows indicate lipid droplets). (F) Jejunum TG in control and CTα^{IKO} mice. (G) Jejunum *Cd36*, *Dgat2*, *Mogat2*, and *Cidec* mRNA levels in control and CTα^{IKO} mice. (H) Weight of control and CTα^{IKO} mice after 4 days of LFD or HFD. 2 h postprandial (I) plasma TG and (J) plasma active GLP-1 in control and CTα^{IKO} mice. Mice were 18-22 weeks fed LFD or HFD for 5 days. Values are reported as ±SEM, n=8-9/group. *P<0.05, **P<0.01, ****P<0.0001.

3.3.3 Lipid malabsorption occurs in isolated intestinal sacs of CTα^{IKO} mice

Changes in gastric emptying can influence the rate at which lipids reach enterocytes before uptake into circulation. Gastric emptying was measured in HFD-fed control and CTα^{IKO} mice to determine whether delayed gastric emptying contributes to lower jejunum and plasma lipid accumulation after a 2 h refeed in CTα^{IKO} mice. Surprisingly, the rate of gastric emptying was higher in CTα^{IKO} mice compared to control mice (Figure 3.5 A) while food intake was similar between groups (Figure 3.5 B). Therefore, lower postprandial jejunum and plasma TG concentrations in CTα^{IKO} mice is not due to delayed gastric emptying.

To determine whether other systemic factors, including neuronal, hormonal, or changes to bile acid homeostasis, influence lipid absorption in CTα^{IKO} mice, an *ex vivo* everted intestinal sac model was used to measure fatty acid uptake. Intestines isolated from either LFD- (Figure 3.5 C-E) or HFD- (Figure 3.5 F-H) fed CTα^{IKO} mice had lower incorporation of radiolabelled oleic acid into PC, PE and TG, and lower radiolabelled choline incorporation into PC compared to control mice. Similar results were also shown in ileal segments of intestines isolated from LFD- and HFD-fed CTα^{IKO} mice (Figure 3.6). These results indicate that fatty acid malabsorption in CTα^{IKO} mice is occurring at the cellular level, independent of systemic factors.

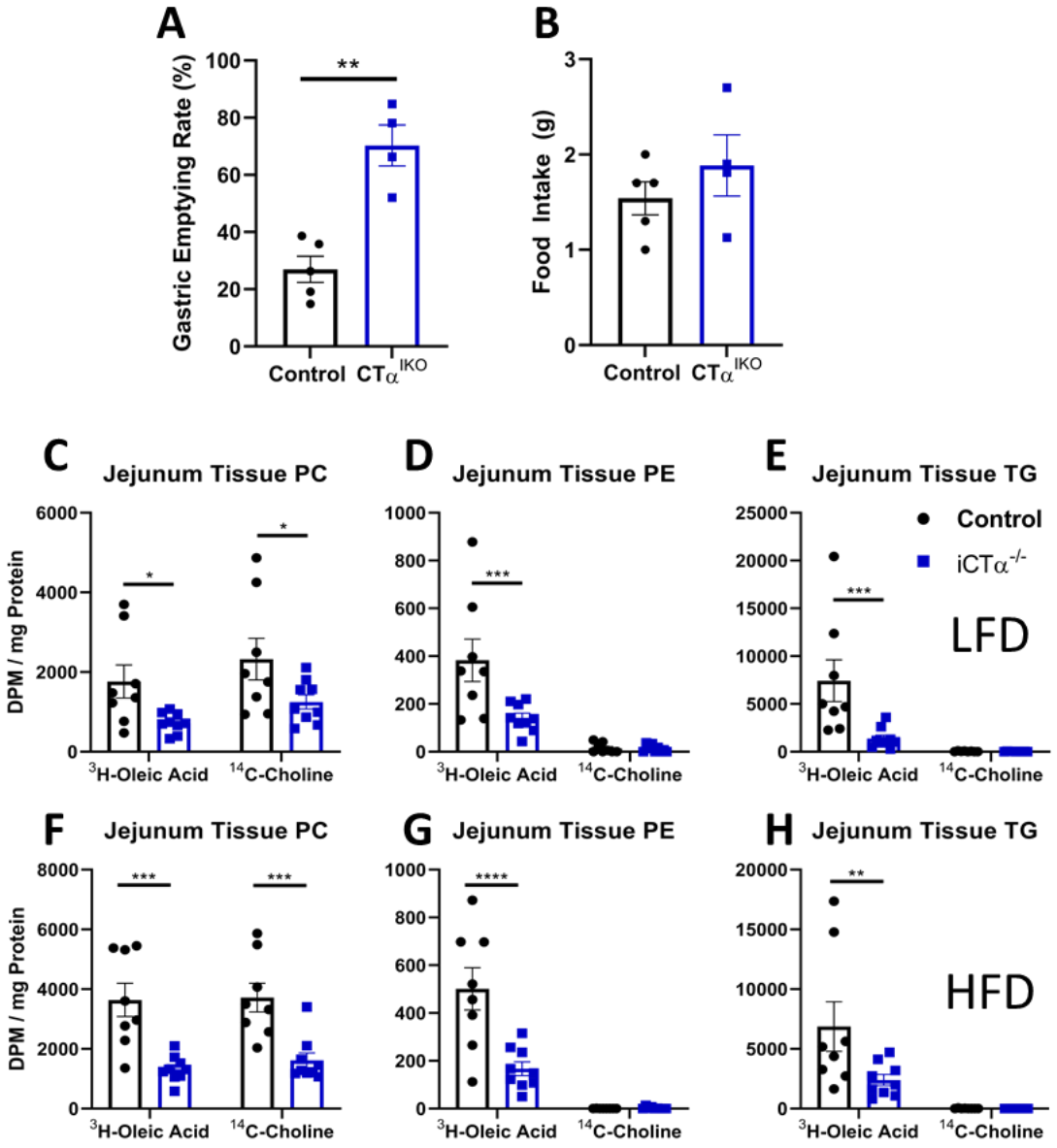

Figure 3.5: Lipid metabolism *ex vivo* in LFD- and HFD-fed control and CTα^{IKO} mice. (A) Gastric emptying rate and (B) food intake in control and CTα^{IKO} mice after overnight fast and 2 h feeding (n=4-5/group). Radiolabelled incorporation of ^{3}H-oleic acid and ^{14}C-choline into (C) PC, (D) PE, and (E) TG of intestinal sacs corresponding to the jejunum isolated from LFD-fed control and CTα^{IKO} mice. Radiolabelled incorporation of ^{3}H-oleic acid and ^{14}C-choline into (F) PC, (G) PE, and (H) TG of intestinal sacs corresponding to the jejunum isolated from HFD-fed control and CTα^{IKO} mice. Mice were 18-22 weeks fed LFD or HFD for 5 days. Values are reported as ±SEM, n=8-9/group unless otherwise stated. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

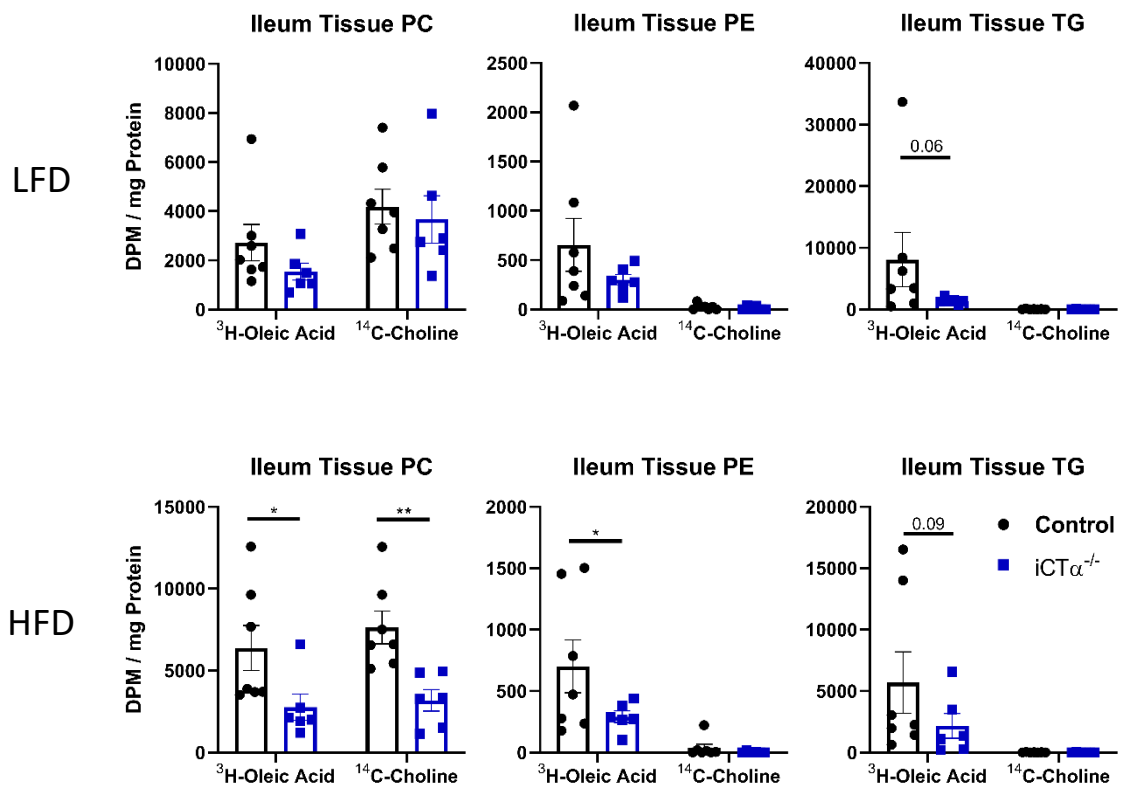

Figure 3.6: Everted intestinal sac *ex vivo* model of ileum lipid absorption. Radiolabelled incorporation of ^{3}H-oleic acid and ^{14}C-choline into (A) PC, (B) PE, and (C) TG of intestinal sacs corresponding to the ileum isolated from LFD-fed control and CTα^{IKO} mice. Radiolabelled incorporation of ^{3}H-oleic acid and ^{14}C-choline into (D) PC, (E) PE, and (F) TG of intestinal sacs corresponding to the ileum isolated from HFD-fed control and CTα^{IKO} mice. Values are reported as ±SEM, n=6-7/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3.3.4 Factors other than dietary fat content contribute to inflammation in CTα^{IKO} mice

To determine whether high dietary fat content drives inflammatory gene expression in the intestines of CTα^{IKO} mice, we compared the transcript abundance of genes related to ER stress and barrier function in LFD- and HFD-fed mice. The mRNA levels of *Atf5* and *Eif4ebp1*, genes associated with ER stress, were higher in the jejunums of CTα^{IKO} mice compared to control mice independent of dietary fat content (Figure 3.7 A). LFD-fed CTα^{IKO} mice had lower mRNA levels of *HspA5,* which is linked to activation of the unfolded protein response, compared to LFD-fed control mice (Figure 3.7 A). Furthermore, higher mRNA levels of *Reg3b*, *Reg3g*, *Duoxa2*, and *Oas1g,* genes induced by microbial invasion of the intestinal epithelium, were observed in CTα^{IKO} mice compared to control mice independent of dietary fat content (Figure 3.7 A). The goblet cell markers *Muc2*, *Tff3*, and *Spdef* were lower in both LFD- and HFD-fed CTα^{IKO} mice compared to control mice (Figure 3.7 B). Accordingly, AB/PAS stained jejunal segments of CTα^{IKO} mice showed fewer goblet cells compared to control mice (Figure 3.7 C and D), which, when quantified, showed higher goblet cell depletion after loss of intestinal CTα (Figure 3.7 E). Furthermore, the mRNA levels of *Zbp1*, which is linked to microbial sensing, and *Ripk3* and *Birc5,* which are linked to cell death, were higher in CTα^{IKO} mice (Figure 3.7 B) and could account for the loss of goblet cells. Therefore, impaired PC synthesis in CTα^{IKO} mice leads to ER stress, cell death, and microbial invasion of the epithelium independent of dietary fat content.

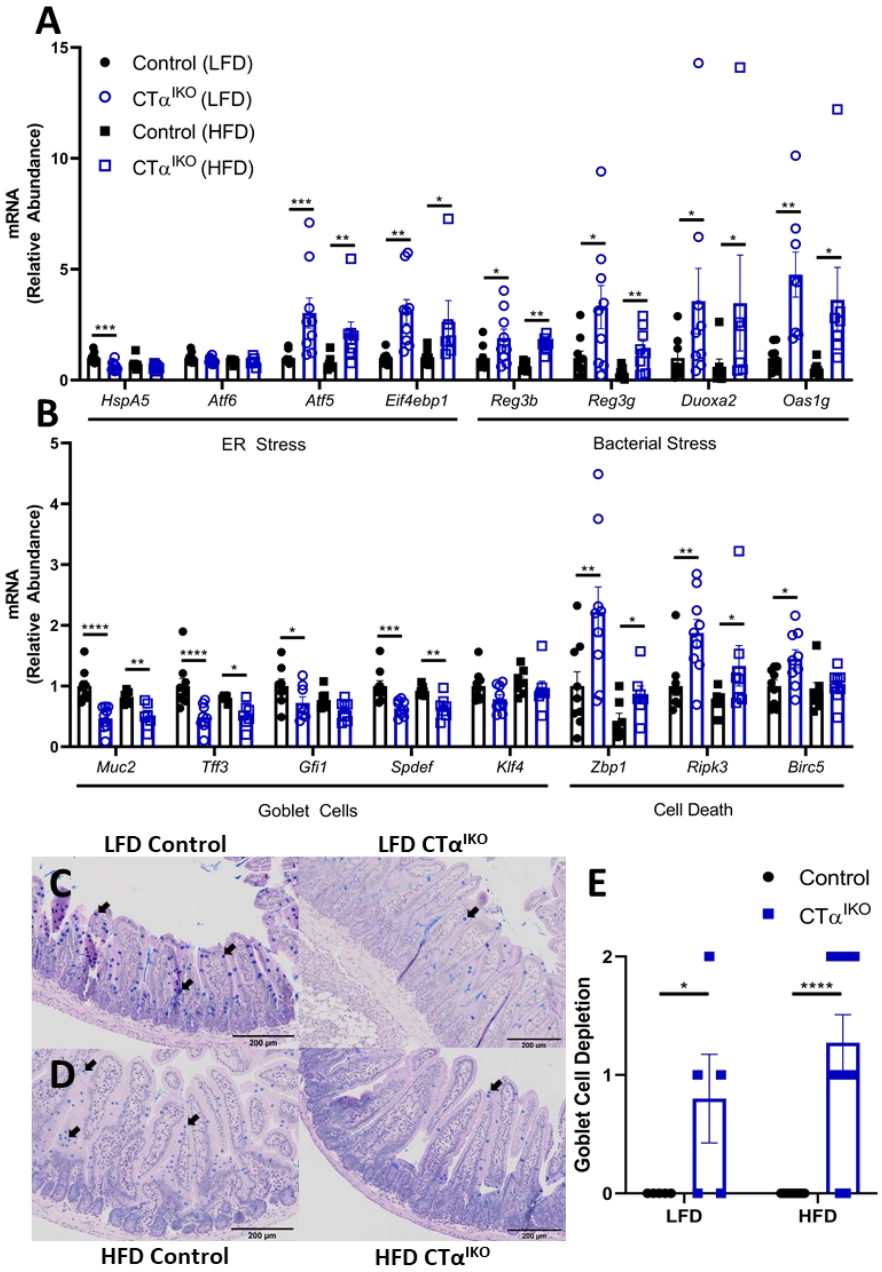

Figure 3.7: Increased cellular stress and cell death in LFD- and HFD-fed control and CTα^{IKO} mice. (A) Jejunum ER stress (*HspA5*, *Atf6*, *Atf5,* and *Eif4ebp1*) and bacterial stress (*Reg3b*, *Reg3g*, *Duoxa2*, and *Oas1g*) mRNA levels in control and CTα^{IKO} mice. (B) Jejunum goblet cell (*Muc2*, *Tff3*, *Gfi1*, *Spdef*, and *Klf4*) and cell death (*Zbp1*, *Ripk3*, and *Birc5*) mRNA levels in control and CTα^{IKO} mice. Representative jejunum AB/PAS stained slides for (C) LFD- and (D) HFD-fed control and CTα^{IKO} mice (arrows indicate goblet cells). (E) Jejunum goblet cell depletion in control and CTα^{IKO} mice (n=5-11/group). Mice were 18-22 weeks fed LFD or HFD for 5 days. Values are reported as ±SEM, n=8-9/group unless otherwise stated. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3.3.5 Providing CTα^{IKO} mice with antibiotics partially improved metabolic function but did not improve cell death

To determine whether impaired host defence against bacteria contributes to lipid malabsorption or acute body weight loss in CTα^{IKO} mice, HFD-fed CTα^{IKO} and control mice were treated with or without broad-spectrum antibiotics, lowering bacterial load in the intestine (Kennelly et al., 2021). While untreated CTα^{IKO} mice lost weight compared to untreated controls as expected, antibiotic treated CTα^{IKO} mice did not lose weight compared to antibiotic treated control mice (Figure 3.8 A). These data suggest that lowering the gut bacterial load prevents acute body weight loss in CTα^{IKO} mice. Antibiotic treated CTα^{IKO} mice had lower jejunal PC levels compared to control mice, while untreated CTα^{IKO} mice had similar jejunum PC levels compared to untreated controls (Figure 3.8 B). Both treated and untreated CTα^{IKO} mice had higher jejunal PE levels and a lower jejunal PC:PE ratio compared to their respective controls (Figure 3.8 C and D). CTα^{IKO} mice had lower plasma TG levels after refeeding both with and without antibiotic treatment (Figure 3.8 E). In contrast, while untreated CTα^{IKO} mice had lower jejunum TG concentrations compared to untreated controls, antibiotic treated CTα^{IKO} mice had comparable jejunum TG concentrations after refeeding to antibiotic treated controls (Figure 3.8 F), suggesting that only chylomicron output capacity remained impaired in antibiotic treated CTα^{IKO} mice. Treated and untreated CTα^{IKO} mice had fewer lipid droplets in jejunal H&E stained slides (Figure 3.8 G and H). Consistent with their comparable jejunal TG levels, antibiotic treated CTα^{IKO} mice had similar mRNA levels of *Dgat2* and *Mogat2* compared to antibiotic treated controls, while these transcripts remained lower in the intestines of untreated CTα^{IKO} mice compared to untreated controls (Figure 3.8 I). Jejunal mRNA levels of *Cidec* remained lowered in treated and untreated CTα^{IKO} mice. Antibiotic treated and untreated CTα^{IKO} mice had higher circulating plasma active

GLP-1 compared to control mice (Figure 3.8 J). Together these results suggests that antibiotic treatment partially restores metabolic function in the intestines of CTα^{IKO} mice, as indicated by normalized body weight and improved intestinal TG after refeeding.

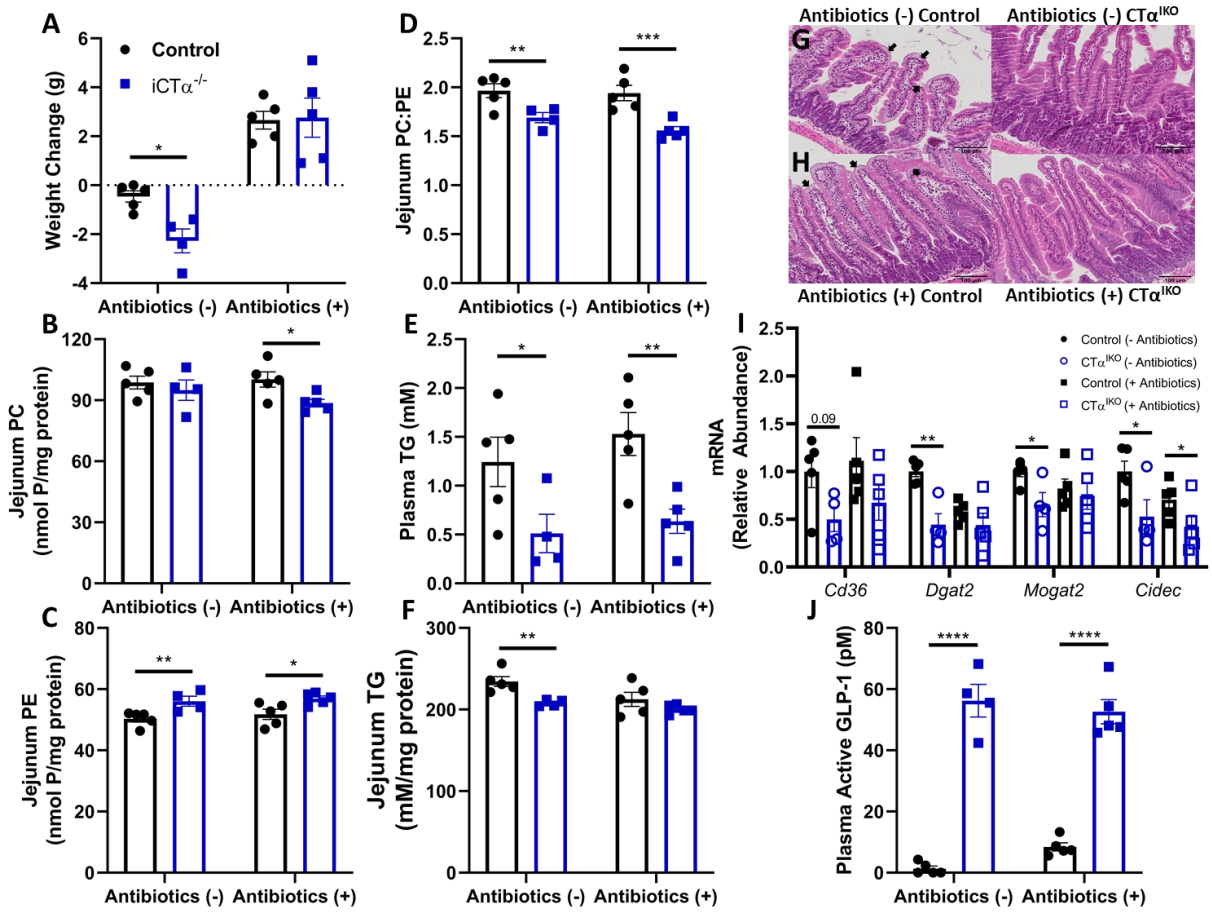

Figure 3.8: Lipid metabolism in control and CTα^{IKO} with or without antibiotic treatment. (A) Weight of control and CTα^{IKO} mice after 4 days of HFD. Jejunum (B) PC, (C) PE, and (D) PC:PE ratio in control and CTα^{IKO} mice. (E) Plasma and (F) jejunum TG in control and CTα^{IKO} mice. Representative jejunum H&E staining in control and CTα^{IKO} mice (G) (-) antibiotics and (H) (+) antibiotics (arrows indicate lipid droplets). (I) Jejunum *Cd36*, *Dgat2*, *Mogat2*, and *Cidec* mRNA levels in control and CTα^{IKO} mice. (J) Plasma active GLP-1 in control and CTα^{IKO} mice. Mice 8-12 with or without antibiotics were fed HFD for 5 days. Values are reported as ±SEM, n=4-5/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

We next determined whether antibiotic treatment would improve ER stress and loss of goblet cells in CTα^{IKO} mice. Untreated CTα^{IKO} mice had 6- and 15-fold higher transcript abundance of *Reg3b* and *Reg3g* respectively, compared to untreated control mice (Figure 3.9 A). Interestingly, antibiotic treatment lowered the abundance of *Reg3b* and *Reg3g* in the jejunum of both control and CTα^{IKO} mice compared to untreated mice, although *Reg3b* and *Reg3g* remained significantly higher in antibiotic treated CTα^{IKO} mice compared to antibiotic treated controls (Figure 3.9 A). As in our initial feeding trial (Figure 3.7), mRNA levels of genes associated with ER stress (*Eif4ebp1*) and cell death (*Ripk3* and *Birc5)* were elevated, while mRNA levels of genes associated with goblet cells (*Muc2*, *Tff3*, *Gfi1* and *Spdef*) were reduced in the intestines of both treated and untreated CTα^{IKO} mice (Figure 3.9 A and B). Accordingly, both antibiotic treated and untreated CTα^{IKO} mice had fewer goblet cells compared to control mice (Figure 3.9 C-D), quantified as increased goblet cell depletion (Figure 3.9 E). Therefore, while antibiotics reduced bacterial stress, they did not improve ER stress, necroptosis, or goblet cell depletion.

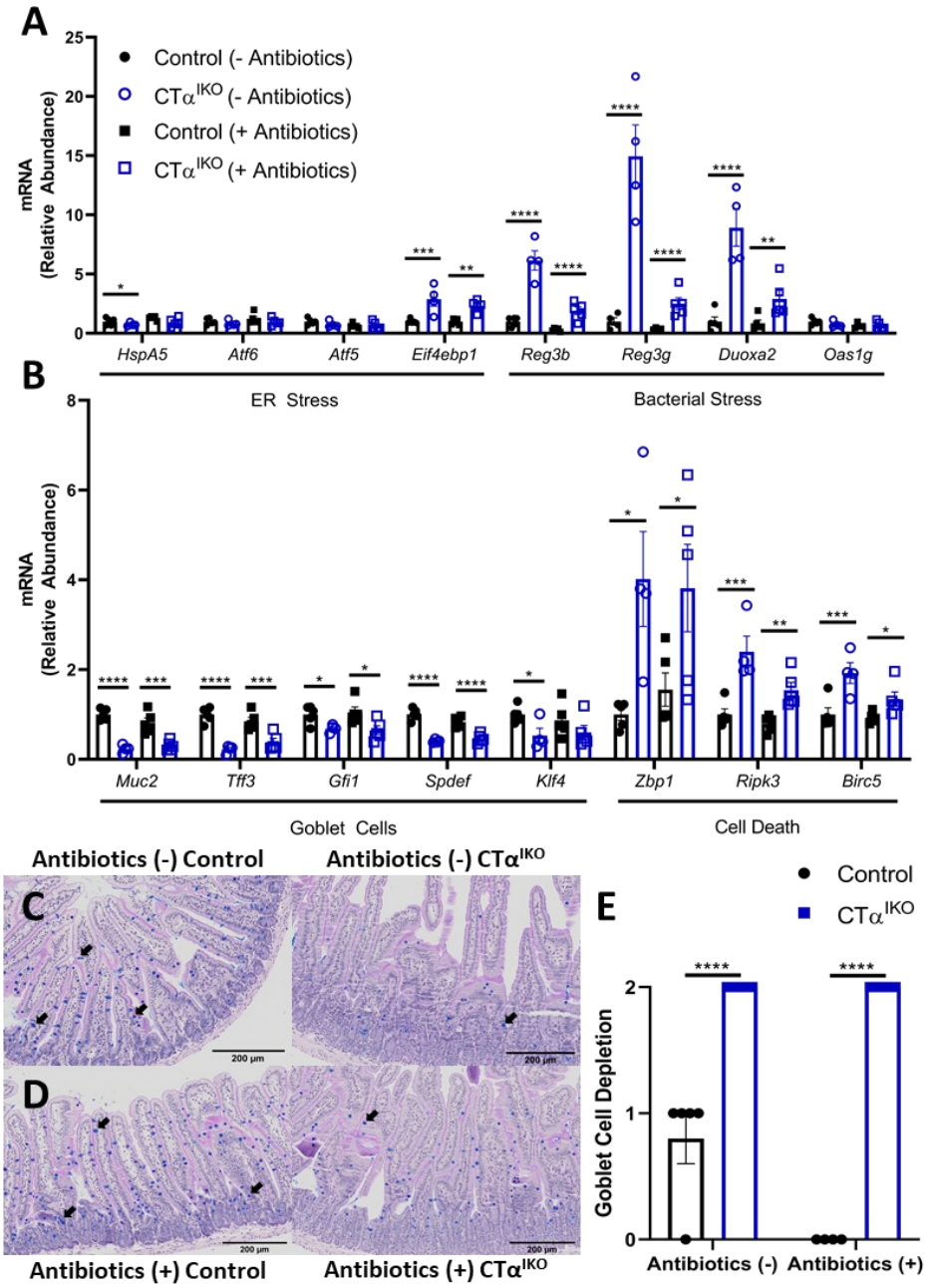

Figure 3.9: Increased cellular stress and cell death in control and CTα^{IKO} mice with or without antibiotic treatment. (A) Jejunum ER stress (*HspA5*, *Atf6*, *Atf5,* and *Eif4ebp1*) and bacterial stress (*Reg3b*, *Reg3g*, *Duoxa2*, and *Oas1g*) mRNA levels in control and CTα^{IKO} mice. (B) Jejunum goblet cell (*Muc2*, *Tff3*, *Gfi1*, *Spdef*, and *Klf4*) and cell death (*Zbp1*, *Ripk3*, and *Birc5*) mRNA levels in control and CTα^{IKO} mice. Representative jejunum AB/PAS stained slides for control and CTα^{IKO} mice (C) (-) antibiotics and (D) (+) antibiotics (arrows indicate goblet cells). (E) Jejunum goblet cell depletion in control and CTα^{IKO} mice. Mice 8-12 weeks with or without antibiotic treatment were fed HFD for 5 days. Values are reported as ±SEM, n=4-5/group unless otherwise stated. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

3.3.6 Supplementing CTα^{IKO} mice with dietary PC restored intestinal PC concentrations and partially restored lipid metabolic function and goblet cell depletion

We next determined whether dietary PC supplementation could improve acute weight loss and intestinal function by feeding control and CTα^{IKO} mice a HFD supplemented with either choline (CSD) or PC (PCSD). While the body weight of CSD-fed CTα^{IKO} mice tended to be lower than CSD-fed control mice, it was not statistically significant (Figure 3.10 A). The PCSD-fed CTα^{IKO} mice had lower weight gain compared to PCSD-fed control mice but did not lose weight compared to CSD-fed control mice (Figure 3.10 A). Consistent with previous experiments, CSD-fed CTα^{IKO} mice had lower jejunal PC levels, higher jejunal PE levels and lower jejunal PC:PE ratio compared to CSD-fed control mice (Figure 3.10 B-D). However, dietary PCSD-fed CTα^{IKO} mice had restored jejunal PC concentrations to levels comparable to PCSD-fed controls (Figure 3.10 B). Furthermore, PCSD-fed CTα^{IKO} mice had slightly lower jejunal PE levels and no difference in jejunal PC:PE ratio compared to controls (Figure 3.10 C-D). The CSD-fed CTα^{IKO} mice had lower plasma TG concentrations after refeeding compared to CSD-fed control mice (Figure 3.10 E). However, PCSD-fed CTα^{IKO} mice had comparable plasma TG concentrations after refeeding compared to PCSD-fed control mice, suggesting that dietary PC supplementation might be able to partially restore chylomicron secretion capacity in CTα^{IKO} mice (Figure 3.10 E). However, both CSD- and PCSD-fed CTα^{IKO} mice had lower jejunal TG levels (Figure 3.10 F) and fewer lipid droplets in H&E stained jejunal slides compared to their respective dietary control mice after refeeding (Figure 3.10 G and H). These data suggest that the acute movement of dietary fatty acids from the intestinal lumen into enterocytes remains impaired in PCSD-fed CTα^{IKO} mice. Furthermore, the mRNA levels of genes associated with lipid metabolism (*Cd36*, *Dgat2*, *Mogat2*, and *Cidec*) were lower in both CSD- and PCSD-fed CTα^{IKO} mice (Figure 3.10 I). Additionally,

circulating levels of plasma active GLP-1 were higher in both CSD- and PCSD-fed CTα^{IKO} mice compared to controls (Figure 3.10 J), showing that normalization of PC concentrations in the intestinal epithelium of CTα^{IKO} mice does not prevent an amplified postprandial secretion of GLP-1. Therefore, while PC supplementation increased IEC PC supply in CTα^{IKO} mice and improved weight loss and postprandial plasma TG levels, CTα^{IKO} mice still had lower TG accumulation in the intestinal epithelium after refeeding compared to control mice.

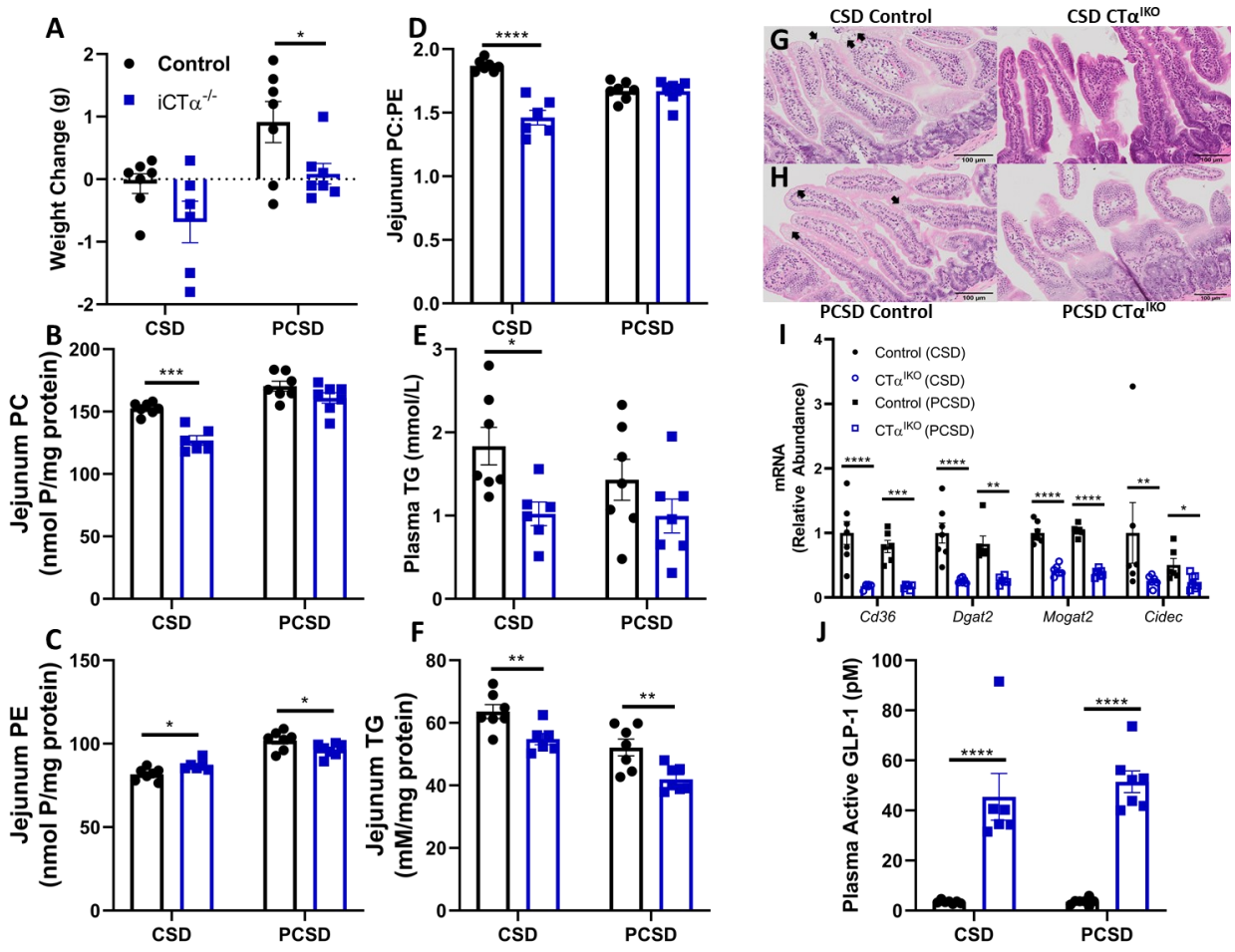

Figure 3.10: Lipid metabolism in CSD- and PCSD-fed control and CTα^{IKO} mice. (A) Weight of control and CTα^{IKO} mice after 4 days of CSD or PCSD. Jejunum (B) PC, (C) PE, and (D) PC:PE ratio in control and CTα^{IKO} mice. (E) Plasma and (F) jejunum TG in control and CTα^{IKO} mice. Representative jejunum H&E staining in (G) CSD- and (H) PCSD-fed control and CTα^{IKO} mice (arrows indicate lipid droplets). (I) Jejunum *Cd36*, *Dgat2*, *Mogat2*, and *Cidec* mRNA levels in control and CTα^{IKO} mice. (J) Plasma active GLP-1 in control and CTα^{IKO} mice. Mice were 18-22 weeks fed CSD or PCSD for 5 days. Values are reported as ±SEM, n=6-7/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

We then determined whether dietary PC supplementation improved markers of ER stress and loss of goblet cells in the small intestines of CTα^{IKO} mice. As in our initial feeding trials (Figure 3.7), the mRNA levels of genes associated with ER stress (*Atf5* and *Eif4ebp1),* microbial defence *(Reg3b*, *Reg3g*, and *Oas1g*), and cell death *(Ripk3* and *Birc5)* were higher in both CSD- and PCSD-fed CTα^{IKO} mice compared to their respective control mice (Figure 3.11 A and B). Furthermore, mRNA levels of the goblet cell markers *Muc2*, *Tff3*, and *Spdef* were lower in the intestines of CSD- and PCSD-fed CTα^{IKO} mice compared to controls (Figure 3.11 B). Jejunal segments of CSD-fed CTα^{IKO} mice had fewer goblet cells compared to control mice by histology (Figure 3.11 C), quantified as increased goblet cell depletion (Figure 3.11 E). Interestingly, only 50% of the PCSD-fed CTα^{IKO} mice showed increased goblet cell depletion despite the reduction in associated genes (Figure 3.11 E). Therefore, PC supplementation was able to improve goblet cell depletion in CTα^{IKO} mice but did not improve transcriptional changes associated with cellular stress.

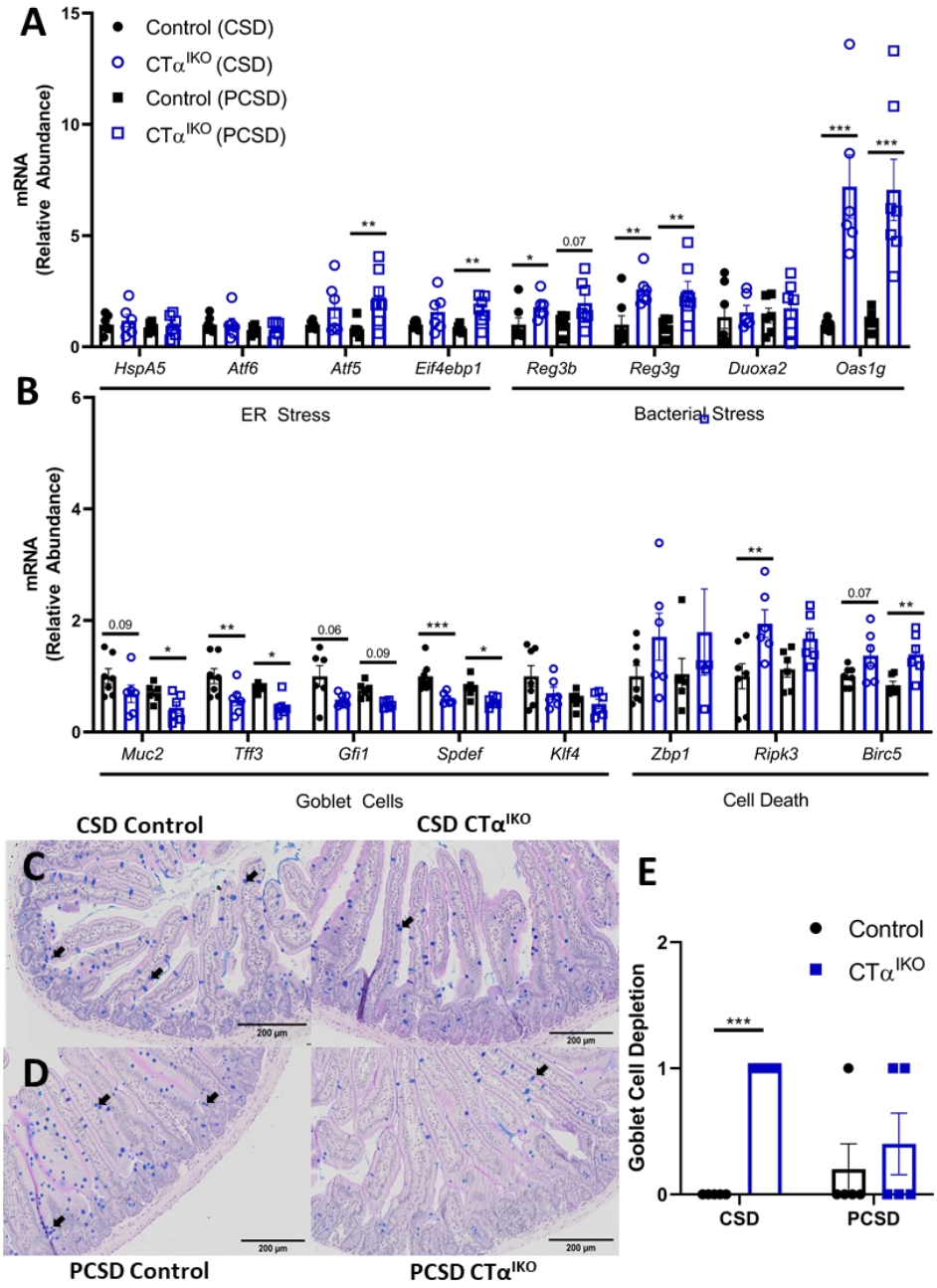

Figure 3.11: Increased cellular stress and cell death in CSD- and PCSD-fed control and CTα^{IKO} mice. (A) Jejunum ER stress (*HspA5*, *Atf6*, *Atf5,* and *Eif4ebp1*) and bacterial stress (*Reg3b*, *Reg3g*, *Duoxa2*, and *Oas1g*) mRNA levels in control and CTα^{IKO} mice. (B) Jejunum goblet cell (*Muc2*, *Tff3*, *Gfi1*, *Spdef*, and *Klf4*) and cell death (*Zbp1*, *Ripk3*, and *Birc5*) mRNA levels in control and CTα^{IKO} mice. Representative jejunum AB/PAS stained slides for (C) CSD- and (D) PCSD-fed control and CTα^{IKO} mice (arrows indicate goblet cells). (E) Jejunum goblet cell depletion in control and CTα^{IKO} mice (n=5/group). Mice were 18-22 weeks fed CSD or PCSD for 5 days. Values are reported as ±SEM, n=6-7/group unless otherwise stated. *P<0.05, **P<0.01, ***P<0.001.

3.4 Discussion

We previously showed that CTα^{IKO} mice fed a short-term HFD have lower intestinal PC concentrations compared to control mice, which is associated with acute weight loss and dietary lipid malabsorption (Kennelly et al., 2018). A key aim of the current study was to understand the changes that occur in the small intestinal epithelium after loss of CTα using transcriptional profiling. We found that impaired *de novo* PC synthesis in the gut is associated with lower abundance of transcripts linked to lipid metabolism and higher abundance of transcripts linked to ER stress, cell death, and inflammation, suggesting that CTα is required to maintain small IEC homeostasis. A further aim of the current study was to determine whether we could rescue acute weight loss in CTα^{IKO} mice by modifying dietary lipid content, luminal PC supply or gut bacterial load. We found that acute body weight loss and postprandial secretion of GLP-1 occurs in CTα^{IKO} mice independent of dietary fat content. Additionally, we found that impaired movement of fatty acids from the intestinal lumen into enterocytes occurs in isolated intestinal sacs from CTα^{IKO} mice compared to control intestinal sacs, independent of potentially confounding influences like bile, circulating hormones, and changes to intestinal motility. Our data show that changes at the level of the enterocyte are responsible for impaired lipid uptake in CTα^{IKO} mice. Antibiotic treatment prevented acute weight loss and normalized jejunum TG concentrations after refeeding but did not alter enhanced postprandial GLP-1 secretion, induction of host defence and ER stress transcripts, or loss of goblet cells in CTα^{IKO} mice. Dietary PC supplementation partially prevented loss of goblet cells but was unable to normalize jejunal TG or plasma GLP-1 concentrations after refeeding in CTα^{IKO} mice, suggesting that there is a specific requirement from *de novo* PC synthesis in maintaining small intestinal homeostasis.

HFD-fed CTα^{IKO} mice lose a significant amount of weight and present with lipid malabsorption. Lipid malabsorption is linked to increased secretion of fat-induced satiety hormone GLP-1, a known modulator of the ileal brake (Cummings & Overduin, 2007). When dietary lipids reach the distal intestine, this signals that absorption is impaired and GLP-1, along with other peptides, are secreted to slow gut motility and enhance lipid absorption (Cummings & Overduin, 2007). Our transcriptional profiling also shows an inverse relationship between transcripts related to lipid metabolism and inflammation in the intestines of CTα^{IKO} mice. Furthermore, it is conceivable that high dietary fat content increases small intestinal PC demand for the assembly of lipid droplets and chylomicrons, and that this demand enhances metabolic stress in CTα^{IKO} mice. Therefore, we hypothesized that HFD-fed CTα^{IKO} mice would have greater weight loss, postprandial GLP-1 secretion, and induction of transcripts related to ER stress, cell death, and host defence compared LFD-fed CTα^{IKO} mice. To our surprise, LFD-fed CTα^{IKO} mice lost comparable amounts of body weight compared to HFD-fed CTα^{IKO} mice. We were also surprised to find that both LFD- and HFD-fed CTα^{IKO} mice had comparable circulating GLP-1 levels after refeeding, suggesting that dietary fat content does not influence the magnitude of GLP-1 release from the intestines of CTα^{IKO} mice. It is therefore conceivable that impaired *de novo* PC synthesis in GLP-1 secreting intestinal epithelial L-cells directly amplifies GLP-1 release, which is an observation that requires further study. Additionally, LFD-fed CTα^{IKO} mice had similar mRNA levels of genes related to ER stress, cell death, and host defence compared to HFD-fed CTα^{IKO} mice. These results together suggest that intestinal metabolic function remains altered in CTα^{IKO} mice regardless of dietary fat content and that factors other than dietary fat contribute to body weight loss in CTα^{IKO} mice.

Intestinal homeostasis is maintained through local and systemic modulators. IEC extrinsic factors that influence lipid absorption include bile (Voshol et al., 2000), hormones (Hsieh et al., 2010), and neuronal cues (Farr, Taher, & Adeli, 2016). For example, neuronal action through the gut-brain axis has been shown to influence intestinal lipid metabolism (Farr et al., 2016). Also, GLP-1 has been shown to reduce gastric emptying and to reduce postprandial chylomicron appearance in circulation (Hsieh et al., 2010; Wettergren et al., 1993). To determine if GLP-1 was slowing gastric emptying and subsequently affecting lipid metabolism in CTα^{IKO} mice, gastric emptying was measured. Surprisingly, CTα^{IKO} mice had increased gastric emptying despite high GLP-1 levels. The rate of gastric emptying is dependent on many factors including diet, neuronal, and hormonal regulation (Goyal, Guo, & Mashimo, 2019) and further study is needed to determine why gastric emptying is increased in CTα^{IKO} mice despite high circulating GLP-1 levels. To determine whether lipid malabsorption still occurred in CTα^{IKO} mice when systemic factors like bile flow, circulating hormones, and the central nervous had been removed, we used an everted intestinal sac technique. The everted intestinal sac model has been optimized to study lipid absorption in the intestines of rodents (W. Strauss, 1963). We incubated the everted intestinal sacs in a buffer containing ^{3}H-oleic acid and ^{14}C-choline and measured the incorporation of the radiolabel into different lipid molecules. Intestinal sacs corresponding to the jejunum of LFD- and HFD-fed CTα^{IKO} mice had lower radiolabel incorporation of ^{3}H-oleic acid and ^{14}C-choline into phosphatidylcholine. The lower ^{14}C-choline incorporation into PC is consistent with the IEC knockout of CTα. Intestinal segments corresponding to the jejunum of LFD- and HFD-fed CTα^{IKO} mice also had lower radiolabel incorporation of ^{3}H-oleic acid into PE and TG, which is consistent with lower ^{3}H-oleic acid movement into the IECs as the synthesis of these molecules is not mediated by CTα. The everted intestinal sac study shows that lipid malabsorption in CTα^{IKO} mice

is due to impaired movement of fatty acids from the intestinal lumen into enterocytes and is independent of potentially confounding influences like changes to bile acid homeostasis, gut motility, and circulating hormone concentrations.

The ratio of PC:PE in cellular membranes has been shown to be a better indicator of membrane homeostasis than total PC levels and alterations in the ratio has been implicated in the development of metabolic disorders such as steatohepatitis (Zhaoyu Li et al., 2006; van der Veen et al., 2017). We found that lower PC concentrations and lower PC:PE ratio in the jejunums of $CT\alpha^{IKO}$ mice leads to the induction of transcriptional programs linked to ER stress (*HspA5*, *Atf5*, and *Eif4ebp1)*, cell death (*Ripk3* and *Birc5*), and host defence (*Reg3b*, *Reg3g*, *Duoxa2*, and *Oas1g*). Furthermore, we found that $CT\alpha^{IKO}$ mice lost goblet cells from the small intestinal epithelium compared to control mice. Induction of these inflammatory programs and loss of goblet cells occurs on both a LFD and a HFD. Alterations to ER phospholipid composition have been shown previously to induce ER stress and the unfolded protein response (Fu et al., 2011; Halbleib et al., 2017; Thibault et al., 2012). Therefore, changes to phospholipid composition of small intestinal membranes after loss CTα likely explains the induction of ER stress in $CT\alpha^{IKO}$ mice. Severe ER stress induction can lead to cell death. Accordingly, $CT\alpha^{IKO}$ mice have higher mRNA levels of genes associated with cell death (*Ripk3* and *Birc5),* which is linked to loss of goblet cells from the intestinal epithelium. ER stress can induce a form of programmed cell death known as necroptosis, an inflammatory cell death, in a RIPK3 protein-dependent manner (Saveljeva, Mc Laughlin, Vandenabeele, Samali, & Bertrand, 2015). Goblet cells in $CT\alpha^{IKO}$ mice appear to be especially impacted by impaired PC synthesis. $CT\alpha^{IKO}$ mice had lower mRNA levels of genes specific to goblet cell development and function including *Muc2*, *Tff3*, and *Spdef,* independent of dietary fat content. The lower abundance of mucus producing goblet cells might leave IECs of

CTα^{IKO} mice susceptible to bacterial interaction. Accordingly, CTα^{IKO} mice had higher mRNA levels of genes associated with host defence against microbes including *Reg3b*, *Reg3g*, *Duoxa2*, and *Oas1g*. *Reg3g* encodes an antibacterial lectin that has been shown to have an important role in maintaining physical distance between bacteria and IECs of the small intestine even with a functional mucus barrier (Vaishnava et al., 2011). The elevation of *Reg3g* mRNA, along with the other host defence genes, is indicative of enhanced microbial interactions with IEC in CTα^{IKO} mice. These results suggest that *de novo* PC synthesis is required to maintain IEC homeostasis. In the absence of CTα, IECs in the small intestine have higher levels of ER stress leading to necroptosis, goblet cell death, and bacterial stress.

Bacterial translocation into the intestinal epithelium leads to both acute and chronic inflammation diseases in the gut (Katayama, Xu, Specian, & Deitch, 1997). The induction of transcriptional programs linked to host defence against microbes, including *Reg3b* and *Reg3g*, suggests that CTα^{IKO} mice have impaired intestinal epithelial barrier function. The induction of Z-DNA binding protein-1 (ZBP-1), a cytosolic Z-DNA sensor that induces necroptosis of IECs by complexing with RIPK3 (Takaoka et al., 2007; Upton, Kaiser, & Mocarski, 2012), could account for loss of goblet cells in the intestinal epithelium of CTα^{IKO} mice (Takaoka et al., 2007). We therefore aimed to determine whether lowering the abundance of bacteria in the intestinal epithelium would prevent the loss of goblet cells or improve metabolic function in the small intestines of CTα^{IKO} mice. Treating CTα^{IKO} mice with antibiotics reduced mRNA levels of genes associated with host defence against microbes (*Reg3b* and *Reg3g*), validating the efficacy of antibiotic treatment. Despite lower bacterial burden in the gut, antibiotic treated CTα^{IKO} mice did not have improved ER stress, or goblet cell depletion. As well, CTα^{IKO} mice treated with antibiotics maintained elevated levels of *Zbp-1* mRNA. There is evidence that ZBP-1 levels can be elevated

from endogenous stimulators in the absence of bacteria (Jiao et al., 2020), therefore ZBP-1 mediated necroptosis does not appear to be initiated by bacterial interaction with IECs in CTα^{IKO} mice. These results indicate that bacterial interaction with IECs is a consequence of lost mucosal integrity and goblet cell depletion and not the primary cause. Interestingly, antibiotic treated CTα^{IKO} mice did not lose weight compared to controls. Furthermore, antibiotic treatment normalized refed jejunum TG levels in CTα^{IKO} mice compared to controls. These data, together with the normalized body weight in CTα^{IKO} mice after antibiotic treatment, suggests that lowering the gut bacterial load at least partially restores metabolic function in CTα^{IKO} mice. However, antibiotic treated CTα^{IKO} mice had lower postprandial plasma TG and higher plasma active GLP-1. This study indicates that reducing bacterial stress in CTα^{IKO} mice does not improve goblet cell depletion, but does improve some aspects of intestinal metabolic function, and prevents body weight loss in CTα^{IKO} mice.

Impaired *de novo* PC synthesis in CTα^{IKO} mice leads to lower total intestinal PC concentrations and lower PC:PE ratio compared to control mice. As discussed above these changes to intestinal phospholipid concentrations is linked to the induction of ER stress and cell death. To determine whether increasing PC availability to IECs of CTα^{IKO} mice through PC supplementation could improve metabolic dysfunction, ER stress, or loss of goblet cells, we fed CTα^{IKO} mice a PCSD. As a control, we fed CTα^{IKO} mice a CSD that had equivalent level of choline as PCSD in the form of free choline. We controlled the amount of choline in the diets as it is involved in many homeostatic pathways including lipid metabolism, cellular signaling, and one-carbon metabolism. Choline and PC are absorbed and metabolised differently in mice, which is a limitation to the current feeding trial. PC supplementation of CTα^{IKO} mice normalized PC concentrations and the ratio of PC:PE in the jejunum. Despite the normalized jejunal PC concentrations, PCSD-fed

CTα^{IKO} mice had lower jejunal TG, fewer lipid droplets in the jejunum, and higher postprandial plasma active GLP-1 compared to PCSD-fed control mice. Additionally, PCSD-fed CTα^{IKO} mice had lower mRNA levels of genes associated with lipid metabolism (*Cd36*, *Dgat2*, *Mogat2*, and *Cidec*) compared to PCSD-fed control mice. Together, these results suggest that fatty acid movement from the intestinal lumen into enterocytes remains impaired in CTα^{IKO} mice. However, PCSD-fed CTα^{IKO} mice had no weight loss compared to CSD-fed control mice and PCSD-fed control and CTα^{IKO} mice had no difference in postprandial plasma TG. This data suggest that dietary PC supplementation can partially restore chylomicron secretion capacity in CTα^{IKO} mice despite impaired movement of fatty acids from the intestinal lumen into enterocytes. It has previously been shown that different sources of intestinal PC have different roles within IECs. *Abcb4*$^{-/-}$ mice have no PC in the bile and accumulate lipids in enterocytes, suggesting that biliary PC is not required for the movement of fatty acids into enterocytes but is required for the assembly or secretion of chylomicrons (Voshol et al., 2000). On the other hand, LPCAT3 knockout mice, which cannot incorporate polyunsaturated fatty acids into PC within the enterocyte, develop lipid malabsorption (Zhiqiang Li et al., 2015; Rong et al., 2015; Wang et al., 2016). These studies highlight the specific roles that different sources of PC have within IEC which could explain why increasing dietary PC levels in CTα^{IKO} mice did not restore the movement of fatty acids from the intestinal lumen into enterocytes. PCSD-fed CTα^{IKO} mice still had elevated mRNA levels of genes associated with ER stress, cell death, and bacterial stress. Surprisingly, PCSD-fed CTα^{IKO} mice did not lose goblet cells from the intestinal epithelium to the same extent a CSD-fed CTα^{IKO} mice. These results show that PC supplementation can partially prevent goblet cell loss in the intestinal epithelium without normalizing transcriptional changes associated with cellular stress.

Our study shows that loss of *de novo* PC synthesis in IECs leads to lipid malabsorption that cannot be rescued by external sources of PC. Whether this inability of exogenous PC to rescue intestinal metabolic function in CTα^{IKO} mice is due to an inability to deliver the PC to specific subcellular compartments within IECs (e.g. delivery of PC to the plasma membrane as opposed to the ER) or the species of PC administered is yet to be determined (Wang et al., 2016). Nonetheless, we found that impaired *de novo* PC synthesis leads to ER stress induction, IEC death, goblet cell depletion, and activation of the host response to microbes in the small intestinal epithelium. In summary, PC derived from *de novo* PC synthesis is an important regulator of small intestinal health.

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Chapter 4

Intestinal phospholipid disequilibrium initiates an ER stress response that drives goblet cell necroptosis and spontaneous colitis in mice

4.1 Introduction

Inflammatory bowel diseases (IBD), comprising Crohn's disease and ulcerative colitis (UC), are increasing in incidence and prevalence worldwide (Ng et al., 2017). Crohn's disease can occur in any area of the gastrointestinal tract and is histologically characterized by transmural inflammation, non-caseating granulomas, and a thickened submucosa (Khor, Gardet, & Xavier, 2011). UC, on the other hand, occurs primarily in the colon and is characterized by superficial damage to the mucosa, cryptitis, and crypt abscesses (Khor et al., 2011). A unique histological feature of UC is the loss of goblet cell mucus granules from the colonic epithelium, although the mechanisms underlying this loss of mucus granules remain unclear (Strugala, Dettmar, & Pearson, 2008). Interestingly, gastrointestinal mucus samples from UC patients show low levels of the major membrane lipid phosphatidylcholine (PC) as compared to mucus from patients with Crohn's disease or people without IBD (Braun et al., 2009; Ehehalt et al., 2004). Furthermore, human clinical trials designed to restore colonic PC concentrations in UC patients have shown promising results (Karner et al., 2014; W. Stremmel et al., 2005; Wolfgang Stremmel, Ehehalt, Autschbach, & Karner, 2007). However, despite these important clinical links between PC and UC, the precise molecular mechanisms linking changes to intestinal PC concentrations (and membrane lipid composition) to intestinal inflammation and features of UC pathology *in vivo* remain unclear.

PC is primarily produced by the CDP-choline pathway in mammalian tissues (van der Veen et al., 2017). The rate-limiting step of the CDP-choline pathway, the conversion of phosphocholine to CDP-choline, is catalyzed by CTP:phosphocholine cytidylyltransferase-α (CTα; encoded by *Pcyt1a*). An adequate supply of PC is required for the prevention and resolution of endoplasmic reticulum (ER) stress in a variety of cell types 10,11, and might be particularly important in intestinal epithelial cells (IECs) due to their high secretory activity and constant exposure to

environmental antigens (Ho, Xu, & Thibault, 2018; Sriburi, Jackowski, Mori, & Brewer, 2004). Furthermore, a relatively low molar ratio of PC to phosphatidylethanolamine (PE) is associated with non-alcoholic steatohepatitis in humans, suggesting that perturbations to membrane lipid composition might influence the initiation or progression of inflammatory diseases (Li et al., 2006). Consistent with an anti-inflammatory role for PC in IECs, exogenous delivery of PC, but not PE, to Caco2 cells after treatment with tumor necrosis factor-α (TNF-α) dampens the induction of pro-inflammatory transcripts (Braun et al., 2009). To date, the link between PC and intestinal inflammation has not yet been thoroughly examined *in vivo*. In addition to *de novo* synthesis, IECs of the small intestine can obtain PC from the diet, bile and circulating lipoproteins. However, since dietary and biliary PC is primarily absorbed in the proximal small intestine, the colon is reliant on *de novo* PC synthesis to maintain membrane lipid composition and thus might be particularly sensitive to dietary or environmental factors that disrupt membrane lipid homeostasis (Nilsson, 1968; Parthasarathy, Subbaiah, & Ganguly, 1974).

To determine the role that PC plays in mucosal barrier function, and to gain insight into the mechanisms by which colonic PC depletion is linked to inflammation in UC patients, we examined mice with IEC-specific deletion of CTα (CTα^{IKO} mice) (Kennelly et al., 2018). We found that inducible loss of CTα in the intestinal epithelium reduces colonic PC concentrations and results in rapid and spontaneous colitis with 100% penetrance in adult mice that is characterized by crypt abscesses, goblet cell depletion, and immune cell infiltration. Colitis development after IEC PC depletion is initiated by a severe and unresolving ER stress response. This ER stress response is linked to the induction of Receptor-interacting serine/threonine-protein kinase 3 (RIP3) and the death of IECs by necroptosis, leading to loss of goblet cells, formation of a thin mucus barrier, infiltration of the epithelium by microbes, and the induction of an array of pro-

inflammatory cytokines. Taken together, our data show that maintaining membrane lipid composition in IECs is crucial for normal colonic barrier function.

4.2 Methods

4.2.1 Animal handling

Mice were housed in a temperature-controlled room with 12-h light/dark cycle and free access to food and water. Generation of *Pcyt1a*$^{LoxP/LoxP}$;villin-CreER^{T2} and *Pcyt1a*$^{LoxP/LoxP}$ mice has been described previously (Kennelly et al., 2018). Cre was induced in age-matched 8-20-week-old female *Pcyt1a*$^{LoxP/LoxP}$;villin-CreER^{T2} (CTα^{IKO}) mice fed a chow diet (5001, Lab Diet, St. Louis, MO) by intraperitoneal injection of tamoxifen (1mg/day in sunflower oil for 5 days), while tamoxifen-treated *Pcyt1a*$^{LoxP/LoxP}$ mice were used as controls. Twenty-four hours after the end of tamoxifen treatment (Time 0), mice were placed on a semi-purified diet (40% fat, 20%, protein, 40% carbohydrate; 16) for either 4 days, 7 days or 7 weeks until termination, as indicated. Colon sections, colon Swiss rolls or cecum sections were fixed in 10% neutral-buffered formalin for histology. Colonic epithelial cells and the overlying mucus layer were collected as previously described (Nenci et al., 2007). Briefly, colons were flushed with a solution containing 0.154 M NaCl and 1 mM dithiothreitol to remove contents. Colons were next ligated, filled with phosphate-buffered saline, and incubated at 37°C for 15 minutes. The phosphate-buffered saline was then replaced with phosphate-buffered saline containing 1.5 mM ethylenediaminetetraacetic acid and 0.5 mM dithiothreitol before being incubated for a further 30 minutes at 37°C. After 30 minutes, one ligature was removed, and colonic epithelial cells were collected. Samples were frozen at minus 80°C before being used for phospholipid analysis or western blotting. Whole blood was collected in ethylenediaminetetraacetic acid-coated tubes containing a protease inhibitor cocktail

(Sigma-Aldrich, St. Louis, MO) for the measurement of Complete Blood Counts on a Siemens ADVIA® 2120i Hematology System, or for the measurement of plasma Lipocalin 2 (R&D Systems, Minneapolis, MN). A group of control mice and $CT\alpha^{IKO}$ mice were given an antibiotic cocktail (bacitracin (500mg/L), neomycin (1g/L) and vancomycin (500mg/L)) in the drinking water or no antibiotics from the time of first tamoxifen injection until termination (10 days), as described previously (Out et al., 2015). A separate group of $CT\alpha^{IKO}$ mice and control mice were administered with 4-phenyl butyric acid sodium salt (PBA, 500 mg per kg body weight, Scandinavian Formulas, USA), dissolved in phosphate buffered saline, or vehicle twice daily by oral gavage from the time of first tamoxifen injection until termination (10 days), as described previously (Cao et al., 2013). A third group of $CT\alpha^{IKO}$ mice and control mice were fed either a diet containing four times the recommended level of choline as PC or a control diet that was matched to the experimental diet for total calories and total choline content (Table 4.1). The University of Alberta's Institutional Animal Care Committee approved all animal procedures, which were in accordance with guidelines of the Canadian Council on Animal Care. All authors have access to the study data and have reviewed and approved the final manuscript.

Table 4.1: Control diet and PC supplemented diet ingredients.

Ingredients (g)	Control Diet	PC Diet
Casein	270	270
Corn starch	170.65	170.65
Sucrose	195.35	195.35
Cellulose	80	80
AIN-93-VX Vitamin mix	19	19
Bernhart-Tomarelli Mineral mix	50	50
Calcium phosphate dibasic	3.4	3.4
myo-Inositol	6.3	6.3
L-cystine	1.8	1.8
Choline bitartrate	10.0	2.5
Crisco Vegetal oil	32	23
Mazola Corn oil	10	10
Lard	155	127
DHAsco	1.5	1.5
Arasco	1.5	1.5
PC (soy lecithin)	0	90

4.2.2 Microscopy

Formalin-fixed, paraffin-embedded tissue slices (5µm) were stained with hematoxylin and eosin (H&E) or Alcian blue/Period acid-Schiff (AB/PAS) and visualized with a light microscope (Zeiss, Zen, AxioCamMR3). TUNEL staining was performed using the In Situ Cell Death Detection Kit (Sigma-Aldrich, MO, USA) and images were obtained with a fluorescence microscope (Olympus, Markham, ON) with Surveyor and Image-Pro Plus software. For electron microscopy, 2 cm colonic rings were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2) and 2% paraformaldehyde. Sections were cryo-sectioned with an ultramicrotome (Ultracut E, Reichert-Jung) and images were obtained using a Philips 410 transmission electron microscope, as previously described (Kennelly et al., 2018). H&E-stained distal colon sections were assessed by an experienced pathologist who was blinded to the experimental groupings. The pathologist assigned a colitis severity score based on a previously established protocol which included

assessment of epithelial hyperplasia, enterocyte injury, and the presence of lymphocytes and neutrophils in the lamina propria, as outlined in Table 4.2 (Madsen et al., 2001). The pathologist also assigned a score for goblet cell depletion using the following scale: 0, no goblet cell depletion; 1, modest goblet cell depletion; 2, substantial goblet cell depletion.

Table 4.2: Histopathologic colitis scoring system.

Group	Description	Score
Enterocytes		
Normal	Rare epithelial lymphocytes	0
Mild	Intraepithelial neutrophils	1
Moderate	Mucosal necrosis and/or luminal pus	2
Severe	Necrosis muscularis mucosa	3
Epithelial hyperplasia		
Normal		0
Mild		1
Moderate		2
Pseudopolyps		3
Lamina propria mononuclear infiltrate		
Normal	One small lymphoid aggregate	0
Slightly increased	More than one small aggregate	1
Markedly increased	Large aggregates and/or greatly increased single cells	2
Lamina propria neutrophil infiltrate		
Normal		0
Slightly increased		1
Markedly increased		2

4.2.3 Lipid analysis

The protein content of colonic epithelial cells was determined by bicinchoninic acid assay (Thermo Scientific, CA, USA), and lipids were extracted from homogenates by the method of Folch (Folch, Lees, & Sloane Stanley, 1957). PC and PE were separated on silica plates (VWR, EM1.05721.0001) by thin layer chromatography using the solvent system chloroform: methanol: acetic acid: water (50:30:8:4). Plates were exposed to iodine for visualization, PC and PE bands were scraped into glass tubes, and lipids were liberated from the silica by heating with perchloric acid (Sigma, 77230) for 60 minutes at 180°C. Subsequently, 0.5 mL of ammonium molybdate (Sigma, 431346) and L-ascorbic acid (Sigma, 255564) were added to the samples before heating at 95°C for 15 minutes. The phosphorous content of samples was determined by measuring the absorbance of the samples at 820 nm on a spectrophotometer (Spectra Max Pro, Molecular devices, USA) and comparing the sample absorbance to the absorbance of a phosphorous standard curve (Sigma, 53139), as described previously (Zhou & Arthur, 1992).

4.2.4 Cytokine and chemokine concentrations

Sections of the distal colon were homogenized in buffer containing a protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO) and dithiothreitol (Sigma-Aldrich, St. Louis, MO). The protein concentration of the supernatant was determined by bicinchoninic acid assay (Thermo Scientific, CA, USA) after centrifugation at 10 000 rpm for 10 min to remove debris. Samples were adjusted to 3 mg protein/ml homogenate, and cytokine and chemokine concentrations were determined using Multiplex LASER Bead Technology (MD31; Eve Technology, Calgary, Canada).

4.2.5 Real-time quantitative PCR analysis

Total RNA was isolated from frozen colonic tissue using Trizol (Invitrogen, CA, USA), as described previously (Kennelly et al., 2018). Superscript II (Invitrogen, CA, USA) was used to reverse-transcribe isolated RNA. Quantitative PCR was run for 40 cycles on a StepOne Plus system (Applied Biosystems, MA, USA) using Power SYBR Green PCR Master Mix (Thermo Fisher Scientific, MA, USA). Quantitation was performed using the standard curves method. Relative mRNA expression was normalized to *Rplp0*. Primer sequences and gene names are listed in Table 4.3.

Table 4.3: Primers for quantitative PCR.

Gene Symbol	Gene Name	Forward Primer Sequence	Reverse Primer Sequence
Mouse			
Muc2	Mucin 2	CCATTGAGTTTGGGAACATGC	TTCGGCTCGGTGTTCAGAG
Tff3	Trefoil factor 3	CTGGGATAGCTGCAGATTACG	CATTTGCCGGCACCATAC
Agr2	anterior gradient 2, protein disulphide isomerase family member	CCTCAACCTGGTCTATGAAACA	ACCGTCAGGGATGGGTCT
Gfi1	Growth factor independent 1 transcriptional repressor	ATGTGCGGCAAGACCTTC	ACAGTCAAAGCTGCGTTCCT
Spdef	SAM pointed domain containing ETS transcription factor	GATGTACTGCATGCCCACCT	GGAGGCGCAGTAGTGAAGG
Klf4	Kruppel like factor 4	CCGTCCTTCTCCACGTTC	GAGTTCCTCACGCCAACG
Zbp1	Z-DNA binding protein 1	CAGGAAGGCCAAGACATAGC	GACAAATAATCGCAGGGGACT
Cldn2	Claudin 2	TGTGAATGAACTGAAGGAAAGC	ATCCTGCACCCAGCTGTATT
Cldn4	Claudin 4	TTTTGTGGTCACCGACTTTG	TGTAGTCCCATAGACGCCATC
Xbp1 (spliced)	X-Box Binding Protein 1 (spliced isoform)	GAGTCCGCAGCAGGTG	GTG TCA GAG TCC ATG GGA
Ddit3	DNA damage inducible transcript 3	GCGACAGAGCCAGAATAACA	GATGCACTTCCTTCTGGAACA
Atf4	Activating transcription factor 4	CTCAGACACCGGCAAGGA	TCATCCAACGTGGTCAAGAG
Atf5	Activating transcription factor 5	GCAGCACCTAGGGTACAGGT	CGCTGGAGACAGACGTACAC
Hspa5	Heat shock protein family A (Hsp70) member 5	CTGAGGCGTATTTGGGAAAG	TCATGACATTCAGTCCAGCAA
Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1	GATGAGCCTCCCATGCAA	AATGTCCATCTCAAATTGTGACTC
Birc5	Baculoviral IAP repeat-containing 5	TGATTTGGCCCAGTGTTTTT	CAGGGGAGTGCTTTCTATGC
Ccnd1	Cyclin D1	GCACAACGCACTTTCTTTCC	TCCAGAAGGGCTTCAATCTG
Rplp0	Ribosomal protein lateral stalk subunit P0	ACTGGTCTAGGACCCGAGAAG	CTCCCACCTTGTCTCCAGTC
Neurog3	Neurogenin 3	ACTGCTGCTTGTCACTGACTG	ATGGTGAGCGCATCCAAG
Sct	Secretin	GCTGTGGTCGAACACTCAGA	GAGACAGGGACCCATCCAG
Insl5	insulin-like 5	GCATTTCCACTCTCAACAAGC	GATGGCTCGTGCCTGTCTA
Pcyt1a	Phosphate cytidylyltransferase 1, choline, alpha isoform	GCTAAAGTCAATTCGAGGAA	CATAGGGCTTACTAAAGTCAACT
Casp4	Caspase 4 (Caspase 11)	GTGGTGAAAGAGGAGCTTACAGC	GCACCAGGAATGTGCTGTCTGA
Gsdmd	Gasdermin D	GGTGCTTGACTCTGGAGAACTG	GCTGCTTTGACAGCACCGTTGT
Bacteria			
UniF340/UniR514	All bacteria	ACTCCTACGGGAGGCAGCAGT	ATTACCGCGGCTGCTGGC

4.2.6 Western blots

Colonic epithelial cells containing 40-50 μg of protein were resolved on a sodium dodecyl sulfate polyacrylamide gel before being transferred to a PVDF membrane and probed with antibodies against CTα (gift from Dr. R.K Mallampalli), spliced XBP1 (D2C1F, Cell Signaling Technology, #12782), PERK (C33E10, Cell Signaling Technology, #3192), ATF6 (Cell Signaling Technology, D4Z8V, #65880S), Sequestosome 1 (p62; Abcam Ab56416), , RIP3 (Biorad, #AHP1797), cleaved caspase 3 (Cell Signaling Technology, #9661), cleaved caspase 8 (Cell Signaling Technology, #8592), β-actin (Cell Signaling Technology, #4967), GAPDH (Abcam, ab8245) and α-tubulin (Sigma-Aldrich, #T6199). Immunoreactive proteins were detected with ECL Western Blot Reagent (Amersham, GE Healthcare, UK), and images were obtained with a Chemi-Doc MP Imager, (Bio-Rad Laboratories, CA, USA).

4.2.7 Paracellular permeability assessment

Mice were fasted for 12 h before, weighed, and orally gavaged with 4 kDA FITC-dextran (FD4, Sigma; 0.44mg/g mouse). Blood was collected by cardiac puncture after 2 h before centrifuging at 2000 g for 5 min to obtain plasma. Plasma was diluted in water (1:1) before fluorescence was measured with an excitation of 485 nm and an emission wavelength of 528 nm on a EnVision Multilabel plate reader (Perkin Elmer, MA, USA). The appearance of the non-digestible FITC-dextran in plasma after oral administration is a measure of paracellular permeability (Woting & Blaut, 2018).

4.2.8 Microbial analysis

DNA was extracted from two fecal pellets collected aseptically from non-antibiotic (Control, n = 14; CTα^{IKO}, n = 11) and antibiotic treated groups (Control + Antibiotics, n = 5; CTα^{IKO} + Antibiotics, n = 5) as previously described (Ju et al., 2017). Real-time PCR was performed to quantify the total bacterial load in feces using primer set UniF340/UniR514 (Table 4.3). The PCR reaction was performed on an ABI StepOne™ real-time System (Applied Biosystems, Foster City, CA) using PerfeCTa SYBR Green Supermix (Quantabio, Gaithersburg, MD). The Amplification program contained an initial denaturation step at 95℃ for 3 min followed by 40 cycles of denaturation at 95℃ for 10 s and annealing at 60℃ for 30 s. The gDNA from a gut commensal *Escherichia coli* strain with genome size of 5,190,098 bp was chosen to create an 8-log-fold standard curve for direct quantification of the total bacteria. The 16S rRNA gene copies were calculated using the formula: (((16S rRNA gene copies in the genome)/(Genome size of the *E. coli* strain))*(DNA concentration at the first serial dilution))/(Average mass of 1 bp dsDNA))*(Avogadro's number))/(1*10^9 ng/g). The Ct value (threshold cycle) was associated with 16S rRNA gene copies (log copy number) to construct a function for quantification of all samples. The total bacterial load was expressed as 16S rRNA gene copies per gram of feces on a base 10 logarithmic scale. Microbial composition was assessed by 16S rRNA gene amplicon sequencing on an Illumina MiSeq platform. Amplicon library construction, paired-end sequencing targeting the V3-V4 region of the 16S rRNA gene, and data analysis has been published previously (Ju et al., 2017).

4.2.9 Statistical analysis

Data are expressed as mean ± standard error of the mean using Graphpad Prism (Version 7). Data were analyzed with a Student's t-test or 2-way ANOVA with Tukey's post-test. A Mann-Whitney test was used to compare histopathologic colitis scores and goblet cell depletion scores between control and CTα^{IKO} mice. For microbiota analysis, permutational multivariate analysis of variance (MANOVA) of the weighted UniFrac distance was conducted to identify the difference in overall microbial structure between groups, using the adonis function in the vegan package with 999 permutations (R v3.4.4). The principal coordinate analysis (PCoA) based on the Bray-Curtis dissimilarity metric was plotted using the phyloseq package (R v3.4.4) (McMurdie & Holmes, 2013). Comparison of individual taxa/OTUs between treatments was performed using the Kruskal-Wallis test with the Dwass, Steel, Critchlow-Fligner multiple comparisons post-hoc procedure (SAS v9.4). Raw sequences of the 16S rRNA gene amplicon data are available through the SRA with accession number PRJNA562603.

4.3 Results

4.3.1 CTα^{IKO} mice have altered colonic phospholipid concentrations

Induction of Cre recombinase with tamoxifen resulted in the generation of adult CTα^{IKO} mice with ~60% lower *Pcyt1a* mRNA (Figure 4.1 A) and ~75% lower CTα protein abundance (Figure 4.1 B-C) in the colon compared to control mice. Residual CTα protein levels were likely due to the presence of non-epithelial cell types including muscle and infiltrating immune cells. Consistent with our previous report, CTα^{IKO} mice experienced rapid body weight loss of varying severity upon Cre induction, while control mice did not experience body weight loss (Figure 4.1 D) (Kennelly et al., 2018). One of 17 CTα^{IKO} mice experienced severe wasting with over 20%

body weight loss and was euthanized on day 5 after Cre induction. Most CTα^{IKO} mice began to regain body weight at day 5 and were a similar body weight to controls by day 7 (Figure 4.1 D). CTα^{IKO} mice had lower PC concentrations in epithelial cells isolated from the colon 7 days after Cre induction compared to epithelial cells isolated from control mice (Figure 4.1 E). PE concentrations in colonic epithelial cells were comparable between groups (Figure 4.1 E), resulting in a significantly lower ratio of PC/PE in epithelial cells of CTα^{IKO} mice compared to control mice (Figure 4.1 F).

Colonic *Pcyt1a* mRNA remained repressed in the colons of CTα^{IKO} mice at 7 weeks following Cre induction (Figure 4.1 G). Additionally, CTα protein levels remained absent in epithelial cells isolated from the ileums of CTα^{IKO} mice after 7 weeks (Figure 4.1 H-I). CTα^{IKO} mice also had lower PC concentrations in ileal epithelial cells after 7 weeks compared to control mice, while PE concentrations were similar (Figure 4.1 J), resulting in a lower ratio of PC to PE in ileal epithelial cells (Figure 4.1 K) at 7 weeks after Cre induction. Furthermore, CTα^{IKO} mice gained less body weight compared to controls over the 7-week follow-up period (Figure 4.1 L), but no additional morbidity was observed after day 8 when one further CTα^{IKO} mouse was euthanized due to excessive body weight loss.

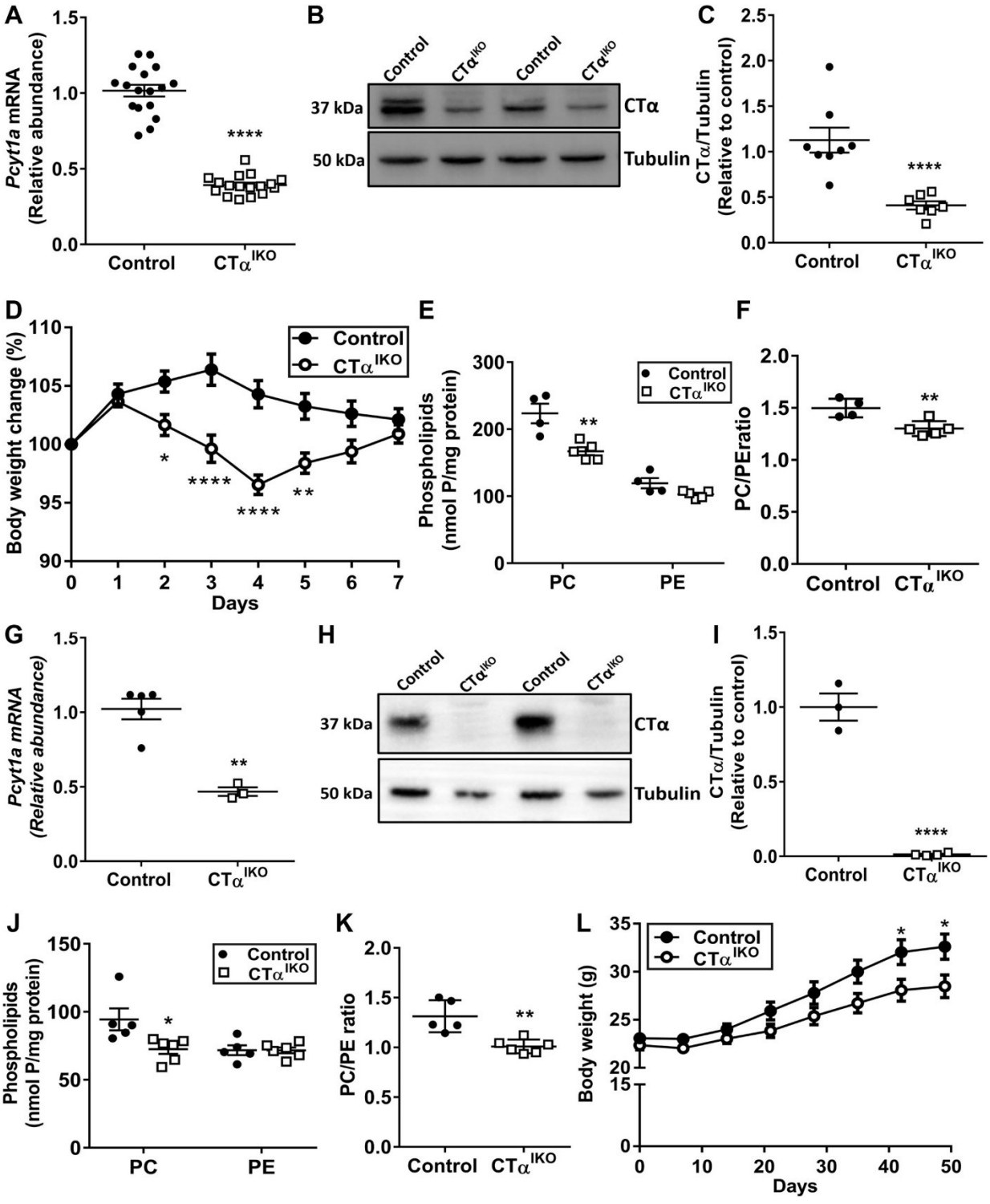
A
Pcyt1a mRNA (Relative abundance)
1.5
1.0
0.5
0.0
****
Control
CTα IKO
B
Control
CTα IKO
Control
CTα IKO
37 kDa
CTα
50 kDa
Tubulin
C
CTα/Tubulin (Relative to control)
2.5
2.0
1.5
1.0
0.5
0.0
****
Control
CTα IKO
D
Body weight change (%)
110
105
100
95
90
Control
CTα IKO
*
****
****
**
0 1 2 3 4 5 6 7
Days
E
Phospholipids (nmol P/mg protein)
300
200
100
0
**
Control
CTα IKO
PC
PE
F
PC/PEratio
2.0
1.5
1.0
0.5
0.0
**
Control
CTα IKO
G
Pcyt1a mRNA (Relative abundance)
1.5
1.0
0.5
0.0
**
Control
CTα IKO
H
Control
CTα IKO
Control
CTα IKO
37 kDa
CTα
50 kDa
Tubulin
I
CTα/Tubulin (Relative to control)
1.5
1.0
0.5
0.0
****
Control
CTα IKO
J
Phospholipids (nmol P/mg protein)
150
100
50
0
Control
CTα IKO
*
PC
PE
K
PC/PE ratio
2.0
1.5
1.0
0.5
0.0
**
Control
CTα IKO
L
Body weight (g)
35
30
25
20
15
0
Control
CTα IKO
*
*
0 10 20 30 40 50
Days

Figure 4.1: CTα^{IKO} mice have low colonic PC concentrations and experience acute body weight loss. (A) The mRNA abundance of *Pcyt1a* in colonic tissue of control mice and CTα^{IKO} mice 7 days after the end of tamoxifen treatment (n = 17/group). (B) Western blot and (C) quantification of CTα relative to tubulin in colonic epithelial cells isolated from control mice and CTα^{IKO} mice 7 days after the end of tamoxifen treatment (n = 8/group). (D) Body mass change relative to body mass at the end of tamoxifen treatment in control mice and CTα^{IKO} mice (n = 17/group). (E) PC and PE concentrations in colonic epithelial cells isolated from control mice and CTα^{IKO} mice 7 days after the end of tamoxifen treatment (n = 4–5/group). (F) The ratio of PC/PE in colonic epithelial cells of control mice and CTα^{IKO} mice 7 days after the end of tamoxifen treatment (n = 4–5/group). (G) The mRNA abundance of Pcyt1a in colonic tissue of control mice and CTα^{IKO} mice 7 weeks after the end of tamoxifen treatment (n = 3–5/group). (H) Western blot and (I) quantification of CTα relative to tubulin in ileal epithelial cells isolated from control mice and CTα^{IKO} mice 7 weeks after the end of tamoxifen treatment (n = 3–4/group). (J) PC and PE concentrations in ileal epithelial cells isolated from control mice and CTα^{IKO} mice 7 weeks after the end of tamoxifen treatment (n = 5–6/group). (K) The ratio of PC/PE in ileal epithelial cells of control mice and CTα^{IKO} mice 7 weeks after the end of tamoxifen treatment (n = 5–6/group). (L) Body mass in control mice and CTα^{IKO} mice over 7 weeks after Cre induction (n = 18–20/group). Mice killed because of excessive weight loss (2 of 35 CTα^{IKO} mice) were not included in any analyses. Values are means ± SEM. $*P < .05$, $**P < .01$, and $****P < .0001$.

4.3.2 CTα^{IKO} mice develop spontaneous colitis

Seven days after Cre induction, the colons of CTα^{IKO} mice weighed ~50% more than those of controls (Figure 4.2 A). Furthermore, red blood cells, hemoglobin and hematocrit were significantly lower in CTα^{IKO} mice, while blood reticulocyte concentrations were higher, which together is indicative of anemia and suggested that CTα^{IKO} mice might have lost blood through the injured bowel (Table 4.4). Additionally, circulating concentrations of the bacteriostatic protein Lipocalin 2 were elevated in CTα^{IKO} mice compared to control mice (Figure 4.2 B). There was extensive damage to the colonic epithelium of CTα^{IKO} mice compared to controls (Figures 4.2 C-H), including crypt abscesses (Figure 4.2 F), lymphocyte infiltration to the lamina propria (Figure 4.2 D), and crypt dysplasia (Figure 4.2 H). Pathology scoring of H&E stained distal colon sections showed that 100% of CTα^{IKO} mice developed spontaneous colitis by day 4 after Cre induction (Figure 4.2 I).

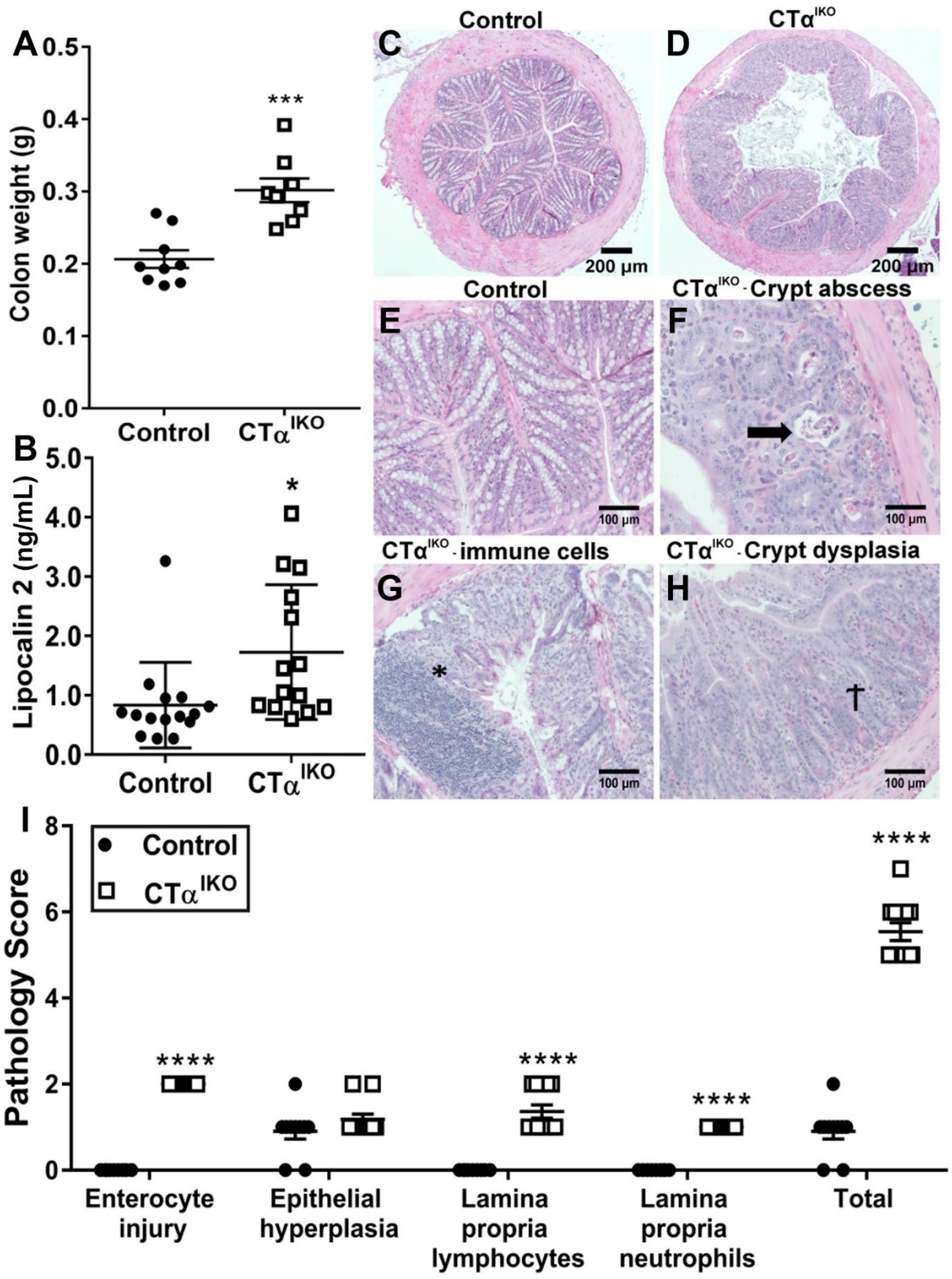
A
Colon weight (g)
0.5
0.4
0.3
0.2
0.1
0.0
***
Control
CTα IKO
B
Lipocalin 2 (ng/mL)
5.0
4.0
3.0
2.0
1.0
0.0
*
Control
CTα IKO
Control
CTα IKO
C
D
200 µm
200 µm
Control
CTα IKO - Crypt abscess
E
F
100 µm
100 µm
CTα IKO - immune cells
CTα IKO - Crypt dysplasia
G
H
*
†
100 µm
100 µm
I
Pathology Score
8
6
4
2
0
Control
CTα IKO
****
****
****
****
Enterocyte injury
Epithelial hyperplasia
Lamina propria lymphocytes
Lamina propria neutrophils
Total

Figure 4.2: CTα^{IKO} mice develop spontaneous colitis. (A) Colon weight in control mice and CTα^{IKO} mice 7 days after the end of tamoxifen treatment (n = 8–9/group). (B) Plasma lipocalin 2 concentrations in control mice and CTα^{IKO} mice 4 days after the end of tamoxifen treatment (n = 14–15/group). (C and D) Representative H&E-stained distal colon sections from control mice and CTα^{IKO} mice 4 days after the end of tamoxifen treatment. (E) Representative H&E-stained distal colon section from control mice 4 days after the end of tamoxifen treatment. (F and H) Representative H&E-stained distal colon sections from CTα^{IKO} mice 4 days after the end of tamoxifen treatment. (F) Arrow indicates crypt abscess. (G) ∗Immune cell infiltration. (H) †Crypt dysplasia. (I) Pathology scores of control mice and CTα^{IKO} mice 4 days after the end of tamoxifen treatment (n = 10–11/group). Values are means ± SEM. ∗$P < .05$, ∗∗∗$P < .001$.

Table 4.4: Complete blood cell count data[1]

Complete Blood Cell Count	Control	CTα^{IKO}
Hematocrit (%)	54.00 ± 1.31	50.32 ± 0.48*
Red blood cells (x10E12 cells/L)	10.38 ± 0.26	9.72 ± 0.11*
Hemoglobin (g/L)	156.70 ± 3.06	147.80 ± 1.8*
Reticulocytes (x10E09 cells/L)	149.40 ± 20.05	295.80 ± 29.50**
Reticulocytes (%)	1.46 ± 0.22	3.05 ± 0.31**
White blood cell count peroxidase method (x10E09 cells/L)	2.85 ± 0.88	2.33 ± 1.10
White blood cell count basophile method (x10E09 cells/L)	3.21 ± 1.03	2.29 ± 0.99
Mean corpuscular volume (fL)	52.02 ± 0.17	51.78 ± 0.29
Mean corpuscular hemoglobin (pg)	15.12 ± 0.10	15.23 ± 0.80
Mean corpuscular hemoglobin concentration (g/L)	290.30 ± 1.86	293.80 ± 1.99
Platelets (x10E09 cells/L)	701.00 ± 11.85	796.20 ± 44.19
Neutrophils (%)	9.90 ± 1.83	11.38 ± 2.51
Lymphocytes (%)	80.32 ± 2.20	79.13 ± 1.86
Monocytes (%)	2.90 ± 0.68	3.32 ± 0.36
Eosinophils (%)	2.55 ± 0.51	3.15 ± 0.61
Large unstained cells (%)	3.95 ± 1.28	2.57 ± 0.71
Basophils (%)	0.47 ± 0.071	0.47 ± 0.1
Neutrophils (x10E09 cells/L)	0.25 ± 0.50	0.21 ± 0.06
Lymphocytes (x10E09 cells/L)	2.65 ± 0.95	1.88 ± 0.87
Monocytes (x10E09 cells/L)	0.07 ± 0.02	0.06 ± 0.2
Eosinophils (x10E09 cells/L)	0.06 ± 0.01	0.05 ± 0.11
Large unstained cells (x10E09 cells/L)	0.15 ± 0.07	0.07 ± 0.04
Basophils (x10E09 cells/L)	0.02 ± 0.01	0.01 ± 0.01

[1]Data are mean ± SEM.

Dietary PC supplementation was unable to rescue body weight loss (Figure 4.3 A) or colitis development (Figure 4.3 B) in CTα^{IKO} mice, as indicated by assessment of pathology scores (Figure 4.3 C) and goblet cell depletion (Figure 4.3 D). The inability of exogenous dietary PC to rescue any metrics of disease pathology in CTα^{IKO} mice likely reflects that dietary PC is efficiently hydrolyzed and absorbed in the proximal small intestine and that very little dietary PC reaches the colon (Parthasarathy et al., 1974).

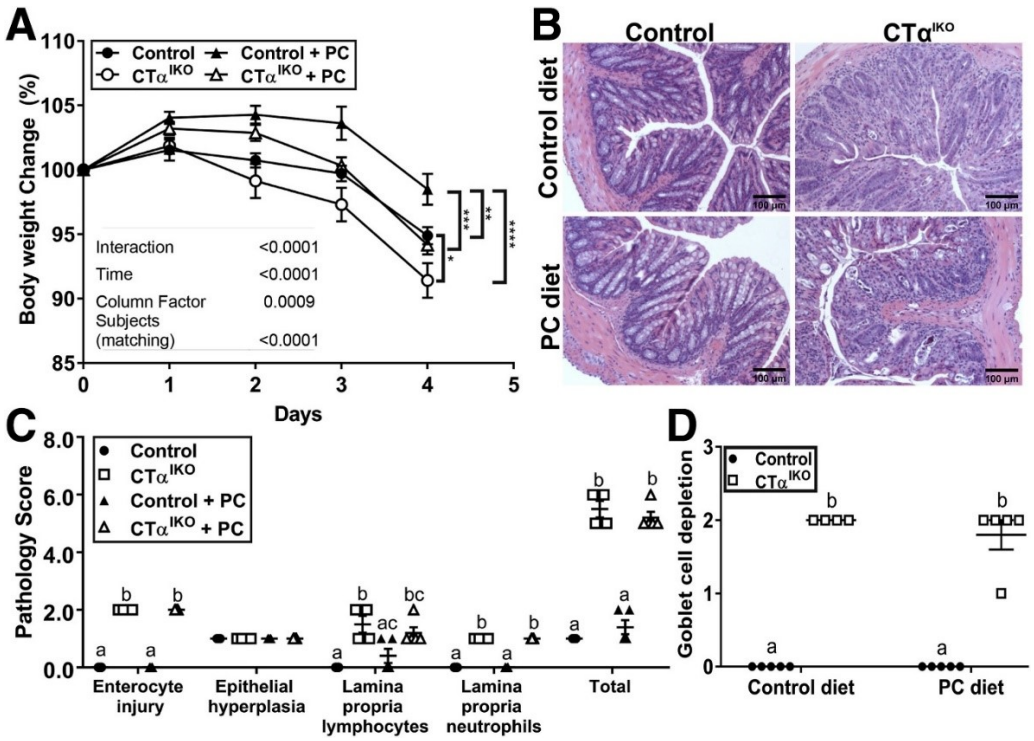

Figure 4.3: Dietary PC supplementation does not rescue body weight loss or colitis development in CTα^{IKO} mice. (A) Body weight change relative to body weight at the end of tamoxifen treatment in control mice and CTα^{IKO} mice treated either with or without supplementary PC in the diet (n = 6–7/group). (B) Representative H&E-stained distal colon sections in control mice and CTα^{IKO} mice treated either with or without supplementary PC in the diet. (C) Pathology scores for control mice and CTα^{IKO} mice treated either with or without supplementary PC in the diet (n = 4–5/group). (D) Goblet cell depletion score in control mice and CTα^{IKO} mice treated either with or without supplementary PC in the diet (n = 4–5/group). (A) ∗Statistical significance in body weight on day 4 after the end of tamoxifen treatment based on 2-way analysis of variance followed by the Tukey post hoc test. (C) Columns that do not share a letter (a, b, or c) are significantly different (α = .05). Values are means ± SEM. ∗$P < .05$, ∗∗$P < .01$,∗∗∗$P < .001$, and ∗∗∗∗$P < .0001$.

Regional analysis of colon Swiss rolls showed similarly high levels of enterocyte injury, epithelial hyperplasia, and lymphocyte infiltration to the lamina propria of the proximal compared to the distal colon of CTα^{IKO} mice, and pathology scores were also higher in the cecum of CTα^{IKO} mice compared to controls (Figure 4.4 A-C). While we initially hypothesized that the weight regain observed in CTα^{IKO} mice at day 5 after Cre induction (Figure 4.4 D) might be linked to improved metrics of disease severity, pathology scoring showed that colitis had not improved by day 7 (Figure 4.4 D), even after most CTα^{IKO} mice were a similar body weight to control mice. Assessment of colon histology 7 weeks after Cre induction showed improved disease pathology relative to 4 days and 7 days after Cre induction in CTα^{IKO} mice (Figure 4.4 E), with infrequent immune cell infiltration, absence of crypt abscesses, and partial restoration of goblet cells. Consistent with the restoration of goblet cells and improvement to the colonic epithelium after 7 weeks in CTα^{IKO} mice, mRNA levels of the goblet cell marker *Muc2* were comparable between groups (Figure 4.4 F) while *Tff3* levels tended to be modestly lower in CTα^{IKO} mice but did not reach statistical significance (P=0.055). The ER stress marker *Atf5* was significantly higher in the colons of CTα^{IKO} mice after 7 weeks, while *Ddit3 (*P=0.063*)*, *Atf4 (*P=0.127*)* and *Eif4bp1 (*P=0.161*)* also tended to be higher but did not reach statistical significance. These observations suggest that epithelial cells of CTα^{IKO} mice activate compensatory pathways to promote mucosal healing following the initial inflammatory response but remain modestly stressed relative to control mice.

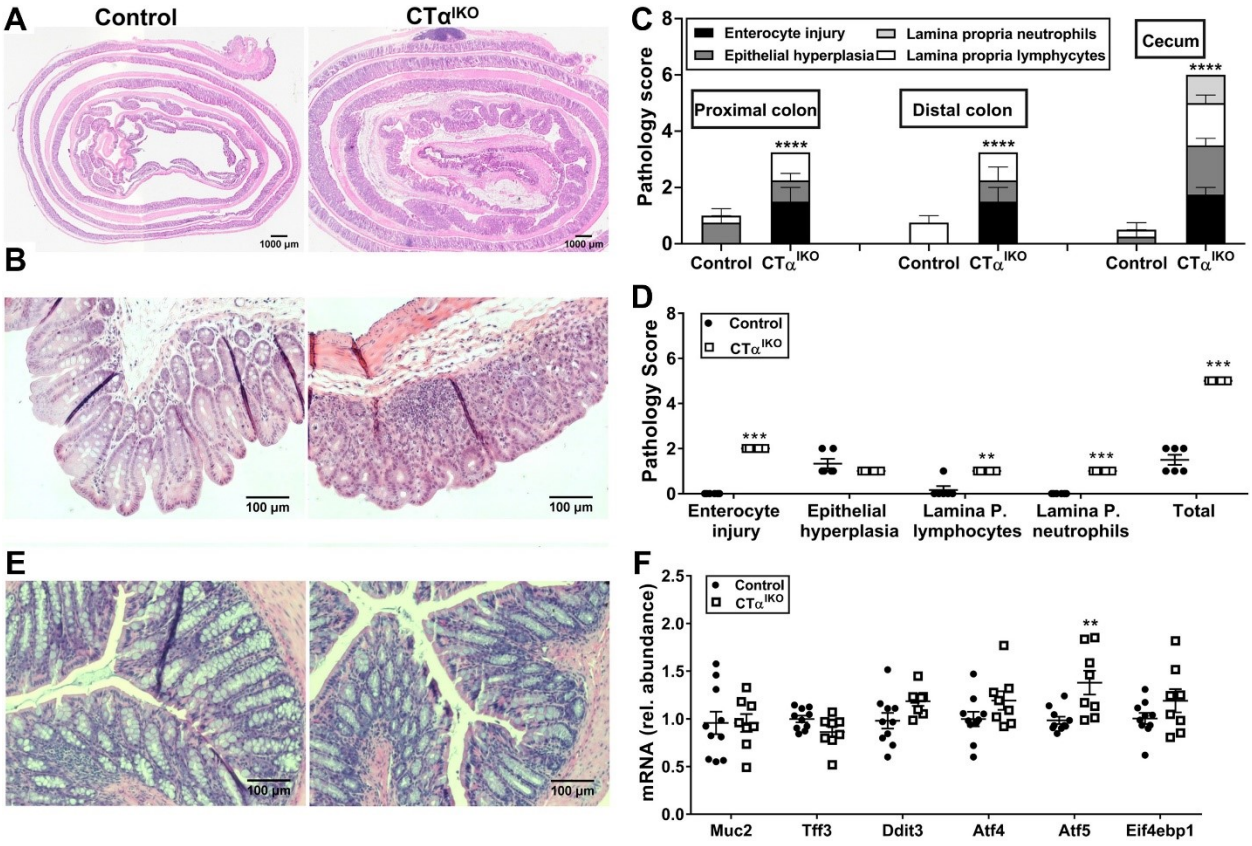

Figure 4.4: Acute inflammation extends to all parts of the colon and cecum in CTα^{IKO} mice but disease severity improves by 7 weeks after Cre induction. (A) Representative H&E-stained colon Swiss rolls from control mice and CTα^{IKO} mice 4 days after the end of tamoxifen treatment. (B) Representative H&E-stained cecum sections from control mice and CTα^{IKO} mice 4 days after the end of tamoxifen treatment. (C) Pathology scores in proximal colon Swiss roll sections, distal colon Swiss roll sections, and cecum sections of control mice and CTα^{IKO} mice 4 days after the end of tamoxifen treatment (n = 4/group). (D) Pathology scores of control mice and CTα^{IKO} mice 7 days after the end of tamoxifen treatment (n = 6–7/group). (E) Representative H&E-stained colon sections from control mice and CTα^{IKO} mice 7 weeks after the end of tamoxifen treatment. (F) The mRNA abundance of *Muc2*, *Tff3*, *Ddit3*, *Atf4*, *Atf5*, and *Eif4ebp1* in the colons of control mice and CTα^{IKO} mice 7 weeks after the end of tamoxifen treatment (n = 8–10/group). Values are means ± SEM. $**P < .01$, $***P < .001$, and $****P < .0001$. P., propria; rel., relative.

4.3.3 CTα^{IKO} mice acutely lose goblet cell mucus granules and have ultrastructural damage to theca in goblet cells

There was a marked decrease in Alcian Blue/Periodic Acid Schiff (AB/PAS) staining, which identifies goblet cell mucus granules, in the colons of CTα^{IKO} mice compared to control mice on day 4 after Cre induction (Figure 4.5 A). Electron microscopy showed that the ultra-structural integrity of mucus-containing theca in CTα-deficient goblet cells was compromised, with loss of mucus granules and infiltration of cellular debris (Figure 4.5 B). This atypical appearance of mucus granules has been observed previously in UC patients (Aachoui et al., 2013). Loss of mucus granules in CTα^{IKO} mice resulted in a significantly higher pathology score for goblet cell depletion compared to control mice (Figure 4.5 C).

The mRNA abundance of the goblet cell markers *Muc2* and *Tff3* were lower in the colons of CTα^{IKO} mice compared to control mice (Figure 4.5 D). However, the mRNA abundance of *Agr2* was not different between groups (Figure 4.5 D), which is a protein encoding gene for a disulfide isomerase that is expressed in Paneth cells and enteroendocrine cells in addition to goblet cells (Park et al., 2009). Furthermore, enteroendocrine cell markers (*Insl5* and *Sct*) were comparable between groups, except for *Neurog3*, which was higher in CTα^{IKO} mice (Figure 4.5 E). These data together suggest that goblet cells might be particularly sensitive to loss of CTα, possibly due to their high secretory activity (Heazlewood et al., 2008). Further consistent with the lower abundance of goblet cells, the mRNA levels of *Gfi1*, *Spdef*, and *Klf4*, which are involved in goblet cell maturation, were lower in the colons of CTα^{IKO} mice compared to control mice (Figure 4.5 F).

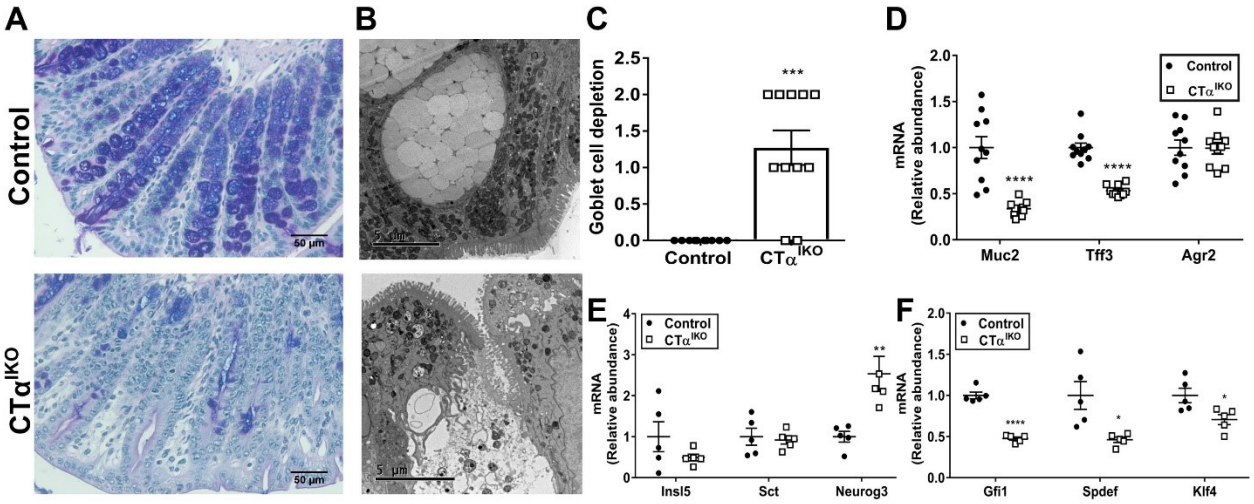

Figure 4.5: Loss of mucus granules and ultrastructural damage to theca in goblet cells of CTα^{IKO} mice. (A) Representative Alcian blue/periodic acid–Schiff staining in colons from control mice and CTα^{IKO} mice. (B) Representative transmission electron micrographs of goblet cell theca in the colons of control mice and CTα^{IKO} mice. (C) Pathology score for goblet cell depletion in the colons of control mice and CTα^{IKO} mice (n = 10–11/group). (D) The mRNA abundance of *Muc2*, *Tff3*, and *Agr2* in the colons of control mice and CTα^{IKO} mice (n = 10/group). (E) The mRNA abundance of enteroendocrine cell markers in the colons of control mice and CTα^{IKO} mice (n = 5/group). (F) The mRNA abundance goblet cell maturation factors in the colons of control mice and CTα^{IKO} mice (n = 5/group). Values are means ± SEM. ∗$P < .05$, ∗∗$P < .01$, and ∗∗∗$P < .001$, ∗∗∗∗$P < .0001$.

4.3.4 CTα^{IKO} mice have a thin mucus layer and enhanced intestinal permeability

The mucus layer of CTα^{IKO} mice was thinner than that of control mice (Figure 4.6 A), and electron microscopy showed that there was extensive damage to the apical brush border of colonic epithelial cells on day 4 after Cre induction (Figure 4.6 B). CTα^{IKO} mice also had a striking increase in intestinal permeability, as assessed by appearance of fluorescein isothiocyanate (FITC)-labeled dextran in circulation following oral administration (Figure 4.6 C). Altered expression of tight junction components is linked to elevated intestinal permeability in UC 19. Accordingly, CTα^{IKO} mice had lower abundance of *Cldn2* transcripts and higher abundance of *Cldn4* transcripts in the colon compared to control mice (Figure 4.6 D). Pyroptotic cell death is triggered in response to Gram-negative bacterial infections and thus plays a crucial role in anti-bacterial innate immune defense (Aachoui et al., 2013; Broz et al., 2012; Kayagaki et al., 2015, 2011; Rathinam et al., 2012). The mRNA levels of *Casp4* (encodes Caspase-11) and *Gsdmd* (encodes gasdermin D), which are markers of pyroptosis, were higher in colonic tissue of CTα^{IKO} mice (Figure 4.6 D), and mRNA levels of the cytosolic bacterial DNA sensor *Zbp1* were also strongly induced (Figure 4.6 D), suggesting that microbes had infiltrated the colonic epithelium after breakdown of the mucosal barrier.

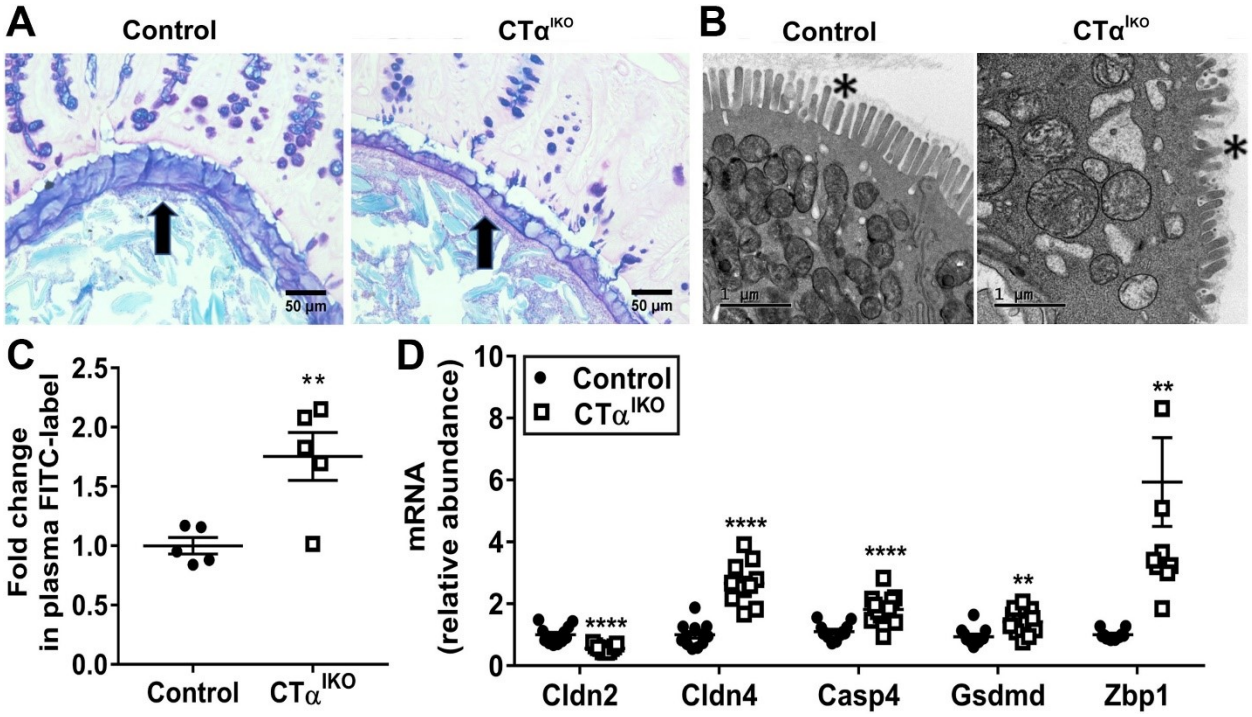

Figure 4.6: Impaired mucus layer integrity and increased intestinal permeability in response to impaired de novo PC synthesis in IECs. (A) Representative light microscope images of the colonic mucus layer (*arrow*) in control mice and CTα^{IKO} mice after Carnoy's fixation and Alcian blue/periodic acid–Schiff staining. (B) Representative electron micrographs of the colonic microvilli (*asterisk*) in control mice and CTα^{IKO} mice. (C) Relative fluorescence in plasma of control mice and CTα^{IKO} mice 2 hours after an oral gavage of FITC-labeled dextran (n = 5/group). (D) The mRNA abundance of *Cldn2*, *Cldn4*, *Casp4, Gsdmd*, and *Zbp1* in the colons of control mice and CTα^{IKO} mice (n = 10–12/group). Values are means ± SEM. $**P < .01$, $****P < .001$.

4.3.5 Antibiotics dampen inflammatory cytokine secretion but do not prevent colitis development in CTα^{IKO} mice

To comprehensively examine the interplay between microbes, the colonic epithelium, and the intestinal immune system of CTα^{IKO} mice, we sequenced the fecal microbiome and profiled colonic cytokine and chemokine concentrations under both untreated and antibiotic-treated conditions on day 4 after Cre induction. Antibiotic treatment reduced fecal bacterial loads in both control mice and CTα^{IKO} mice compared to untreated mice (12-18% decrease in 16S rDNA copy numbers; Figure 4.7 A). CTα^{IKO} mice had lower richness of the fecal microbiota compared to control mice as indicted by chao1 index, and antibiotic treatment ameliorated these differences between groups (Figure 4.7 B), in line with previous studies showing a decrease in gut microbial diversity in both humans and mice with UC (Qin et al., 2010; Wohlgemuth, Haller, Blaut, & Loh, 2009). Shannon index also indicated that loss of intestinal CTα tended to reduce richness and evenness of the microbiome, although this did not reach statistical significance (Figure 4.7 C). While there was a clear clustering of samples according to antibiotic treatment status (Figure 4.7 D), there was no difference in the beta-diversity between CTα^{IKO} mice and control mice under untreated (adinos, $R^2 = 0.703$, $P = 0.090$) or antibiotic-treated ($R^2 = 0.380$, $P = 0.822$) conditions. However, CTα^{IKO} mice showed a marked expansion of Proteobacteria, including the genus unclassified_*Enterobacteriaceae* (Table 4.5), which has previously been linked to intestinal inflammation (Lupp et al., 2007). Additionally, the genus *Akkermansia* was significantly increased, while anaerobic bacteria belonging to the family *Ruminococcaceae* were reduced, in CTα^{IKO} mice compared to control mice (Table 4.5). Antibiotic treatment prevented the expansion of *Enterobacteriaceae and Akkermansia* in CTα^{IKO} mice (Table 4.5). Thus, CTα^{IKO} mice have

changes to gut microbes that reflect increased intestinal inflammation including lower richness of fecal microbiome and increased abundance of *Enterobacteriaceae.*

Table 4.5: Relative abundance of predominant fecal bacterial phyla and genera under untreated or antibiotic-treated conditions[1].

	Control	CTα^{IKO}	Control + antibiotics	CTα^{IKO} + antibiotics	SEM	*P* value	FDR_*P* value
Phylum							
p__Actinobacteria	0.44	0.43	0.24	0.44	0.05	0.385	0.405
p__Bacteroidetes	41.12^{ab}	43.53^{a}	14.98^{c}	33.20^{bc}	2.14	0.001	0.001
p__Firmicutes	33.06^{b}	11.42^{c}	64.92^{a}	43.43^{ab}	3.43	<0.001	<0.001
p__Proteobacteria	11.18^{a}	16.79^{a}	10.25^{a}	7.74^{b}	0.98	0.003	0.003
p__Verrucomicrobia	13.05^{b}	26.35^{a}	0.46^{c}	0.06^{c}	2.03	<0.001	<0.001
p__Tenericutes	0.17^{c}	0.24^{bc}	1.54^{a}	0.66^{ab}	0.15	0.002	0.003
p__Deferribacteres	0.48^{b}	0.93^{a}	0.04^{c}	0.04^{c}	0.13	<0.001	<0.001
p__TM7	0.21^{a}	0.04^{b}	0.08^{ab}	0.20^{ab}	0.03	0.021	0.025
p__Spirochaetes	0.02^{b}	0.01^{c}	3.43^{a}	8.59^{a}	0.57	<0.001	<0.001
p__Lentisphaerae	ND^{b}	ND^{b}	0.42^{a}	1.00^{a}	0.12	0.008	0.012
Actinobacteria							
g__*Bifidobacterium*	0.33^{a}	0.22^{a}	ND^{b}	0.01^{b}	0.05	<0.001	<0.001
f_*Coriobacteriaceae*;g_	0.01^{b}	0.10^{ab}	0.06^{ab}	0.17^{a}	0.02	0.010	0.017
g__*Adlercreutzia*	0.10^{a}	0.10^{a}	ND^{b}	ND^{b}	0.01	<0.001	<0.001
g__*Collinsella*	ND^{b}	ND^{b}	0.16^{a}	0.22^{a}	0.02	<0.001	<0.001
Bacteroidetes							
o__*Bacteroidales*;f__;g__	5.74	4.17	2.97	6.84	0.48	0.086	0.120
g__*Bacteroides*	4.59^{b}	14.06^{a}	0.24^{c}	0.16^{c}	1.25	<0.001	<0.001
g__*Parabacteroides*	4.71^{a}	8.21^{a}	1.36^{b}	3.46^{ab}	0.82	0.002	0.004
g__*Prevotella*	0.08^{b}	0.03^{c}	5.72^{a}	11.93^{a}	0.83	<0.001	<0.001
g__*[Prevotella]*	1.41	2.48	1.63	3.60	0.37	0.202	0.220
f__*Rikenellaceae*;g__	7.21^{a}	7.00^{a}	0.05^{b}	0.01^{b}	0.65	<0.001	<0.001
g_*AF12*	0.33^{a}	0.19^{a}	ND^{b}	ND^{b}	0.04	<0.001	<0.001
f_*S24-7*;g_	10.60^{a}	3.71^{b}	1.16^{b}	3.58^{b}	0.77	<0.001	<0.001
g__*Odoribacter*	6.44^{a}	3.67^{a}	0.01^{b}	ND^{b}	0.57	<0.001	<0.001
Firmicutes							
f__*Bacillaceae*;g__	0.01	0.03	0.04	ND	0.01	0.093	0.128
g__*Enterococcus*	0.01^{b}	0.06^{ab}	0.35^{a}	0.59^{a}	0.04	<0.001	<0.001
g__*Lactobacillus*	1.50^{b}	1.43^{b}	1.76^{ab}	3.90^{a}	0.23	0.015	0.023
g__*Lactococcus*	1.12	0.16	45.62	3.92	3.62	0.054	0.078
g__*Turicibacter*	0.26^{a}	0.01^{b}	0.03^{b}	0.04^{b}	0.03	<0.001	<0.001
o__*Clostridiales*;f__;g__	13.36^{a}	3.67^{b}	3.53^{b}	7.05^{ab}	1.05	<0.001	0.001
f__*Christensenellaceae*;g__	0.01^{b}	0.01^{b}	1.02^{a}	2.50^{a}	0.17	<0.001	<0.001
f__*Clostridiaceae*;g__	0.02^{b}	0.14^{ab}	0.25^{a}	0.55^{a}	0.04	0.003	0.006
g__*Clostridium*	0.03^{b}	0.02^{b}	0.04^{ab}	0.10^{a}	0.01	0.015	0.024
g__*Dehalobacterium*	0.33^{a}	0.08^{a}	ND^{b}	0.02^{ab}	0.03	<0.001	<0.001
f__*Lachnospiraceae*;g__	1.80^{a}	0.64^{b}	0.84^{ab}	1.66^{a}	0.13	<0.001	0.001
g__*Coprococcus*	0.69^{a}	0.12^{b}	0.40^{ab}	0.63^{a}	0.06	<0.001	0.001
g__*Dorea*	0.21^{a}	0.03^{b}	0.21^{ab}	0.40^{a}	0.03	<0.001	<0.001
g__*[Ruminococcus]*	0.89^{a}	0.20^{b}	ND^{c}	ND^{c}	0.08	<0.001	<0.001
f__*Peptococcaceae*;g__	0.07^{a}	0.02^{ab}	ND^{b}	0.01^{b}	0.01	<0.001	<0.001
f__*Ruminococcaceae*;Other	1.21^{a}	0.53^{b}	0.01^{c}	0.01^{c}	0.10	<0.001	<0.001
f__*Ruminococcaceae*;g__	4.28^{b}	1.28^{c}	5.23^{abc}	11.17^{a}	0.67	<0.001	0.001
g__*Oscillospira*	5.31^{a}	1.23^{b}	0.56^{b}	1.41^{b}	0.40	<0.001	<0.001
g__*Ruminococcus*	0.94^{a}	0.40^{b}	0.36^{b}	0.63^{ab}	0.06	<0.001	0.001
g__*Megasphaera*	ND^{b}	0.01^{b}	0.31^{a}	0.78^{a}	0.05	<0.001	<0.001
g__*Anaerovorax*	0.04^{a}	0.04^{a}	ND^{b}	ND^{b}	0.00	<0.001	<0.001
f__*Erysipelotrichaceae*;g__	0.17	0.06	0.04	0.21	0.03	0.124	0.141
g__*Allobaculum*	0.70^{a}	1.13^{a}	0.01^{b}	ND^{b}	0.14	<0.001	<0.001
g__*[Eubacterium]*	ND^{b}	0.01^{b}	0.15^{a}	0.15^{a}	0.02	<0.001	<0.001

Proteobacteria							
o__*RF32*;f__:g__	0.59^{ab}	2.83^{a}	0.28^{b}	0.59^{ab}	0.53	0.013	0.020
g__*Sutterella*	0.02^{b}	3.25^{a}	3.40^{a}	0.20^{ab}	0.37	<0.001	<0.001
f__*Desulfovibrionaceae*;g__	8.46^{a}	0.65^{ab}	0.19^{b}	ND^{b}	0.78	<0.001	<0.001
g__*Desulfovibrio*	0.10	0.09	0.05	0.17	0.01	0.069	0.098
g__*Campylobacter*	0.01^{b}	ND^{b}	1.50^{a}	3.22^{a}	0.22	<0.001	<0.001
g__*Flexispira*	1.04	1.55	0.18	0.23	0.40	0.634	0.649
g__*Helicobacter*	0.38^{a}	0.05^{b}	0.23^{a}	0.25^{a}	0.04	0.001	0.001
f__*Enterobacteriaceae*;g__	0.16^{c}	6.88^{a}	2.75^{b}	0.64^{b}	0.76	0.001	0.003
Verrucomicrobia							
g__*Akkermansia*	13.05^{b}	26.35^{a}	0.42^{c}	0.02^{c}	2.03	<0.001	<0.001
Tenericutes							
f__*Mycoplasmataceae*;g__	0.03^{ab}	0.02^{ab}	1.31^{a}	ND^{b}	0.14	0.012	0.020
Deferribacteres							
g__*Mucispirillum*	0.48^{b}	0.93^{a}	0.04^{c}	0.04^{c}	0.13	<0.001	<0.001
TM7							
f__*F16*;g__	0.21^{a}	0.08^{b}	0.08^{ab}	0.20^{a}	0.03	0.021	0.032
Spirochaetes							
g__*Treponema*	0.01^{bc}	0.01^{c}	2.76^{ab}	7.28^{a}	0.48	<0.001	<0.001
Lentisphaerae							
f__*Victivallaceae*;g__	ND^{b}	ND^{b}	0.10^{a}	0.39^{a}	0.03	<0.001	<0.001
f__*R4-45B*;g	ND^{b}	ND^{b}	0.32^{a}	0.61^{a}	0.05	<0.001	<0.001

[1]The relative abundance data (%) are presented as mean ± pooled standard error of the mean (SEM). The non-parametric Kruskall Wallis test with the Dwass, Steel, Critchlow-Fligner multiple comparisons post-hoc procedure was used to compare the differences between treatment groups. The *P* value and false discovery rate (FDR)-adjusted *P* value are shown. ND, not detected. Control, n = 14; CTα^{IKO}, n = 11; Control + antibiotics, n = 5; CTα^{IKO} + antibiotics, n = 5. Means that do not share a common letter (a, b or c) are significantly different. α = 0.05.

We hypothesized that a thinner mucus layer allowed microbes to infiltrate the intestinal epithelium of CTα^{IKO} mice, and that lowering the abundance of gut microbes with antibiotics might reduce microbial infiltration and associated inflammation. The master inflammatory cytokines Interleukin (IL)-1β, IL-1α and Interferon-γ (IFNγ; *P*=0.06) were higher in the colons of untreated CTα^{IKO} mice compared to control mice, and antibiotics partially ameliorated their induction (Table 4.3). Furthermore, the pro-inflammatory factors granulocyte-macrophage colony-stimulating factor (GM-CSF) and leukemia inhibitory factor (LIF) were strongly induced, while monocyte chemoattractant protein-1 (MCP-1) and TNF-α also tended to be higher, in the colons of CTα^{IKO} mice compared to control mice (Table 4.6). Antibiotic treatment partially blunted LIF induction, did not influence the induction of GM-CSF or TNF-α (Table 4.3), and further stimulated the secretion of MCP-1 in CTα^{IKO} mice. Colonic concentrations of IL-10, and a panel of other cytokines and chemokines, were not different between groups (Table 4.6). Therefore, changes to

colonic PC concentrations induce the secretion of a specific array of inflammatory factors, and antibiotics can dampen the induction of some, but not all, of these factors. Notably, antibiotic treatment did not improve the loss of goblet cells (Figure 4.7 E) or histopathologic colitis scores in CTα^{IKO} mice (Figure 4.7 F) suggesting that, while luminal bacteria can exacerbate inflammation in CTα^{IKO} mice, non-bacterial inflammatory stimuli (e.g. inflammatory signaling cascades initiated within the IECs as a result of perturbations to membrane phospholipid composition) are likely the primary drivers of inflammation in these mice.

Table 4.6: Colonic cytokines and chemokines in control and CTα^{IKO} mice with and without antibiotics[1,2].

Protein Name	Control (pg/mg protein)	CTα^{IKO} (pg/mg protein)	Control + Antibiotics (pg/mg protein)	CTα^{IKO} + Antibiotics (pg/mg protein)	*P Genotype*	*P Treatment*	*P Interaction*
IL-1β	3.00 ± 1.45^{a}	24.49 ± 6.32^{b}	5.87 ± 1.42^{a}	13.27 ± 1.72^{ab}	0.0002	0.18	0.03
IL-1α	12.53 ± 4.09^{a}	32.98 ±4.64^{b}	10.52 ± 3.53^{a}	23.41 ± 4.48^{ab}	0.001	0.19	0.38
IFNγ	0.88 ± 0.06	2.86 ± 0.88	1.31 ± 0.36	2.27 ± 0.49	0.009	0.87	0.32
GM-CSF	1.02 ± 0.1^{a}	2.23 ± 0.26^{b}	1.02 ± 0.1^{a}	4.64 ± 0.44^{b}	<0.0001	0.001	0.001
LIF	1.14 ± 0.33^{a}	20.69 ± 7.69^{b}	1.38 ± 0.42^{a}	8.44 ± 1.25^{ab}	0.001	0.08	0.07
MCP-1	4.68 ± 1.71^{a}	10.23 ± 1.72^{ab}	5.18 ± 1.19^{a}	15.6 ± 2.32^{b}	0.0005	0.12	0.2
TNF-α	0.35 ± 0.09^{a}	0.78 ± 0.17^{ab}	0.35 ± 0.06^{a}	0.96 ± 0.13^{b}	0.0004	0.39	0.53
IL-10	3.82 ± 1.42	6.04 ± 1.07	5.27 ± 1.4	7.15 ± 0.94	0.12	0.32	0.89
IL-2	2.26 ± 0.69^{a}	0.78 ± 0.21^{ab}	0.44 ± 0.21^{b}	0.29 ± 0.11^{b}	0.06	0.01	0.12
IL-3	0.46 ± 0.23	0.66 ± 0.21	0.68 ± 0.16	0.83 ± 0.12	0.53	0.46	0.8
IL-5	0.29 ± 0.12	0.38 ± 0.07	0.22 ± 0.07	1.42 ± 0.88	0.19	0.32	0.26
IL-6	1.37 ± 0.54	1.88 ± 0.54	1.48 ± 0.4	2.29 ± 0.29	0.16	0.57	0.74
IL-9	16.03 ± 8.58	3.23 ± 2.88	0.50 ± 0.13	0.30 ± 0.1	0.19	0.07	0.2
IL-12 (p40)	6.40 ± 1.18	6.81 ± 2.23	7.12 ± 1.16	12.00 ± 2.94	0.21	0.16	0.29
IL-12 (p70)	2.41 ± 1.29	5.19 ± 1.42	4.08 ± 0.79	6.99 ± 2.15	0.08	0.27	0.97
MIP-1α	7.00 ± 2.81	12.29± 2.04	8.07 ± 1.44	12.95 ± 1.85	0.03	0.69	0.92
MIP-2	32.40 ± 4.48	50.31± 5.72	38.48 ± 3.14	63.55 ± 16.13	0.04	0.32	0.71
RANTES	1.60 ± 0.77	1.24 ± 0.43	0.93 ± 0.27	0.65 ± 0.10	0.51	0.2	0.94
G-CSF	0.85 ± 0.09	1.29 ± 0.22	0.96 ± 0.07	1.97 ± 0.52	0.03	0.2	0.35
M-CSF	1.63 ± 0.72	5.78 ± 3.02	1.08 ± 0.26	3.03 ± 1.11	0.051	0.27	0.46
Eotaxin	4.47 ± 2.62	3.94 ± 3.47	1.25 ± 0.79	2.10 ± 0.82	0.94	0.24	0.75

[1]Data are mean ± SEM. A 2-way ANOVA was used with Tukey's post-test was used. Columns that do not share a letter (a or b) are significantly different (α=0.05).
[2]Samples that were lower than the limit of detection were assigned a value of half the limit of detection (the lowest point obtained from the standard curve).

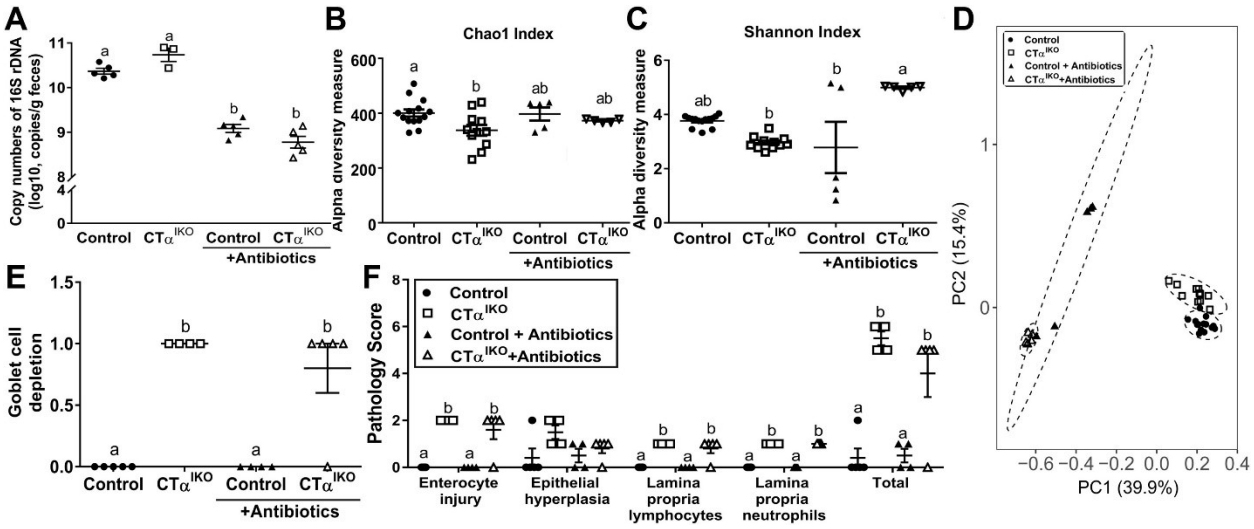

Figure 4.7: Loss of intestinal CTα changes the microbiome but depletion of gut microbes with antibiotics does not prevent colitis development in CTα^{IKO} mice. (A) Copy number of 16S rRNA in feces of control mice and CTα^{IKO} mice treated with and without antibiotics (n = 3–5/group). (B) Chao1 and (C) Shannon indexes of gut microbiota from control mice and CTα^{IKO} mice treated with and without antibiotics (control, n = 14; CTα^{IKO}, n = 11; control + antibiotics, n = 5; CTα^{IKO} + antibiotics, n = 5). (D) Principle component analysis plots of the bacterial communities based on the Bray–Curtis distance matrix. Each point represents an individual mouse (control, n = 14; CTα^{IKO}, n = 11; control + antibiotics, n = 5; CTα^{IKO} + antibiotics, n = 5). (E) Pathology score for goblet cell depletion in the colons of control mice and CTα^{IKO} mice treated with and without antibiotics (n = 4–5/group). (F) Pathology scores of control mice and CTα^{IKO} mice treated with and without antibiotics (n = 4–5/group). Columns that do not share a letter (a, b, or c) are significantly different (α = .05).

4.3.6 PC depletion in IECs leads to ER stress and UPR activation

Electron microscopy showed that the ER in colonic IECs of CTα^{IKO} mice appeared dilated and distended (Figure 4.8 A). The unfolded protein response (UPR) can be activated independently of unfolded proteins in the ER by perturbations to membrane lipid composition resulting in lipid bilayer stress (Halbleib et al., 2017; Ho et al., 2020; Thibault et al., 2012; Volmer, Van Der Ploeg, & Ron, 2013). Accordingly, both mRNA (Figure 4.8 B) and protein levels (Figure 4.8 C-D) of spliced X-Box Binding Protein 1 (XBP1), the major downstream target of the UPR initiator Inositol-requiring enzyme 1-α (IRE1α), were higher in the colons of CTα^{IKO} mice compared to control mice. Furthermore, protein levels of the ER stress sensors Protein kinase R-like ER kinase (PERK) and Activating transcription factor 6 (ATF6) were significantly elevated in the colons of CTα^{IKO} mice (Figure 4.8 C-D). The mRNA levels the downstream UPR targets *Ddit3, Atf4*, *Atf5*, *Eif4ebp1* were also robustly higher in the colons of CTα^{IKO} mice (Figure 4.8 B and E), while *Hspa5* levels were higher in some, but not all, experiments (Figure 4.8 B and I). Furthermore, colonic epithelial cells of CTα^{IKO} mice had higher P62 protein levels than those of control mice (Figure 4.8 F-G) and contained numerous autophagic vesicles (Figure 4.8 H), as has been reported previously during pathological UPR activation (Lee et al., 2012). Thus, perturbations to membrane phospholipid composition results in ER stress and activation of the UPR in IECs of CTα^{IKO} mice.

We next treated mice with PBA, a chemical chaperone that alleviates ER stress driven by misfolded protein accumulation but that fails to alleviate ER stress arising due to perturbations to membrane lipid composition (Cao et al., 2013; Ho et al., 2020; Tam et al., 2018). PBA failed to improve total pathology scores (Figure 4.8 I), goblet cell depletion (Figure 4.8 J), or the colonic ER stress markers sXBP1, *Ddit3* or *Hspa5* (Figure 4.8 K) in CTα^{IKO}. PBA also further increased the levels of GM-CSF, lowered IL-10 and IL-12(p40) concentrations and did not affect colonic

TNFα or LIF in CTα^{IKO} mice compared to control mice (Table 4.7). However, colonic IL-1β concentrations, which were 10-fold higher in untreated CTα^{IKO} mice compared to control mice, were normalized with PBA treatment (Table 4.7). Furthermore, MCP-1 concentrations, which were elevated in CTα^{IKO} mice compared to controls, were equalized between groups after PBA treatment (Table 4.7). Surprisingly, PBA treatment completely prevented the increase in colon weight observed in untreated CTα^{IKO} mice (Figure 4.8 L). Together, these data show that PBA is largely ineffective at improving colitis pathology in CTα^{IKO} mice, which is consistent with a primary role for lipid bilayer stress, as opposed to protein misfolding, in the initiation of ER stress in CTα^{IKO} mice. However, impaired protein folding, likely occurring secondary to changes in ER function, appears to exacerbate inflammation after perturbation to IEC phospholipid composition because PBA treatment blunted the induction of IL-1β and MCP-1 in the colons of CTα^{IKO} mice.

Table 4.7: Colonic cytokines and chemokines in control and CTα^{IKO} mice with and without PBA[1,2].

Protein	Control (pg/mg protein)	CTα^{IKO} (pg/mg protein)	Control + PBA (pg/mg protein)	CTα^{IKO} + PBA (pg/mg protein)	*P Genotype*	*P Treatment*	*P Interaction*
IL-1β	6.94 ± 2.93^{a}	48.86±15.41^{b}	9.16 ± 4.35^{a}	8.32 ± 2.92^{a}	0.03	0.4	0.2
MCP-1	2.13 ± 1.28^{a}	12.02 ± 2.4^{b}	6.57 ± 3.24^{a}	6.88 ± 2.3^{a}	0.04	0.88	0.05
GM-CSF	0.58 ± 0.0^{a}	2.53 ± 0.75^{a}	0.87 ± 0.29^{a}	5.13 ± 0.6^{b}	<0.0001	0.01	0.04
IL-10	2.86 ± 0.97^{ab}	6.26 ± 1.37^{a}	6.74 ± 0.44^{a}	2.21 ± 0.41^{b}	0.57	0.93	0.002
IL-12 (p40)	5.33 ± 2.28^{ab}	8.31 ± 2.80^{ab}	12.50 ± 0.78^{a}	1.38 ± 0.45^{b}	0.07	0.95	0.006
TNF-α	0.28 ± 0.13^{a}	1.75 ± 0.49^{b}	0.45 ± 0.17^{a}	1.86 ± 0.25^{b}	0.0004	0.63	0.91
LIF	0.71 ± 0.3^{a}	7.45 ± 1.65^{b}	0.58 ± 0.12^{a}	5.27 ± 0.53^{b}	<0.0001	0.23	0.28
IL-12 (p70)	0.64 ± 0.48	4.17 ± 2.19	6.41 ± 3.18	0.1 ± 0.02	0.41	0.61	0.01
IL-6	0.85 ± 0.41	2.18 ± 0.70	2.76 ± 1.12	1.18 ± 0.33	0.84	0.47	0.04
IL-2	2.88 ± 0.66	2.11 ± 0.66	1.52 ± 0.68	4.21 ± 0.64	0.18	0.59	0.03
IL-3	0.24 ± 0.15	0.53± 0.19	0.73 ± 0.31	0.09 ± 0.01	0.34	0.88	0.02
M-CSF	0.43 ± 0.22	1.36 ± 0.33	0.94 ± 0.38	0.52 ± 0.02	0.35	0.54	0.03
MIP-1α	2.99 ± 0.75	4.07 ± 0.92	2.44 ± 1.01	5.36 ± 0.42	0.03	0.65	0.27
Eotaxin	5.07 ± 2.51	22.15 ± 10.87	0.61 ± 0.26	19.3 ± 3.73	0.01	0.56	0.9
IFNγ	0.41 ± 0.08	0.96 ± 0.19	0.77 ± 0.37	0.62 ± 0.10	0.29	0.98	0.07
G-CSF	1.23 ± 0.33	1.91 ± 0.43	1.07 ± 0.25	1.50 ± 0.23	0.13	0.42	0.72
IL-1α	20.14 ± 1.90	26.57 ± 2.89	22.04 ± 7.77	25.99 ± 5.38	0.26	0.88	0.78
IL-5	0.38 ± 0.12	0.20 ± 0.09	0.08 ± 0.05	0.28 ± 0.10	0.92	0.32	0.08
IL-9	16.91 ± 4.77	8.00 ± 2.83	11.62 ± 9.76	18.17 ± 6.27	0.84	0.68	0.21
MIP-2	28.70 ± 8.55	59.90 ± 11.34	42.53 ± 3.31	41.02 ± 1.86	0.15	0.98	0.11
RANTES	1.57 ± 0.87	2.73 ± 0.92	1.22 ± 0.21	2.26 ± 0.53	0.18	0.65	0.99

[1]Data are mean ± SEM. A 2-way ANOVA with Tukey's post-test was used. Columns that do not share a letter (a or b) are significantly different (α=0.05).
[2]Samples that were lower than the limit of detection were assigned a value of half the limit of detection (the lowest point obtained from the standard curve)

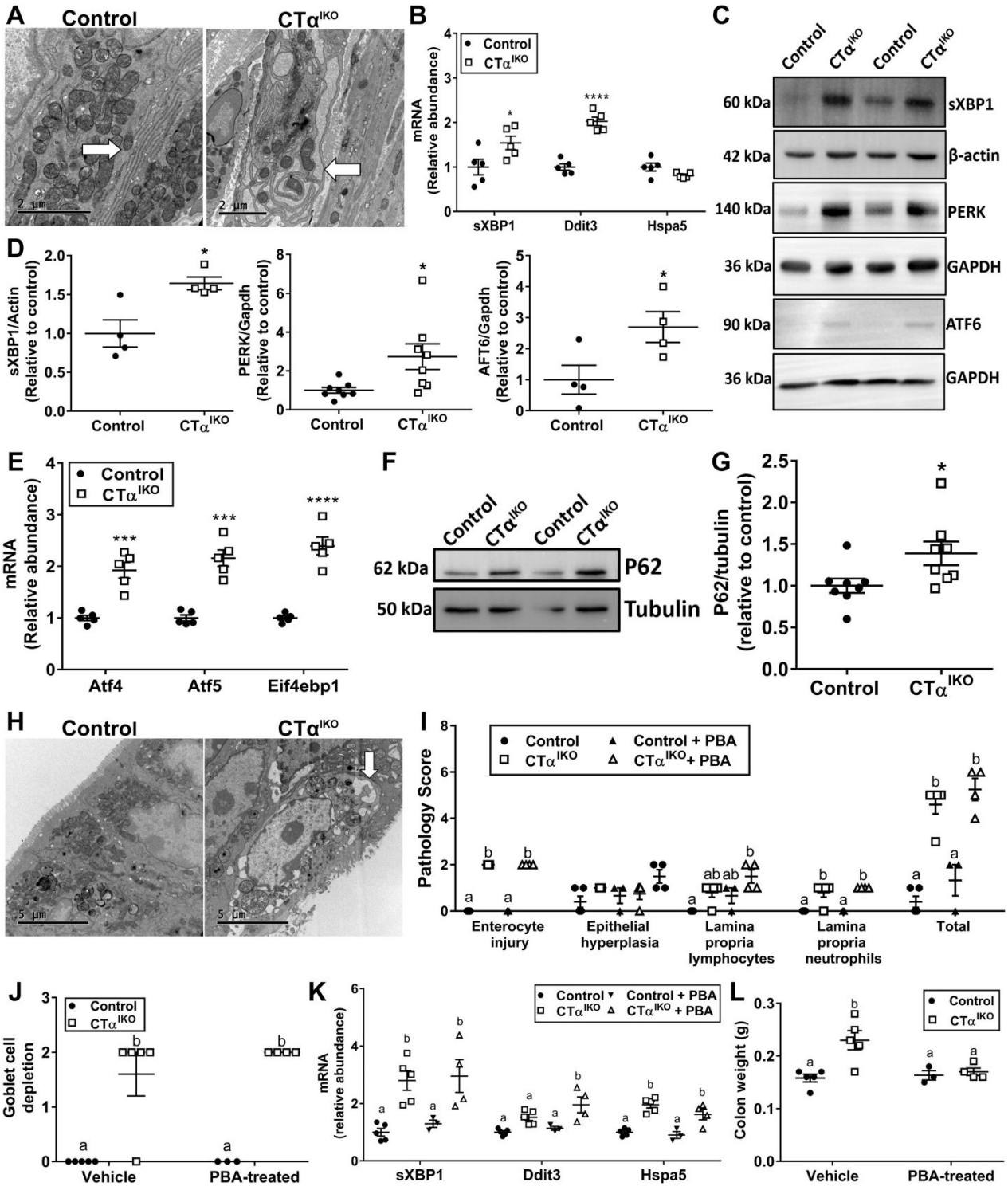
A
Control
CTα^IKO
2 μm
B
Control
CTα^IKO
mRNA (Relative abundance)
sXBP1
Ddit3
Hspa5
C
Control
CTα^IKO
Control
CTα^IKO
60 kDa
sXBP1
42 kDa
β-actin
140 kDa
PERK
36 kDa
GAPDH
90 kDa
ATF6
36 kDa
GAPDH
D
sXBP1/Actin (Relative to control)
PERK/Gapdh (Relative to control)
AFT6/Gapdh (Relative to control)
Control
CTα^IKO
E
mRNA (Relative abundance)
Atf4
Atf5
Eif4ebp1
F
62 kDa
P62
50 kDa
Tubulin
G
P62/tubulin (relative to control)
H
5 μm
I
Control
CTα^IKO
Control + PBA
CTα^IKO+ PBA
Pathology Score
Enterocyte injury
Epithelial hyperplasia
Lamina propria lymphocytes
Lamina propria neutrophils
Total
J
Goblet cell depletion
Vehicle
PBA-treated
K
mRNA (relative abundance)
sXBP1
Ddit3
Hspa5
L
Colon weight (g)
Vehicle
PBA-treated

Figure 4.8: Phosphatidylcholine depletion in IECs leads to ER stress and UPR activation. (A) Representative electron micrographs of the ER in colonic epithelial cells of control mice and CTα^{IKO} mice. *Arrow* indicates ER. (B) The mRNA abundance of sXBP1, *Ddit3*, and *Hspa5* in the colons of control mice and CTα^{IKO} mice (n = 5/group). (C) Representative Western blots and (D) quantification of spliced XBP1, PERK, and ATF6 relative to loading controls in the colons of control mice and CTα^{IKO} mice (n = 4–8/group). (E) The mRNA abundance of *Atf4*, *Atf5*, and *Eif4ebp1* in the colons of control mice and CTα^{IKO} mice (n = 5/group). (F) Representative Western blot and (G) quantification of P62 relative to tubulin in the colons of control mice and CTα^{IKO} mice (n = 8/group). (H) Representative electron micrographs of autophagic vesicles in colonic epithelial cells of control mice and CTα^{IKO} mice. *Arrow* indicates autophagosome. (I) Pathology scores for control mice and CTα^{IKO} mice treated with and without PBA (n = 3–5/group). (J) Goblet cell depletion scores for control mice and CTα^{IKO} mice treated with and without PBA (n = 3–5/group). (K) The mRNA abundance of sXBP1, *Ddit3*, and *Hspa5* in the colons of control mice and CTα^{IKO} mice treated with and without PBA (n = 3–5/group). (L) Colon weight of control mice and CTα^{IKO} mice treated with and without PBA (n = 3–5/group). Values are means ± SEM. (*I–L*) Columns that do not share a letter (a or b) are significantly different (α = .05). ∗$P < .05$, ∗∗∗$P < .001$, and ∗∗∗∗$P < .0001$. GAPDH, glyceraldehyde-3-phosphate dehydrogenase; sXBP1, spliced XBP1.

4.3.7 ER stress induced by altered membrane lipid composition drives IEC necroptosis

Terminal deoxynucleotidyl transferase dUTP nick end (TUNEL) staining, which detects DNA fragmentation caused by apoptosis, necroptosis or pyroptosis, was more intense in the colons of CTα^{IKO} mice compared to control mice (Figure 4.9 A-C). TUNEL staining was especially prominent in colonic crypts, which are typically filled with goblet cells (Figure 4.9 C). Pyroptotic cell death induced after microbial invasion of IECs likely contributes to TUNEL-positive staining in the colons of CTα^{IKO} mice (Figure 4.9 D). However, since makers of pyroptosis were only modestly elevated in CTα^{IKO} mice, we investigated whether other forms of cell death were involved in the loss of goblet cells prior to microbial invasion of the epithelium. Surprisingly, protein levels of both cleaved caspase-3 and cleaved caspase-8, critical mediators of apoptosis, tended to be lower in the colons of most CTα^{IKO} mice compared to controls (Figure 4.9 E-G). Furthermore, mRNA levels of the anti-apoptotic (pro-survival) factors *Birc5* (encodes Survivin) and *Ccnd1* (encodes Cyclin D1) were higher in the colons of CTα^{IKO} mice compared to controls

(Figure 4.9 H). These data suggest that apoptosis is not a major contributor to TUNEL-positive staining in the colons of CTα^{IKO} mice.

Necroptosis, a form of programmed cell death that shares the subcellular characteristics of necrosis, is negatively regulated by caspases, and is initiated by RIP3 (encoded by *Ripk3*), has recently been implicated in human IBD (Pierdomenico et al., 2014; Wang et al., 2020). Hallmarks of necroptosis were immediately evident in IECs of CTα^{IKO} mice by electron microscopy (Figure 4.9 I), including swollen mitochondria and ER, disrupted plasma membranes, and many cytoplasmic vacuoles. The lack of chromatin condensation in the nuclei of cells with swollen mitochondria further suggested that the cells were necroptotic as opposed to apoptotic (Günther, Neumann, Neurath, & Becker, 2013). Importantly, the mRNA and protein levels of the major mediator of necroptosis, RIP3, were strongly induced in the colons of CTα^{IKO} mice compared to control mice (Figure 4.9 J-L). Swollen mitochondria and cytoplasmic vacuoles were prominent in cells containing damaged mucus granules (Figure 4.9 I). Together, these data show that phospholipid imbalance in IECs induces an ER stress response that promotes IEC death by necroptosis, loss of barrier function, microbial infiltration, and spontaneous colitis in mice.

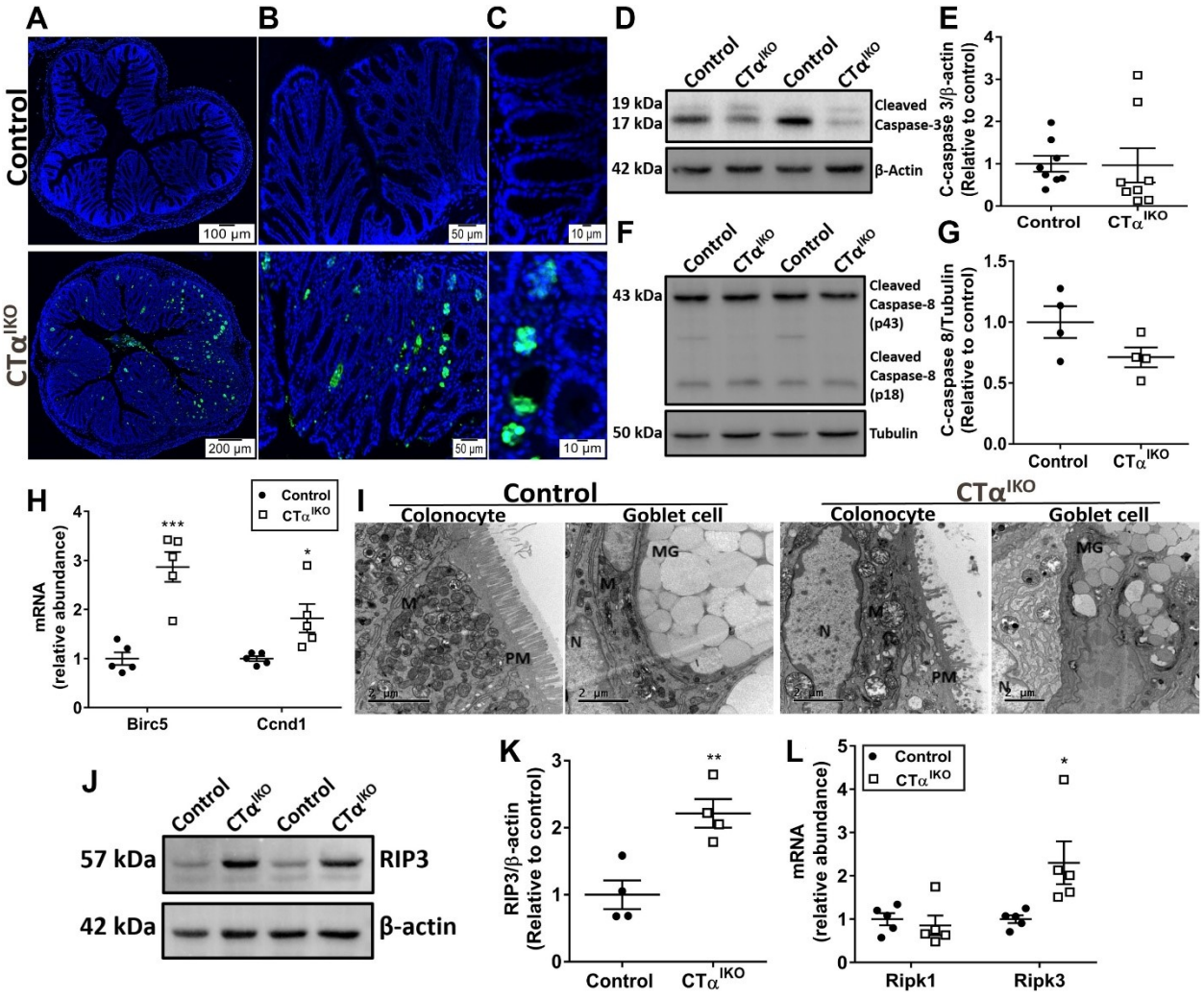

Figure 4.9: ER stress induced by altered membrane lipid composition drives IEC necroptosis in CTαIKO mice. (*A–C*) Representative TUNEL staining in the colons of control mice and CTα^{IKO} mice. (*D*) Representative Western blot of cleaved caspase-3 and (*E*) quantification of cleaved caspase-3 relative to β-actin in the colons of control mice and CTα^{IKO} mice (n = 4/group). (*F*) Representative Western blot of cleaved caspase-8 and (*G*) quantification of cleaved caspase-8 relative to tubulin in the colons of control mice and CTα^{IKO} mice (n = 8/group). (*H*) The mRNA abundance of *Birc5* and *Ccnd1* in the colons of control mice and CTα^{IKO} mice (n = 5/group). (*I*) Representative electron micrographs of necroptotic features in colonocytes and goblet cells of CTα^{IKO} mice compared with control mice. Note swollen mitochondria (M), ruptured plasma membrane (PM), and nuclei (N) without chromatin condensation. (*J*) Representative Western blot of RIP3 and (*K*) quantification of RIP3 in the colons of control mice and CTα^{IKO} mice (n = 4/group). (*L*) The mRNA abundance of *Ripk1* and *Ripk3* in the colons of control mice and CTα^{IKO} mice (n = 5/group). Values are means ± SEM. ∗P < .05, ∗∗P < .01, ∗∗∗P < .001, and ∗∗∗∗P < .0001.

4.4 Discussion

The present study shows that disturbances to membrane phospholipid composition in IECs leads to an ER stress response that drives spontaneous colitis in mice. The spontaneous nature of colitis development in CTα^{IKO} mice (i.e. in the absence of aggravating factors such as dextran sodium sulfate) points to a particularly important role for membrane PC content in maintaining gut barrier function. CTα^{IKO} mice experience severe colonic ER stress that results in IEC necroptosis, a form of cell death that has recently been implicated in human IBD (Günther et al., 2011; Negroni et al., 2017; Pierdomenico et al., 2014; Wang et al., 2020). IEC death is linked to goblet cell depletion, crypt abscess formation, elevated intestinal permeability, microbial invasion of the intestinal epithelium, immune cell infiltration, and augmented inflammatory cytokine secretion in CTα^{IKO} mice. Thus, phospholipid disequilibrium in murine IECs leads to a form of colitis that closely resembles that seen in humans (Khor et al., 2011). An important role for PC in maintaining goblet cell function correlates with human clinical observations, as UC patients typically have low colonic PC concentrations with goblet cell mucus granule depletion (Braun et al., 2009; Ehehalt et al., 2004; Strugala et al., 2008).

Impaired ER homeostasis can arise for many reasons including viral infections, drug toxicity, impaired calcium or redox balance, and lipid accumulation or depletion (Hetz, 2012; Ho et al., 2018). Eukaryotes have evolved the UPR to relay information regarding stressful conditions in the ER to the nucleus where adaptive changes to cellular gene expression or cell fate decisions occur (Hetz, 2012). Accumulation of unfolded or misfolded proteins in the lumen of the ER is a well-characterized activator of the UPR, as its name suggests (Hetz, 2012). In the colon, accumulation of misfolded MUC2 promotes ER stress and UC-like inflammation in mice(Heazlewood et al., 2008). Furthermore, slowing the accumulation of misfolded proteins with

the chemical chaperones PBA or tauroursodeoxycholic acid reduces colitis severity in several genetically modified mouse models (Cao et al., 2013). In addition to misfolded protein accumulation, the UPR can be activated directly by perturbations to membrane lipid composition resulting in lipid bilayer stress (Halbleib et al., 2017; Ho et al., 2020; Volmer et al., 2013). Our experiments show that perturbations to IEC phospholipid composition lead to ultrastructural changes to the ER, activation of PERK, ATF6 and IRE1α, and colitis development. These data highlight the physiological consequences of lipid bilayer stress in the colon. The ER stress sensors IRE1α and PERK sense unfolded proteins with their luminal domains but sense perturbations to membrane lipid composition by an alternative mechanism involving their transmembrane domains (Ho et al., 2020; Volmer et al., 2013). Furthermore, the UPR activator ATF6 responds to proteotoxic and lipotoxic stress by distinct mechanisms (Tam et al., 2018). Importantly, PBA is largely ineffective at resolving the form of ER stress that arises due to perturbations to membrane lipid composition (Ho et al., 2020; Tam et al., 2018). PBA did not improve colitis scores or rescue goblet cell depletion in $CT\alpha^{IKO}$ mice, consistent with a primary role for lipid bilayer stress in colitis development after IEC PC depletion. However, the accumulation of misfolded proteins secondary to changes in ER structure might exacerbate inflammation in $CT\alpha^{IKO}$ mice because PBA ameliorated the induction of a subset of pro-inflammatory cytokines including IL-1β. PBA treatment also prevented the increase in colon mass observed in $CT\alpha^{IKO}$ mice, an effect that might be linked to its lowering of colonic levels of the pro-survival chemokine MCP-1 (McClellan et al., 2012). Anti-hypertrophic effects of PBA have been reported previously in pressure overload-induced myocardial hypertrophy (Luo, Chen, & Wang, 2015).

Consistent with our results highlighting the importance of maintaining membrane lipid equilibrium in IECs, pancreatic beta cells loaded with saturated fatty acids, macrophages loaded

with cholesterol, and the livers of mice fed a high fat diet have high levels of ER stress (Cunha et al., 2013; Feng et al., 2003; Fu et al., 2011). Lipids that increase membrane saturation (i.e. reduce membrane fluidity), in particular, have been shown to promote UPR activation (Ariyama, Kono, Matsuda, Inoue, & Arai, 2010; Cunha et al., 2013; Kitai et al., 2013; Volmer et al., 2013). For example, loading cells with palmitate increases the content of saturated fatty acid-containing phospholipids in cellular membranes before induction of ER stress response pathways and cell death (Borradaile et al., 2006). Similarly, CTα^{IKO} mice have a lower ratio of PC:PE in IEC membranes (a change predicted to increase membrane saturation) relative to control mice, which is linked to a strong induction of the UPR and cell death. Overexpression of spliced XBP1 in fibroblasts increases CTα activity and PC synthesis, linking XBP1 to PC synthesis for membrane expansion during ER stress (Sriburi et al., 2004). In CTα^{IKO} mice, impaired PC synthesis triggers XBP1 splicing, but CTα deficiency in these mice does not allow subsequent membrane expansion. The uncontrolled inflammation that develops in CTα^{IKO} mice highlights the importance of PC synthesis for the prevention and resolution of colonic ER stress. Although immune cell-derived cytokines likely exacerbate ER stress in CTα^{IKO} mice 49, the use of a villin promotor traces colonic pathology in CTα^{IKO} mice to IECs (Zhang & Kaufman, 2008). Furthermore, while our data show that dietary phospholipid treatment might not be a viable way to improve colitis in CTα^{IKO} mice, likely due to phospholipid absorption in the proximal small intestine, future studies should examine the effects of local phospholipid delivery on ER stress and associated inflammation in UC.

We found using H&E staining, Alcian Blue staining, electron microscopy, qPCR of goblet cell markers, and assessment of the mucus layer that CTα^{IKO} mice lose goblet cells from the colonic epithelium by 4 days following Cre induction. A primary reason for this loss of goblet cells appears

to be induction of IEC necroptosis, as indicated by TUNEL-positive staining localized to colonic crypts, electron micrographs of necroptotic goblet cells, and the induction of RIP3 without induction of cleaved caspase-3 in colonic epithelial cells of CTα^{IKO} mice. Pyroptosis might also contribute to IEC death following breakdown of the mucosal barrier and microbial infiltration of the epithelium because mRNA levels of *Casp4* and *Gsdmd* were modestly elevated in the colons CTα^{IKO} mice (Aachoui et al., 2013; Broz et al., 2012; Kayagaki et al., 2015, 2011; Rathinam et al., 2012). It has traditionally been reported that unresolved ER stress leads to cell death by activation of the intrinsic mitochondrial apoptotic pathway (Hetz, 2012). However, it is increasingly appreciated that severe ER stress can also induce necroptosis (Saveljeva, Laughlin, Vandenabeele, Samali, & Bertrand, 2015; Zhu et al., 2018). Importantly, necroptosis is active in human IBD (Günther et al., 2011; Negroni et al., 2017; Pierdomenico et al., 2014; Wang et al., 2020). It should be noted that goblet cells are not the only IEC type to undergo necroptosis in the colons CTα^{IKO} mice because electron microscopy also showed necroptotic features in IECs without mucus granules. However, loss of goblet cells is predicted to be particularly detrimental to mucosal barrier function due to their role in mucus production and secretion of various protective factors including TFF3 (Johansson & Hansson, 2016). Electron microscopy of goblet cells that remained in the colons of CTα^{IKO} mice showed that the integrity of mucus-containing theca appeared compromised, with loss of mucus granule structure and infiltration of cellular debris and vacuoles. Reasons for subcellular damage to goblet cell theca in CTα^{IKO} mice might include impaired intracellular membrane integrity or swelling of organelles upon initiation of necroptosis. It is conceivable that impairments to ER function might be especially detrimental to goblet cells because they must continuously produce, fold, and secrete massive quantities of the MUC2 glycoprotein, as has been suggested previously (Halbleib et al., 2017). Furthermore, CTα^{IKO} mice

appear unable to replace mature goblet cells due to transcriptional repression of the goblet cell maturation factors *Gfi1*, *Klf4* and *Spdef*. An inability to produce mature IECs is a feature of colitis-associated cancer 53 that, when also considering the 50% increase in colon mass within 7 days of Cre induction, might be due high mucosal concentrations of the pro-survival factors LIF and MCP-1 in CTα^{IKO} mice (McClellan et al., 2012; Reya & Clevers, 2005; Yu et al., 2014).

Given that UC onset typically occurs in young adulthood, the use of a tamoxifen-inducible Cre-recombinase system allowing us to delete CTα in young adult mice was an advantage in these experiments. CTα^{IKO} mice rapidly lose weight upon Cre induction before beginning to regain body weight at day 5 and arriving at a similar body weight controls by day 7, although pathology scoring showed that severe colitis remained in CTα^{IKO} mice at day 7. The colonic epithelium of CTα^{IKO} mice was, however, substantially less damaged at 7 weeks after Cre induction when compared 4 days or 7 days after Cre induction. These observations suggest that epithelial cells of CTα^{IKO} mice activate compensatory pathways to promote mucosal healing following the initial inflammatory response. A similar pattern of acute and rapid weight loss followed by weight regain and colonic restitution has been observed previously in mice infected by *Citrobacter rodentium*, a murine pathogen that can subvert the colonic mucus barrier and cause colitis (Mundy, MacDonald, Dougan, Frankel, & Wiles, 2005). While some of the epithelial repair mechanisms induced after *Citrobacter* infection, such as production of anti-inflammatory or antimicrobial factors might apply to CTα^{IKO} mice, a rewiring of cellular lipid metabolism is likely also required (Buffie & Pamer, 2013; Mundy et al., 2005). An example of a cellular adaptation to limited PC supply was reported previously with the slowing of PC catabolism in the brains of mice fed a diet deficient in choline, the dietary precursor of PC (Li, Agellon, & Vance, 2007). The course of severe and debilitating colonic inflammation followed by gradual epithelial restitution observed in CTα^{IKO}

mice is comparable to that seen in human IBD and will make CTα^{IKO} mice a useful model for studying mechanisms of epithelial repair and return to homeostasis following bouts of colitis.

We reported in our previous manuscript that the small intestines of CTα^{IKO} mice look overtly normal and, unlike the colons of CTα^{IKO} mice, do not have obvious structural damage. Severe colonic pathology with overtly normal small intestinal morphology has also been observed previously in MUC2-deficient mice (Ng et al., 2017). It is conceivable that the colons of CTα^{IKO} mice are more severely affected by disturbances to PC synthesis than their small intestines because there is a constant supply of PC to the small intestine in bile, while very little biliary PC reaches the colon (Nilsson, 1968; Parthasarathy et al., 1974). Additionally, we reported previously that CTα^{IKO} mice have small intestinal lipid malabsorption (Kennelly et al., 2018). There is evidence from other murine models that intestinal inflammation and lipid malabsorption are linked. For example, mice lacking B cells (which secrete IgA to restrict bacterial interaction with the epithelium) have enhanced interferon-related immune responses in the gut, which is linked to lipid malabsorption (Shulzhenko et al., 2011). Furthermore, enteric infection of mice with *Citrobacter rodentium* has been shown to induce diarrhea and water efflux to promote pathogen clearance (Tsai et al., 2017). Impaired dietary lipid absorption has also been described in patients with UC (Andersson, Dotevall, Gillberg, Jagenburg, & Kock, 1971; Chakravarti, Sehgal, Chakravarti, & Chnuttani, 1973; Salem & Truelove, 1965). Future studies will investigate the mechanisms underlying the relationship between intestinal inflammation and dietary lipid malabsorption in CTα^{IKO} mice.

While most CTα^{IKO} mice experience body weight loss upon Cre induction, a minority of mice also experience severe wasting that requires euthanasia. This striking response to colonic PC depletion might be due in part to loss of blood through the injured bowel, as indicated by low

circulating levels of red blood cells, hemoglobin, and hematocrit. However, additional inflammatory stimuli likely also play a role. Firstly, necroptotic cells undergo plasma membrane permeabilization and release their cellular contents (including cytokines such as IL-1α) to amplify local inflammation and recruit neutrophils and lymphocytes to affected tissues (Kaczmarek, Vandenabeele, & Krysko, 2013). Furthermore, ER stress has been shown to independently activate inflammatory signaling pathways and pro-inflammatory cytokine secretion (Zhang & Kaufman, 2008). Blockage of protein translation by induction of *Eif4ebp1* (likely leading to lower secretion of mucus components) could also contribute towards disease pathogenesis in CTα^{IKO} mice. High concentrations of colonic pro-inflammatory cytokines promote tight junction dysfunction, as indicated by altered expression of Claudins and substantially elevated gut permeability to FITC-labeled dextran in CTα^{IKO} mice. Elevated intestinal permeability, combined with a thin mucus layer, allows microbes to infiltrate the epithelium, as indicated by higher circulating levels of Lipocalin and high colonic concentrations of the cytosolic bacterial DNA sensor ZBP1 in CTα^{IKO} mice, generating uncontrolled inflammation and further immune cell recruitment to the colon (Ariyama et al., 2010; Khor et al., 2011; Kitai et al., 2013). Consistent with a role for microbes in exacerbating inflammation in CTα^{IKO} mice, microbe depletion by antibiotic treatment reduces colonic IL-1β and LIF concentrations in CTα^{IKO} mice without affecting goblet cell depletion, total pathology scores or the induction of GM-CSF or MCP-1. Therefore, ER stress, necroptotic signals, cytokines, enhanced intestinal permeability, microbes, and mucus layer depletion promote inflammation in CTα^{IKO} mice.

In conclusion, the present study shows that *de novo* PC synthesis is required to maintain the intestinal mucosal barrier by protecting IECs against ER stress and subsequent non-apoptotic cell death. CTα^{IKO} mice will be a useful model for studying aspects of UC pathogenesis.

4.5 References

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Chapter 5

Intestinal *de novo* phosphatidylcholine synthesis is important for gallbladder regulation through maintaining cholecystokinin levels

5.1 Introduction

Enterohepatic circulation encompasses simultaneous communication and coordination between the intestine, liver, and gallbladder for the maintenance of biliary homeostasis (Ridgway & McLeod, 2008; Russell, 2009). Primary bile acids are the most abundant constituent of bile and are synthesized in hepatocytes where they are then secreted into the canaliculus by the bile salt export pump (BSEP) (Gerloff et al., 1998; O'Máille, Richards, & Short, 1965). In humans, primary bile acids include cholic acid and chenodeoxycholic acid whereas in mice primary bile acids include cholic acid and muricholic acid (Ridgway & McLeod, 2008). The second most abundant constituent of bile is phosphatidylcholine (PC), which is added to bile by the multidrug resistant protein 2 (MDR2) flippase protein (Smit et al., 1993). Finally, cholesterol is added to bile by the ATP-binding cassette sub-family G member 5/8 (ABCG5/8) heterodimer (Yu et al., 2002).

Bile in the canaliculus travels to the main bile duct where entry into the small intestine is controlled by the sphincter of Oddi. In the fasted state, the sphincter of Oddi is contracted and bile backs up in the main bile duct until it reaches the cystic duct and enters the gallbladder (Torsoli, Corazziari, Habib, & Cicala, 1990). The gallbladder is acted upon by fibroblast growth factor 15 (FGF15) in mice and FGF19 in humans to relax and fill with bile (Choi et al., 2006; Sayin et al., 2013). In the postprandial state, when nutrients reach the duodenum, they stimulate the release of cholecystokinin (CCK) from the small intestine which acts to relax the sphincter of Oddi and contract the gallbladder releasing bile into the lumen of the intestine (Krishnamurthy & Brown, 2002; Lanzini, Jazrawi, & Northfield, 1987; Helen H. Wang et al., 2010). When bile acids reach the terminal ileum, they are absorbed and secreted back into portal circulation where they are taken up by the liver to be used for bile production once more (Dawson et al., 2003, 2005; Reichen & Paumgartner, 1976; Ridgway & McLeod, 2008; Weinberg, Burckhardt, & Wilson, 1986). The

absorption of bile acids in the ileum also stimulates the release of FGF15/19 into portal circulation which then signals for the storage of bile once more (Choi et al., 2006; Sayin et al., 2013). Around 5 % of bile acids are not absorbed and reach the colon where they are metabolized by the microbiota into secondary bile acids. Some of the secondary bile acids are excreted in feces whereas others are absorbed in the colon and returned to the liver increasing the diversity of the bile acid pool (Björkhem, Danielsson, Einarsson, & Johansson, 1968; Hofmann, 1984).

Digestion, absorption, and processing of nutrients relies on the presence and appropriate composition of bile. When there are alterations to the enterohepatic circulation, and subsequent biliary composition, lipid metabolism can be severely affected. To maintain proper PC levels in bile, the liver can synthesize PC through two mechanisms. The first is the CDP-choline pathway which synthesizes PC from choline with CTP phosphocholine cytidylyltransferase alpha (CTα) as the rate limiting enzyme. The second pathway, which accounts for 30 % of total hepatic PC synthesis, involves the conversion of phosphatidylethanolamine (PE) to PC by phosphatidylethanolamine *N*-methyltransferase (PEMT). When PEMT was acutely knocked out of high fat diet (HFD) fed mice, they developed cholestasis and had reduced biliary bile acid and PC secretion (Wan et al., 2019).

The small intestine also has an important role in maintaining enterohepatic circulation and lipid metabolism. The small intestine is only capable of synthesizing *de novo* phosphatidylcholine through the CDP-choline pathway. Our lab has previously developed an inducible, intestinal specific, CTα knockout ($CT\alpha^{IKO}$) mouse which has lost the ability to synthesize *de novo* PC through the CDP-choline pathway (Kennelly et al., 2018). These mice can only obtain intestinal PC from the lumen (dietary and biliary sources) or from uptake from circulating lipoproteins. HFD-fed $CT\alpha^{IKO}$ mice present acute weight loss and lipid malabsorption.

Through an incidental finding, we noted that CTα^{IKO} mice, when euthanized in the postprandial state, have enlarged gallbladders. Additionally, we found that CTα^{IKO} mice had reduced circulating levels of CCK as well as reduced duodenal mRNA expression of the *Cck* gene indicating that the release of bile into the duodenum in the postprandial state was impaired. Next, we wanted to determine whether improving biliary homeostasis could alter the weight loss or lipid malabsorption phenotype observed in CTα^{IKO} mice. To try and improve gallbladder contraction, and release of bile, we treated CTα^{IKO} mice with exogenous CCK. Exogenous CCK improved the acute weight loss of CTα^{IKO} mice despite minimal improvement in lipid absorption. As well, we fed CTα^{IKO} mice a diet supplemented with bile acids to try and improve the digestion of lipids. Bile acid supplementation did not improve weight loss or lipid malabsorption in CTα^{IKO} mice. In summary, we found that CTα^{IKO} mice have reduced gallbladder emptying and treatments to improve gallbladder function or lipid digestion were unable to compensate for the loss of intestinal PC synthesis.

5.2 Methods

5.2.1 Animal handling

Experimental mice were caged in a temperature-controlled environment with a 12h light/dark cycle. Only female mice were used and were given free access to water and a standardized chow diet prior to experimentation. *Pcyt1a*$^{loxP/loxP}$ and *Pcyt1a*$^{loxP/loxP}$;villin-Cre-ER^{T2} mice were bred to generate experimental mice as described previously (Kennelly et al., 2018). CTα intestinal knockout (CTα^{IKO}) mice were generated by giving *Pcyt1a*$^{loxP/loxP}$;villin-Cre-ER^{T2} mice five days of intraperitoneal injections of tamoxifen (1mg/day) dissolved in sunflower oil. Control mice were generated by treating aged matched 8-21 weeks old *Pcyt1a*$^{flox/flox}$ mice with tamoxifen as

described. Mice were continued on chow diet during tamoxifen injection then were switched to a specialized diet for 4 days. Mice were euthanized by cardiac puncture following 16 h fast and 2 h refeed of specialized diet. EDTA, dipeptidyl peptidase 4 inhibitor (EMD Millipore, MA) and complete general protease inhibitor (Sigma) containing tubes were used to store blood and were spun at 3000 g for 10 min at room-temperature to collect plasma. Small intestines were excised and flushed with PBS and protease inhibitor cocktail (Sigma) then segmented into a length ratio of 1:3:2 representing the duodenum, jejunum, and ileum respectively. Intestinal epithelial cells were collected by opening the segments longitudinally and scrapping them off the intestinal layers below and snap frozen in liquid nitrogen. Gallbladders were collected and were either snap frozen in liquid nitrogen or were stored in 10 % neutral buffered formalin for histology. Experiments were approved following guidelines set by the Canadian Council on Animal Care by the University of Alberta's Institutional Animal Care Committee.

5.2.2 Experimental trials

Unless stated otherwise, the specialized diet fed to all mice was a 40 % fat/calorie high-fat diet (HFD) (Table 5.1). For the bile acid supplementation study, control and CTα^{IKO} mice were randomly assigned to HFD or HFD containing 0.05 % of cholic acid and 0.05 % of chenodeoxycholic acid (5 mice/group) for 4 days following tamoxifen injection. For the cholecystokinin (CCK) injection study, mice were injected intraperitoneally with 0.1 µg/Kg of CCK-8 twice daily for 5 days following the tamoxifen injections.

Table 5.1: Composition of experimental diet.

Ingredients	HFD (g)
Casein	270.0
Corn Starch	170.7
Sucrose	195.4
Cellulose	80.0
Vitamin Mix	19.0
Mineral Mix	50.0
Calcium Phosphate Dibasic	3.4
Inositol	6.3
L-cysteine	1.8
Choline Bitartrate	4.2
PC (soy lecithin)	0
Crisco Vegetal Oil	32.0
Mazola Corn Oil	10.0
Lard	155.0
DHAsco	1.5
ARAsco	1.5

5.2.3 Microscopy

Gallbladders were collected from control and CTα^{IKO} mice fed the standardized chow diet for 4 days following tamoxifen injections. Gallbladders were collected after a 16 h fast and a 2 h refeed. Gallbladders stored in 10 % neutral buffered formalin were paraffin embedded prior to slicing (5µm thick). Hematoxylin and eosin (H&E) stained slides were visualized under light-microscopy.

5.2.4 Lipid analysis

Plasma triacylglycerol (TG) was measured using a Sekisui Diagnostics kit and plasma CCK was measured using an enzyme-linked immunosorbent assay (RayBiotech, Inc., Norcross, GA). Protein levels of jejunal IEC homogenates were analyzed by bicinchoninic acid assay. 1 mg jejunal protein homogenate was mixed in a chloroform and methanol (2:1) solution containing batyl alcohol (200μg) and PDME (50μg) as an internal standard to extract lipids. While vortexing after each addition, 1.25 mL of mildly acid 0.9 % sodium chloride and 1.25 mL of chloroform were added. The bottom layer was removed after a 3000 rpm centrifugation for 10 min. The lipid extraction was dried down and redissolved in 100 μL of chloroform and isooctane (1:1) before analysis by HPLC.

5.2.5 Real-time quantitative PCR analysis

Duodenal, jejunal and gallbladder samples were homogenized in TRIzol (15596018; Invitrogen). Total RNA was isolated in duodenal and jejunal samples using RNEasy Mini (74104; Qiagen) and treated with DNase 1 (18068-015; Invitrogen). Total RNA was isolated in gallbladder samples using RNEasy Micro (74004; Qiagen) which contains a DNase 1 treatment step. Isolated RNA from all samples were reverse transcribed using oligo(dT)12–18 primers (18418-012; Invitrogen), random primers (48190011; Thermo Fisher Scientific), and Superscript II (18064-173 014; Invitrogen) generating cDNA. Samples containing Power SYBR Green PCR Master Mix (4367659; Thermo Fisher Scientific) was analyzed by quantitative PCR run on a StepOne Plus system (Applied Biosystems, MA, USA) for 40 cycles. Intestinal data was normalized to mRNA expression of *Rplp0* and gallbladder data was normalized to mRNA expression of *B-actin.* Primer sequences for all genes are in Table 5.2.

Table 5.2: Primers for quantitative PCR.

Gene	Gene Name	Forward Primer	Reverse Primer
Rplp0	Ribosomal protein lateral stalk subunit P0	ACT GGT CTA GGA CCC GAG AAG	CTC CCA CCT TGT CTC CAG TC
Cck	Cholecystokinin	GCTGATTTCCCCATCCAAA	GCTTCTGCAGGGACTACCG
Pcyt1a	Phosphate cytidylyltransferase 1, choline, α isoform	GCT AAA GTC AAT TCG AGG AA	CAT AGG GCT TAC TAA AGT CAA CT
Cd36	Cluster-determinant 36	TGG CTA AAT GAG ACT GGG ACC	ACA TCA CCA CTC CAA TCC CAA G
Dgat2	Diacylglycerol O-Acyltransferase 2	GGC TAC GTT GGC TGG TAA CTT	TTC AGG GTG ACT GCG TTC TT
Mogat2	Monoacylglycerol O-Acyltransferase 2	TAC AGC TTT GGC CTC ATG C	AGG GCT GTG GTG TCA TCT G
Cidec	Cell Death Inducing DFFA Like Effector C	CAC TGC TAC AAG GCC AAG C	GGT GGC ATC CAG GAA CTG
B-actin	Beta actin	GCT CTG GCT CCT AGC ACC AT	GCC ACC ATC CAC ACA GAG T
Cck-1r	Cholecystokinin Receptor	CAA CCT GCT CAA GGA TTT CAT CT	CAC GGA AGT GCC CAT GAA GT
Acat2	Acetyl-CoA Acetyltransferase 2	CCA GCT TCG GAG GAG AGA A	AGT CTG GGG TTC CGT GTG T
Npc1l1	NPC1 like intracellular cholesterol transporter 1	TGG ACT GGA AGG ACC ATT TCC	GTG CCC CGT AGT CAG CTA T
Muc2	Mucin 2	CCA TTG AGT TTG GGA ACA TGC	TTC GGC TCG GTG TTC AGA G
Muc5ac	Mucin 5ac	GTC TGG CAG AAA CAG TGG AGA TT	TCG TGG CTT CTC ACA GAA CTT G
Muc5b	Mucin 5b	TCT TGC CCT GAT GTA TCC AA	TGC ACT TGA CAG GTA CTT GAG TC

5.2.6 Statistical analysis

Graph Pad Prism 9 was used for statistical analysis. Data was expressed as mean ± SEM and statistical significance ($p<0.05$) was determined by student's T-test when analyzing two independent groups and by two-way ANOVA with uncorrected Fisher's Least Significant Difference test when comparing more than two independent groups.

5.3 Results

5.3.1 CTα^{IKO} mice have enlarged postprandial gallbladders and reduced CCK signaling

When CTα^{IKO} mice were euthanized in the postprandial state we were surprised to find that they had a full gallbladder compared to control mice euthanized in the postprandial state (Figure 5.1 A-B). When histology slides of these gallbladders were stained with H&E we noted that the gallbladders of CTα^{IKO} mice were extremely stretched and had lost all folds found in the contracted gallbladders of control mice (Figure 5.1 C-D). To investigate the cause of the enlarged postprandial gallbladders in CTα^{IKO} mice, we measured cholecystokinin (CCK) levels in the duodenum and

plasma. CCK is a signal sent from the duodenum under times of feeding to the gallbladder, stimulating contraction. We found that duodenal mRNA levels and plasma protein levels of CCK were reduced in CTα^{IKO} mice compared to controls (Figure 5.1 E-F). These results indicate that the enlarged gallbladders of CTα^{IKO} mice in the postprandial state could be due to improper gallbladder signaling and an altered gut-liver axis.

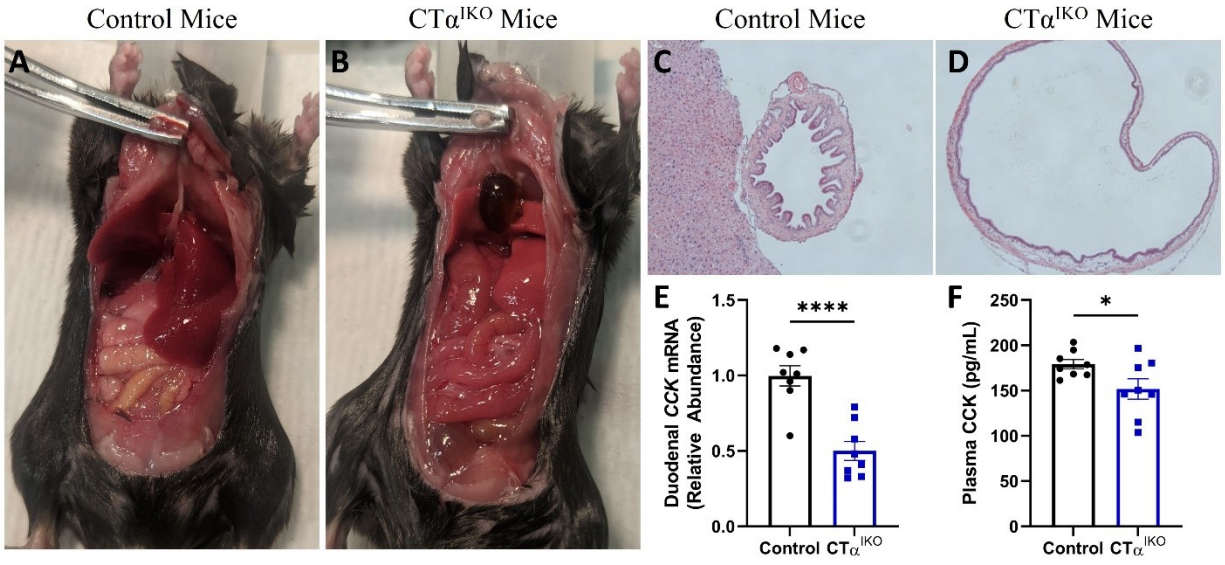

Figure 5.1: CTα^{IKO} mice have enlarged postprandial gallbladders. Postprandial gallbladders of (A) control and (B) CTα^{IKO} mice. Representative gallbladder H&E staining in (C) control and (D) CTα^{IKO} mice fed chow diet for 5 days. (E) Duodenal mRNA and (F) plasma CCK levels in control and CTα^{IKO} mice. Mice were 8-22 weeks fed HFD for 5 days. Values are reported as ±SEM, n=8/group. *P<0.05, ****P<0.0001.

5.3.2 CCK-8 injections improved weight loss but did not affect postprandial lipid metabolism

Previous work has shown that CTα^{IKO} mice present with weight loss, reduced plasma lipids and lipid malabsorption (Kennelly et al., 2018). To determine whether exogenous CCK could improve gallbladder contraction and increase lipid absorption in CTα^{IKO} mice, HFD-fed control and knockout mice were injected twice daily with CCK-8. CCK-8 is the most abundant active circulating isoform of CCK and exogenous CCK-8 injections have been shown to induce gallbladder emptying (H. H. Wang, Liu, Portincasa, Tso, & Wang, 2016). Interestingly, CCK-8 injected CTα^{IKO} mice did not lose weight compared to CCK-injected control mice (Figure 5.2 A). Despite the improved weight, CCK-8 injected CTα^{IKO} mice maintained reduced plasma TG (Figure 5.2 B). Jejunum TG was also unaltered between control and CTα^{IKO} mice independent of treatment group, possibly due to the large interindividual variability observed (Figure 5.2 C). CCK-8 injections also did not improve jejunal phospholipid levels as CCK-8 injected CTα^{IKO} mice maintained a reduced jejunal PC level and PC:PE ratio (Figure 5.2 D-F). Together, these results indicate that exogenous CCK can improve weight loss, but not lipid absorption in CTα^{IKO} mice.

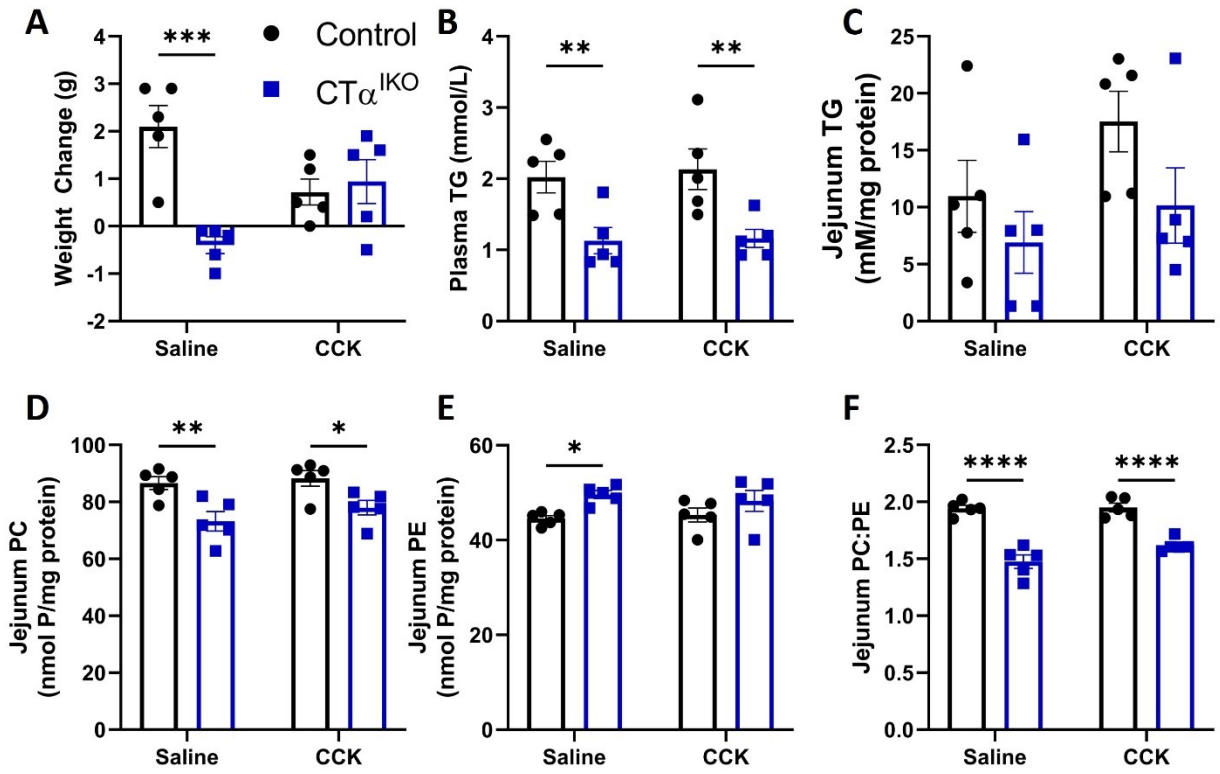

Figure 5.2: CCK injections normalize weight gain in CTα^{IKO} mice but does not improve lipid metabolism. (A) Weight change in control and CTα^{IKO} mice. (B) Plasma and (C) jejunum TG in control and CTα^{IKO} mice. Jejunal (D) PC, (E) PE and (F) PC:PE ratio in control and CTα^{IKO} mice. Mice were 8-12 weeks injected with saline or CCK fed HFD for 5 days. Values are reported as ±SEM, n=5/group. *P<0.05, **P<0.01, ***P<0.001, ****P<0.0001.

5.3.3 CCK injections had minimal effect on jejunal and gallbladder mRNA levels

To determine whether CCK-8 injections improved lipid metabolism in CTα^{IKO} mice, jejunal mRNA levels of genes known to be altered in CTα^{IKO} mice were measured. CCK-8 injected CTα^{IKO} mice maintained a reduction in mRNA levels of *Cd36*, *Dgat2*, and *Cidec* compared to controls(Figure 5.2 A). These results corroborate that CCK-8 injections were unable to improve lipid absorption and metabolism in CTα^{IKO} mice. To determine whether CCK-8 injections improved gallbladder function in CCK-8 injected CTα^{IKO} mice, mRNA levels of genes known to be altered in CCK knockout mice were measured (H. H. Wang et al., 2016). Saline injected CTα^{IKO} mice had reduced gallbladder mRNA levels of *CCK-1r*, *Acat2*, and *Npc1l1* compared to control mice (Figure 5.3 B). CCK-8 injected CTα^{IKO} mice maintained a reduced gallbladder mRNA level of *CCK-1r* but had no difference in *Acat2* or *Npc1l1* mRNA levels (Figure 5.3 B). These results indicate that CCK-8 injections were able to improve certain gallbladder gene expression in CTα^{IKO} mice.

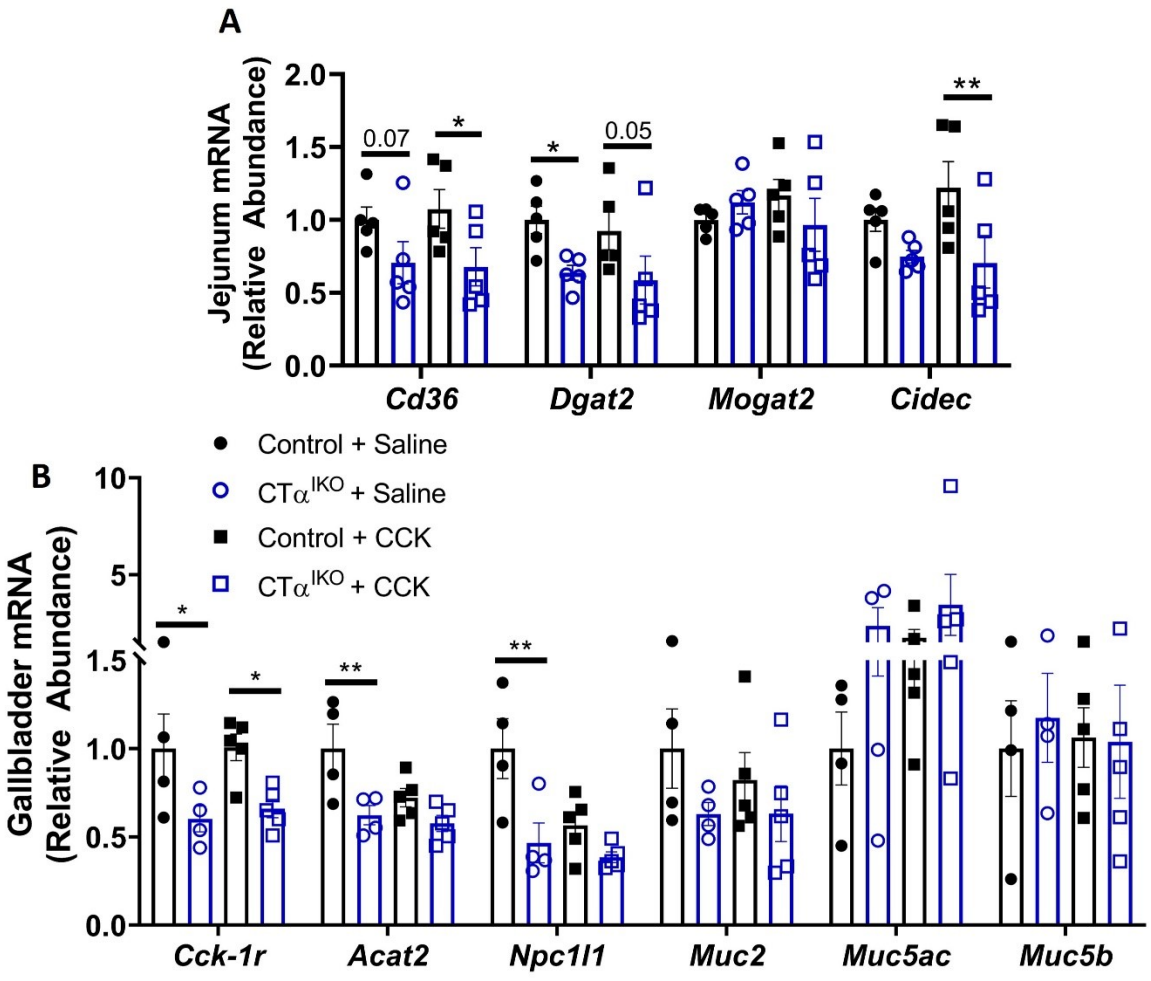

Figure 5.3: CCK injections only mildly improved gene expression in CTα^{IKO} mice. (A) Jejunum mRNA levels of *Cd36*, *Dgat2*, *Mogat2*, and *Cidec*. (B) Gallbladder mRNA levels of *Cck-1r*, *Acat2*, *Npc1l1*, *Muc2*, *Muc5ac*, and *Muc5b*. Mice were 8-12 weeks injected with saline or CCK fed HFD for 5 days. Values are reported as ±SEM, n=4-5/group. **P*<0.05, ***P*<0.01.

5.3.4 Bile acid supplementation to CTα^{IKO} mice did not improve weight loss or postprandial lipid metabolism

To determine whether lipid malabsorption observed in CTα^{IKO} mice was due to reduced bile reaching the intestine, control and CTα^{IKO} mice were fed a bile acid supplemented or control 40 % HFD (Figure 5.4 A-C). Bile acid supplemented CTα^{IKO} mice maintained reduced weight, plasma TG and jejunal TG compared to control mice. As well, bile acid supplemented CTα^{IKO} mice maintained a reduction in jejunal PC levels and PC:PE ratio (Figure 5.4 D-F). In conclusion, bile acid supplementation was unable to improve weight loss or lipid absorption in CTα^{IKO} mice.

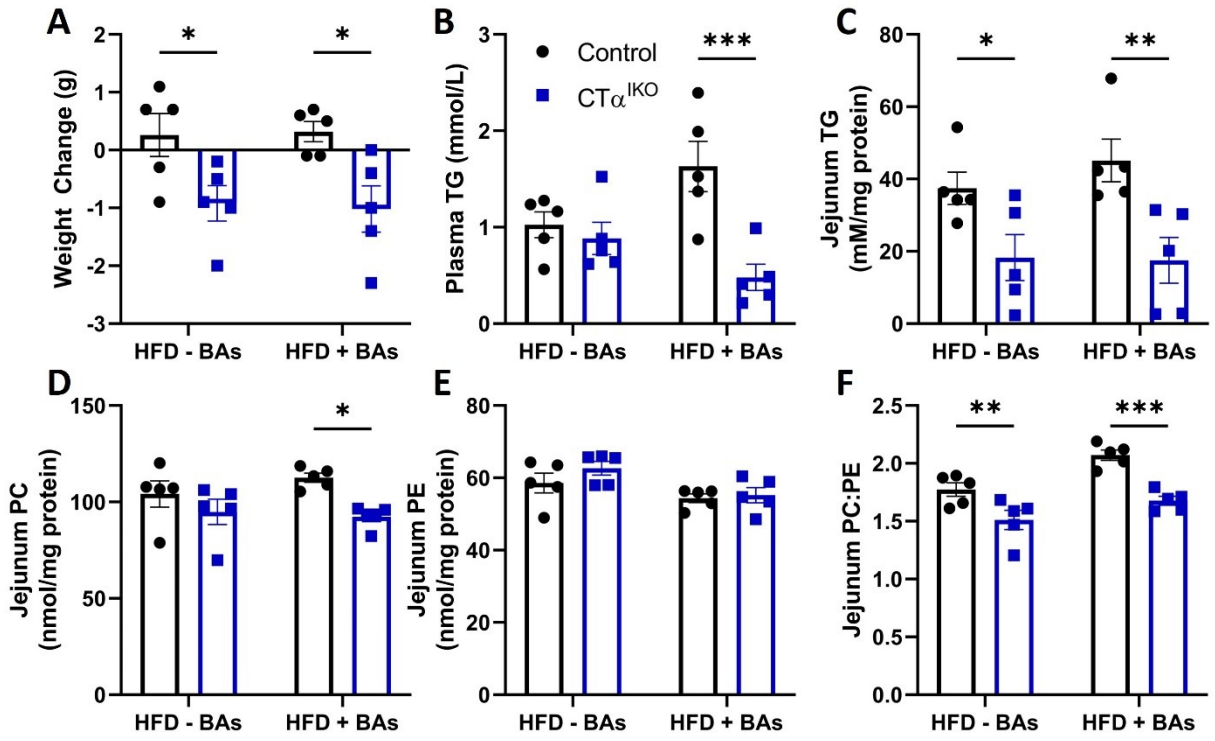

Figure 5.4: Bile acid supplementation does not improve weight loss nor lipid metabolism in CTα^{IKO} mice. (A) Weight change in control and CTα^{IKO} mice. (B) Plasma and (C) jejunum TG in control and CTα^{IKO} mice. Jejunal (D) PC, (E) PE and (F) PC:PE ratio in control and CTα^{IKO} mice. Mice were 8-12 weeks fed a control (-BAs) or bile acid supplemented (+BAs) HFD for 5 days. Values are reported as ±SEM, n=5/group. *P<0.05, **P<0.01, ***P<0.001.

5.3.5 Bile acid supplementation had minimal affect on jejunal and gallbladder mRNA levels

To determine whether gene expression was improved with bile acid supplementation, jejunal and gallbladder mRNA levels were analyzed as in Figure 5.3. The only jejunal mRNA level altered in bile acid supplemented CTα^{IKO} mice was no significant difference in *Cd36* (Figure 5.5 A). The only gallbladder mRNA levels altered in bile acid supplemented CTα^{IKO} mice were no significant difference in *Acat2* and a significant decrease in *Muc2* (Figure 5.5 B). Together, these results show minimal changes, corroborating the conclusion that bile acid supplementation was unable to improve lipid metabolic changes in CTα^{IKO} mice.

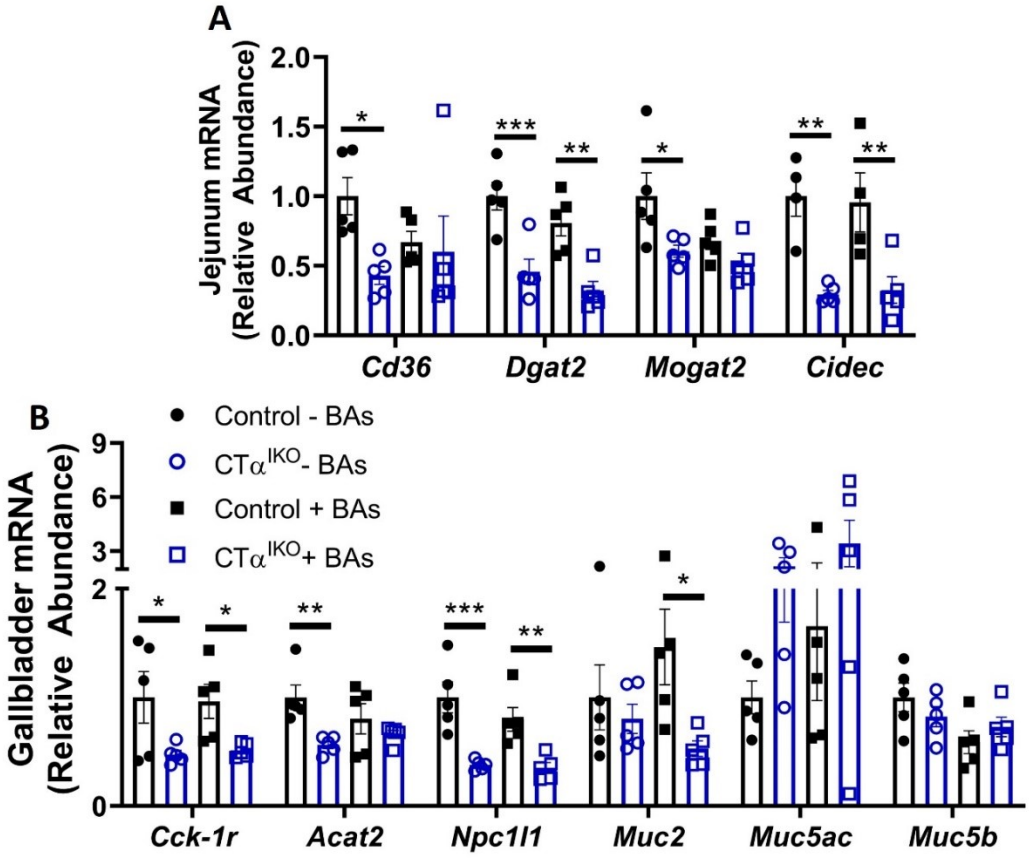

Figure 5.5: Bile acid supplementation only mildly improved gene expression in CTα^{IKO} mice. (A) Jejunum mRNA levels of *Cd36*, *Dgat2*, *Mogat2*, and *Cidec*. (B) Gallbladder mRNA levels of *Cck-1r*, *Acat2*, *Npc1l1*, *Muc2*, *Muc5ac*, and *Muc5b*. Mice were 8-12 weeks fed a control (-BAs) or bile acid supplemented (+BAs) HFD for 5 days. Values are reported as ±SEM, n=5/group. **P*<0.05, ***P*<0.01, ****P*<0.001.

5.4 Discussion

The gallbladder is the storage organ of bile and contracts under hormonal control from CCK to release bile into the duodenum to aid in the digestion and absorption of lipids (Krishnamurthy & Brown, 2002; Lanzini et al., 1987; Helen H. Wang et al., 2010). Incidentally, we discovered that the gallbladders of CTα^{IKO} mice were enlarged in the postprandial state compared to control mice whose gallbladders were fully contracted. Upon further investigation, we found that CTα^{IKO} mice had reduced circulating CCK levels and reduced duodenal *Cck* mRNA levels, indicative of alterations in hormonal regulations which could account for the enlarged gallbladder size. Previous studies in CTα^{IKO} mice have shown that they present with weight loss and lipid malabsorption (Kennelly et al., 2018). We hypothesize that the weight loss and lipid malabsorption is due to reduced contractions of gallbladders in CTα^{IKO} mice, leading to reduced bile reaching the duodenum and an inability to digest dietary lipids. In an attempt to improve gallbladder contractions, and subsequently increase bile reaching the duodenum, we injected CTα^{IKO} mice with a CCK analogue. CCK-injected CTα^{IKO} mice had improved weight gain despite appearing to have maintained lipid malabsorption. As CCK injections were unable to restore lipid absorption in CTα^{IKO} mice, we fed CTα^{IKO} mice a diet supplemented with bile acids to improve lipid digestion and absorption. Bile acid supplemented CTα^{IKO} mice still lost weight and had reduced lipid absorption. These results indicate that exogenous CCK and bile acid supplementation are unable to fully compensate for the intricate roles of intestinal derived *de novo* PC.

The effects of reduced intestinal PC synthesis on biliary homeostasis are complex. CTα^{IKO} mice that have undergone a gallbladder cannulation have increased bile flow with a significant increase in bile acid, PC, and cholesterol secretion with only minimal alterations to biliary composition or hydrophobicity (Kennelly et al., 2018). This led to the conclusion that there was

an increase in bile reaching the duodenum under times of feeding. Interestingly, gallbladder cannulation bypasses the hormonal control of the gallbladder and therefore may only represent bile synthesized and secreted by the liver but not bile reaching the duodenum. As CTα^{IKO} mice have enlarged gallbladders and reduced circulating CCK, the increased hepatic bile flow may actually represent a feedback mechanism from reduced bile reaching the intestine.

Alterations in biliary composition and homeostasis lead to altered lipid absorption and impaired lipid metabolic processes. When BSEP is knocked out of mice, bile acid secretion into bile is significantly reduced and lipid absorption is impaired (Fuchs et al., 2020; R. Wang et al., 2016). Interestingly, Na+ taurocholate cotransporting polypeptide (NTCP), the hepatic transporter that takes up circulating bile acids from plasma is also important for proper lipid absorption. NTCP deficient mice have reduced intestinal lipid absorption and are protected from diet induced obesity (Donkers et al., 2019). Finally, alteration in hepatic PC concentrations also affects lipid metabolism. When MDR2 is knockout out of mice, PC in bile is essentially eliminated (Smit et al., 1993). The reduction in biliary phospholipids leads to a reduction in plasma appearance of TG due to an accumulation within small intestinal enterocytes (Voshol et al., 2000). Therefore, the lipid malabsorption, and subsequent weight loss, in CTα^{IKO} mice may be caused by reduced bile reaching the duodenum.

CCK knockout (KO) mice have enlarged gallbladders in the postprandial state, indicating the importance of CCK in biliary release to the duodenum (H. H. Wang et al., 2016). Additionally, CCK KO mice injected with CCK-8, an exogenous form of CCK, had improved gallbladder contractility and subsequent release of bile. To determine whether the weight loss and lipid malabsorption observed was caused by reduced biliary secretion from the gallbladders, CTα^{IKO} mice were injected with CCK-8. Exogenous CCK was able to normalize weight gain in CTα^{IKO}

mice but was unable to improve lipid absorption. Another finding in CCK KO mice was altered gallbladder mRNA levels. A lack of CCK lead to reduced *Cck-1r*, the CCK receptor encoding gene, as well as an increase in mRNA levels of genes associated with mucin production. Surprisingly, CCK KO mice also had an increase in gallbladder *Acat2*, and a decrease in gallbladder *Npc1l1*, protein encoding genes involved in cholesterol regulation (H. H. Wang et al., 2016). We measured gallbladder mRNA levels of these genes in CTα^{IKO} mice to determine if exogenous CCK improved their expression. While exogenous CCK did not increase gallbladder gene expression of *Cck-1r*, it did normalize the levels of *Acat2* and *Npc1l1*. These results indicate that exogenous CCK has an effect on the gallbladders of CTα^{IKO} mice, despite being unable to fully restore gene expression. Together, exogenous CCK was unable to fully compensate for the loss of intestinal PC synthesis.

Future work will involve attempting to understand how a reduction in small intestinal PC synthesis leads to both reduced *Cck* gene expression and circulating protein levels. Additionally, future studies will involve determining whether the reduction in CCK also has an affect on pancreatic secretions to the small intestine. CCK regulates not only gallbladder contraction but also the relaxation of the sphincter of Oddi, which is important for the release of both bile and pancreatic juices into the small intestine. In summary, intestinal *de novo* PC synthesis is required for maintaining biliary homeostasis. CTα^{IKO} mice have reduced bile reaching the small intestine caused by reduced gallbladder contraction and reduced circulating levels of CCK. Attempts to attenuate the effects of reduced intestinal PC through exogenous CCK or bile acid supplementation were unable to improve lipid absorption.

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Chapter 6

Conclusion and future directions

6.1 Conclusion

Phosphatidylcholine (PC) is a cylindrical, amphipathic phospholipid ideal for spontaneously forming micelles and membrane bilayers (Van Meer, Voelker, & Feigenson, 2008). PC is the most abundant phospholipid found in the membranes of mammalian cells and is involved in maintaining cellular structure. PC has a variety of other cellular and systemic roles including cell signalling, and lipid digestion and systemic lipid metabolism (Alvaro et al., 1986; Exton, 1994; Skipski et al., 1967). The body is capable of synthesizing PC *de novo* through two independent processes, the phosphatidylethanolamine N-methyltransferase (PEMT) pathway and the cytidine diphosphate (CDP)-choline pathway. The PEMT pathway occurs primarily in the liver and involves the conversion of phosphatidylethanolamine (PE) to PC. The PEMT pathway synthesizes 30 % of hepatic PC under physiologic conditions while the CDP-choline pathway synthesizes the other 70 % of hepatic PC (Bremer, Figard, & Greenberg, 1960; DeLong, Shen, Thomas, & Cui, 1999; Sundler & Akesson, 1975a, 1975b; Tasseva et al., 2016). The CDP-choline pathway occurs in all nucleated cells and utilizes cytidine triphosphate:phosphocholine cytidylyltransferase (CT) as the rate limiting enzyme (Kennedy and Weiss, 1956). CT has two isoforms, CTα which is ubiquitously expressed, and CTβ which is only significantly utilized in gonadal and neuronal tissues under physiologic conditions (Cornell & Ridgway, 2015; Karim, Jackson, & Jackowski, 2003; Lykidis, Baburina, & Jackowski, 1999). The aim of this thesis was to better understand how PC synthesis affects both the organs involved in the gut-liver axis and the communication between them.

Maintaining appropriate hepatic PC levels is important for regulating lipid metabolism and preventing the development of disease states such as non-alcoholic fatty liver disease (NAFLD). The role of PEMT-derived PC synthesis is especially important under times of liver stress. After

10 weeks of HFD feeding, PEMT knockout mice ($PEMT^{-/-}$) mice are resistant to diet induced obesity but accumulate hepatic TG and develop NASH (Jacobs et al., 2010; Van Der Veen et al., 2019). HFD-fed $PEMT^{-/-}$ mice maintain the ability to synthesize PC through the CDP-choline pathway, so $PEMT^{-/-}$ mice have also been fed a choline-deficient diet to minimize alternative PC synthesis. Choline-deficient $PEMT^{-/-}$ mice go into liver failure after only 3 days (Walkey, Yu, Agellon, & Vance, 1998). The role of CTα-derived PC synthesis in lipid metabolism has been analyzed through knockout mouse models. Chow-fed CTα permanent liver knockout ($CT\alpha^{PLKO}$) mice have a reduction in hepatic PC levels despite an increase in enzymes involved in alternative PC synthesis pathways including CTβ and PEMT (Jacobs, Devlin, Tabas, & Vance, 2004). Additionally, chow-fed $CT\alpha^{PLKO}$ mice have reduced plasma TG and reduced HDL and VLDL levels in the fasted state. Interestingly, only female chow-fed $CT\alpha^{PLKO}$ mice had an accumulation of hepatic TG (Jacobs et al., 2004). HFD-fed $CT\alpha^{PLKO}$ mice develop NASH within 1 week, including an increase in hepatic TG accumulation, inflammation, and oxidative stress (Niebergall, Jacobs, Chaba, & Vance, 2011).

In Chapter 2 we determined the effect of an acute CTα liver knockout ($CT\alpha^{LKO}$) on hepatic function. Chow-fed $CT\alpha^{LKO}$ mice lost weight, accumulated hepatic TG, and had markers of NASH after only 1 week of inducing the knockout. When $CT\alpha^{LKO}$ mice were fed a HFD for 1 week, they had a greater reduction in body weight, developed hepatomegaly along with hepatic TG accumulation, and developed NASH. Neither chow-fed nor HFD-fed $CT\alpha^{PLKO}$ mice developed hepatomegaly or lost weight (Jacobs et al., 2004; Niebergall et al., 2011). Additionally, $CT\alpha^{LKO}$ mice had reduced mRNA levels of *Pemt* and *Pcytlb* (gene encoding CTβ) involved in alternative sources of PC synthesis. Interestingly, HFD-fed $PEMT^{-/-}$ mice do lose weight, therefore the reduction in *Pemt* mRNA levels may be contributing to the weight loss observed in $CT\alpha^{LKO}$ mice

(Jacobs et al., 2010; Wan, van der Veen, et al., 2019). In conclusion, knocking out CTα acutely leads to a more severe presentation of altered hepatic function, independent of dietary lipid content. The increased severity may be due to reduced compensation of total PC levels through alternative synthetic pathways (Figure 6.1).

Chapter 2 also focused on how a reduction in hepatic PC synthesis affect lipid metabolism in the small intestine as these organs are in constant communication. Chow- and HFD-fed CTα^{LKO} mice had a surprising reduction in postprandial chylomicron secretion. The reduced appeared of plasma postprandial lipid levels was attributed to reduced lipid absorption, as no lipids accumulated in enterocytes of CTα^{LKO} mice. Interestingly, chow-fed CTα^{LKO} mice also had reduced intestinal PC levels. During the formation of chylomicron particles, there is an increased demand for PC (Lee & Ridgway, 2018). When chow-fed CTα^{LKO} mice are given a single lipid load, it may have depleted the PC stores in the enterocytes. One important communication between the gut-liver axis involves the regulation of enterohepatic circulation (Plauth, Raible, Gregor, & Hartmann, 1993). Enterohepatic circulation encompasses the synthesis and secretion of bile acids from the liver and the subsequent absorption of bile acids from the small intestine to be returned to the liver for future use (Ridgway & McLeod, 2008). Reductions in hepatic PC synthesis induce alterations to biliary homeostasis. HFD-fed PEMT$^{-/-}$ mice have reduced biliary secretion of bile acids and PC, and develop cholestasis (Wan, Kuipers, et al., 2019). Additionally, alterations to the enterohepatic circulation lead to impaired small intestinal lipid metabolism (Fuchs et al., 2020; Voshol et al., 2000). However, CTα^{LKO} mice had similar bile acid secretion levels and increased PC secretion and therefore biliary changes are probably not contributing to the lipid malabsorption (Figure 6.1).

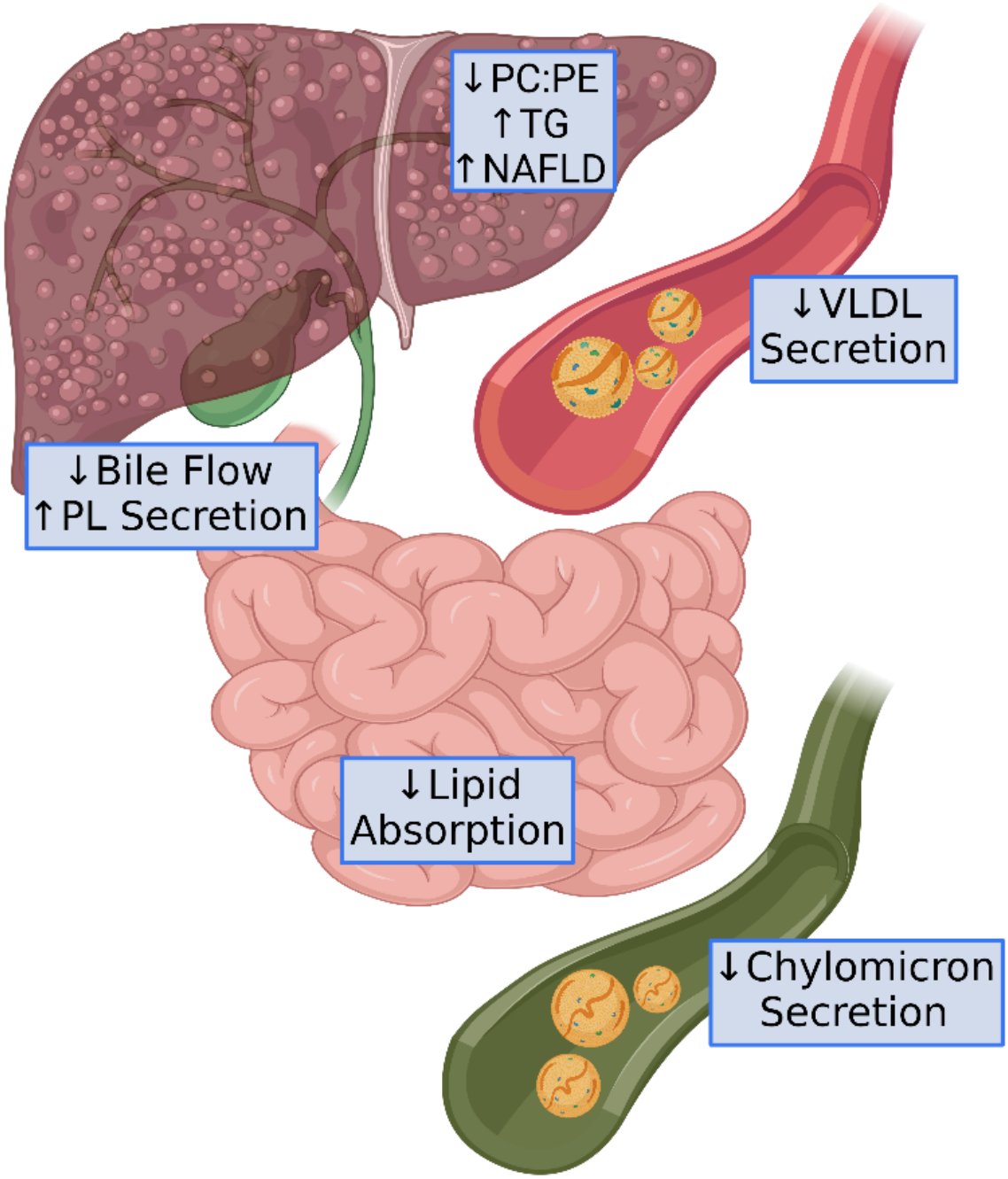

Figure 6.1: The effects of CTα^{LKO} on systemic lipid metabolism. When CTα is knockout out of the liver it leads to a significant reduction in the hepatic PC:PE ratio. CTα^{LKO} mice accumulate hepatic TG leading to an increased liver weight and the development of NAFLD. Additionally, CTα^{LKO} mice have reduced VLDL secretion in the fasted state. CTα^{LKO} mice also have reduced lipid absorption and reduced biliary flow despite having normal bile acid secretion and increased PC secretion into bile. Finally, CTα^{LKO} mice have reduced chylomicron secretion in the postprandial state.

The role of CTα-derived PC has also been analyzed in the intestine. Our lab has created an inducible, CTα intestinal specific knockout ($CT\alpha^{IKO}$) mouse. The Cre-Lox system was used to create knockout mice specific to the intestinal epithelial cells (IECs) of the intestine. IECs include enterocytes, goblet cells, enteroendocrine cells, and Paneth cells (Roda et al., 2010). Enterocytes are the nutrient absorbing cells and are comprised of 80 % of all IECs (Cheng & Leblond, 1974). Goblet cells secrete mucous that helps create the mucous barrier in the lumen of the intestine (Sicard, Bihan, Vogeleer, Jacques, & Harel, 2017). The enteroendocrine cells are the hormone secreting cells, including the secretion of glucagon-like peptide 1 (GLP-1) (Baggio & Drucker, 2007). Finally, Paneth cells secrete antimicrobial peptides and are also involved in the antimicrobial function of the mucosal barrier (Sicard et al., 2017). IECs are only capable of synthesizing PC through the CDP-choline pathway, therefore $CT\alpha^{IKO}$ mice receive all intestinal PC through absorption from the lumen (biliary and dietary sources) and from uptake of lipoproteins from circulation (Mansbach II & Arnold, 1986). Within one week of inducing the CTα knockout, HFD-fed $CT\alpha^{IKO}$ mice lose 15 % of their body weight (Kennelly et al., 2018). The weight loss is accompanied by lipid malabsorption and alterations in intestinal hormonal signaling including an increase in active circulating GLP-1 levels (Kennelly et al., 2018).

In chapter 3 we aimed to determine the pathophysiology of weight loss, lipid malabsorption, and alteration in hormonal signaling that occur in $CT\alpha^{IKO}$ mice. Utilizing transcriptomics, we were able to determine that the down-regulated genes in the IECs of $CT\alpha^{IKO}$ mice involved lipid metabolism – including lipid absorption, lipid droplet regulation, and chylomicron secretion. As previous studies showing weight loss, lipid malabsorption and increased GLP-1 signalling occurred in HFD-fed $CT\alpha^{IKO}$ mice, we aimed to determine whether these changes would occur in low fat diet (LFD)-fed $CT\alpha^{IKO}$ mice. Lipid malabsorption is known to induce the ileal break, a

negative feedback loop that leads to the secretion of GLP-1 to slow gastric emptying and delay intestinal mobility in an attempt to improve lipid absorption upstream (Cummings & Overduin, 2007). We were surprised to see that LFD-fed CTα^{IKO} mice still lost a significant amount of weight and had increased active circulating GLP-1 indicating that the weight loss and hormone secretion is independent of dietary lipid intake and the ileal brake. Lipid absorption is known to be affected by many systemic processes including biliary, hormonal and neuronal influences (Farr, Taher, & Adeli, 2016; Hsieh et al., 2010; Voshol et al., 2000). To determine whether the increased GLP-1, or the other extrinsic factors listed above, were leading to the reduced lipid absorption in our CTα^{IKO} mice we performed an everted intestinal sac experiment which has been historically utilized in rodents to analyze lipid absorption (Figure 3.2) (Strauss, 1963). We found that when the everted intestinal sacs were incubated with radiolabelled oleic acid, there was reduced absorption of the oleic acid leading to reduced incorporation of the radiolabel into lipid metabolites – including TG and PE (Figure 6.2). The everted intestinal sac experiment showed that the limiting factor in lipid absorption of CTα^{IKO} mice was the movement of fatty acids into the IECs and was not solely due to extrinsic factors.

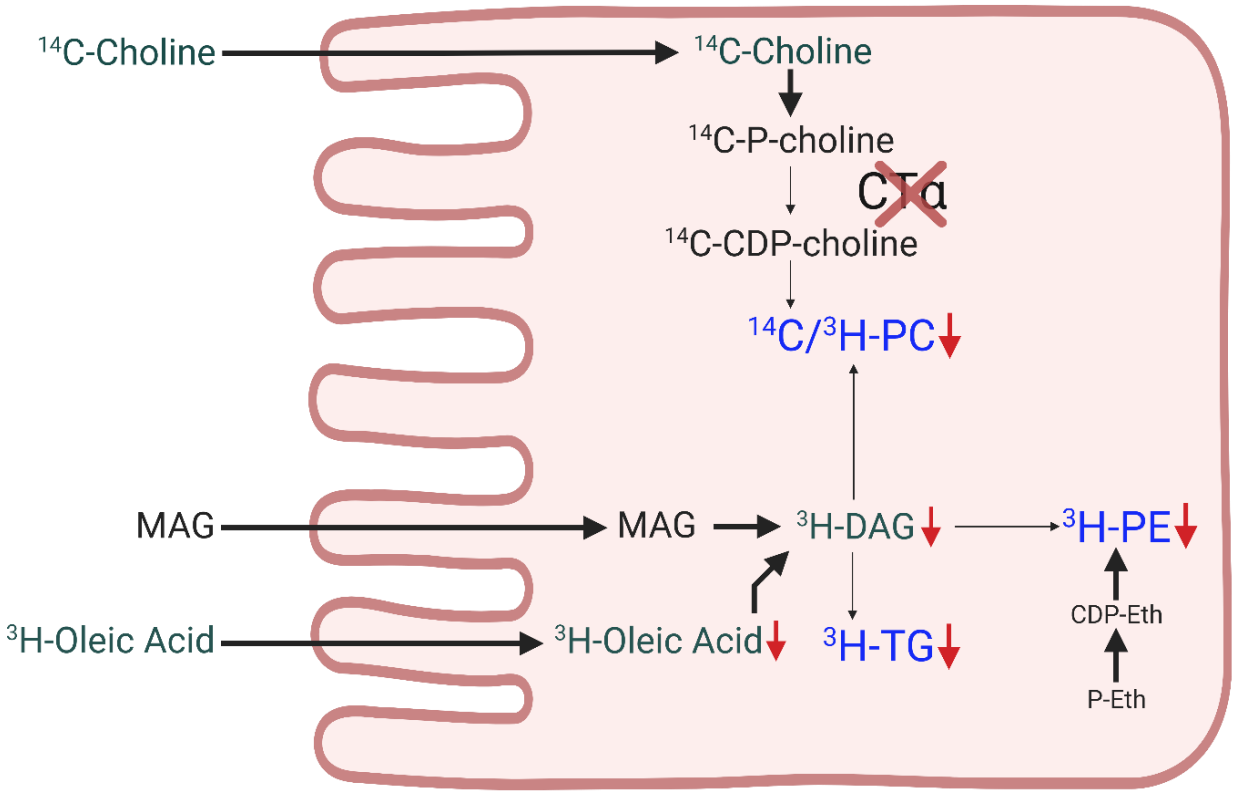

Figure 6.2: Radiolabel incorporation into enterocyte lipid molecules from the everted intestinal sac experiment. Radiolabel experiments show that enterocytes from CTα^{IKO} mice had reduced ^{14}C incorporation into PC molecules. The reduction can be explained by the CTα knockout as the total amount of synthesized PC is reduced despite ^{14}C-choline availability within the cell. The experiments also showed reduced ^{3}H incorporation into TG and PE molecules which can only be explained by reduced lipid absorption. Reduced oleic acid absorption leads to a reduced ^{3}H-oleic acid pool within enterocytes. Subsequently, there is a reduction in the amount of ^{3}H-DAG produced. DAG is the precursor for a variety of lipid molecules including PE and TG explaining the reduction in ^{3}H radiolabelled TG and PE.

Chapter 3 also aimed to determine what was occurring at the cellular level that may be leading to the lipid malabsorption. Transcriptomic of the IECs of CTα^{IKO} mice showed that the upregulated genes were involved in injury and cell abnormalities – including stress, cell death, and antibacterial defense systems consistent with increased microbial interaction (Atarashi et al., 2015). Interestingly, altered ER phospholipid ratios, including alterations in the PC:PE ratio, have been shown to induce ER stress and activate the unfolded protein response (Fu et al., 2011; Gao et al., 2015; Halbleib et al., 2017; Thibault et al., 2012). This is thought to be occurring in the IECs CTα^{IKO} mice as they have increased gene expression of ER stress markers. ER stress can lead to the activation of programmed cell death, including necroptosis mediated by RIPK3, a gene that is also elevated in CTα^{IKO} mice (Saveljeva, Mc Laughlin, Vandenabeele, Samali, & Bertrand, 2015). The death of IECs appears to target goblet cells as CTα^{IKO} mice had both a reduction in genes involved in goblet cell function and development as well as total goblet cell numbers. Alterations in the mucosal barrier can lead to bacterial translocation, therefore it was not surprising that CTα^{IKO} mice also had increased genes involved in defense against microbes (Figure 6.3).

Bacterial translocation into IECs can lead to an acute induction of the immune system and cell death via necroptosis (Katayama, Xu, Specian, & Deitch, 1997; Takaoka et al., 2007; Upton, Kaiser, & Mocarski, 2012). Therefore, we wanted to determine whether bacterial stress was a consequence of an altered mucosal layer through goblet cell death or a cause. When CTα^{IKO} mice were treated with antibiotics they had a reduction in the indications of bacterial stress but did not have an improvement in ER stress or goblet cell death. Interestingly, antibiotic-treated CTα^{IKO} mice did not lose weight and had improved lipid absorption indicating that bacterial translocation impacts the metabolic function of IECs. As the driving factor to the dysregulated IECs is a reduction in PC availability from the CDP-choline pathway, we fed CTα^{IKO} mice a PC

supplemented diet (PCSD) in order to try and increase total cellular PC. PCSD-fed CTα^{IKO} mice had a normalization of total IEC PC. The increased total PC led to improved postprandial plasma TG levels despite having a reduction in lipid absorption as well as increased active circulating GLP-1 levels. Additionally, PCSD-fed CTα^{IKO} mice had a reduction in goblet cell loss despite still having ER stress, necroptosis, and bacterial stress. These results indicate that the source of PC is an important factor in maintaining intestinal homeostasis (Figure 6.3). Previous research also strengthens this conclusion. For example, when biliary PC is reduced, lipids accumulate in enterocytes and chylomicron secretion is reduced (Voshol et al., 2000). Also, when lyso-PC acyltransferase 3 is knockout out of IECs (the enzyme responsible for adding a polyunsaturated fatty acid to PC molecules), the mice develop lipid malabsorption (Li et al., 2015; Rong et al., 2015; B. Wang et al., 2016).

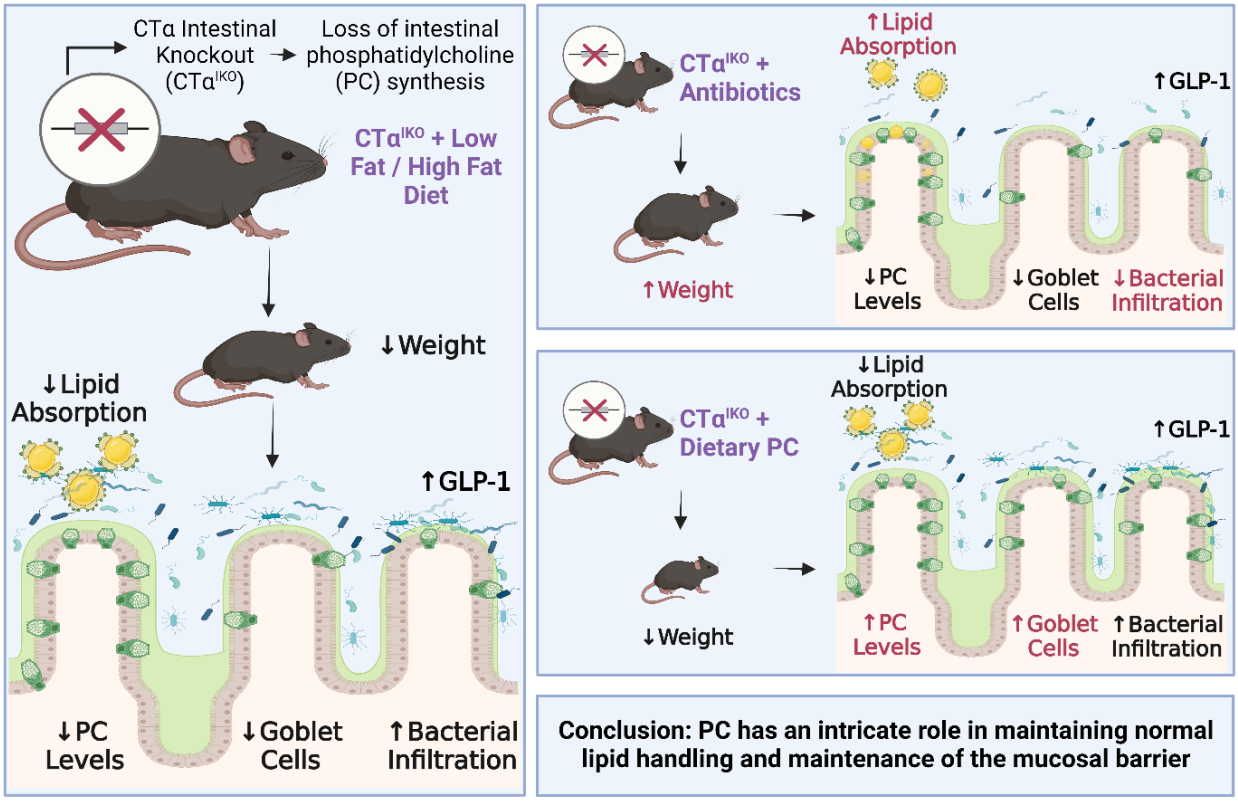

Figure 6.3: The consequences of CTα^{IKO} mice in small intestinal homeostasis. When CTα is knocked out of small intestinal epithelial cells (IEC) there is a loss of *de novo* phosphatidylcholine (PC) synthesis and a subsequent reduction in intestinal PC availability. Regardless of dietary fat content, CTα^{IKO} mice present with weight loss, reduced IEC PC level, reduced lipid absorption, reduced goblet cell number, bacterial infiltration, and increased GLP-1 secretion. When CTα^{IKO} mice are treated with antibiotics they have improved weight gain, improved lipid absorption, and reduced bacterial infiltration. Despite these improvements, antibiotic-treated CTα^{IKO} mice maintain reduced IEC PC level, reduced lipid absorption, reduced goblet cell number, and increased GLP-1 secretion. When CTα^{IKO} mice are fed a diet supplemented with PC they have normalized IEC PC level and an improved goblet cell count. Despite these improvements, PC supplemented CTα^{IKO} mice have reduced weight gain, reduced lipid absorption, bacterial infiltration, and increased GLP-1 secretion. In conclusion, PC has an intricate role in maintaining normal lipid handling and maintenance of the mucosal barrier that cannot be fully compensated by either antibiotic treatment or PC supplementation.

The colon, much like the small intestine is comprised of IECs, mostly colonocytes (the absorptive cells of the colon), goblet cells, and enteroendocrine cells (Robert Ehehalt, Braun, Karner, Füllekrug, & Stremmel, 2010; McCauley & Guasch, 2015; Parikh et al., 2019; Shamsuddin, Phelps, & Trump, 1982; Sjölund, Sandén, Håkanson, & Sundler, 1983). These cells work together to create the mucosal barrier and protect against bacterial translocation and inflammation. Alterations to the mucosal barrier can lead to colonic inflammation and ultimately disease states, such as colitis. Inflammatory bowel diseases, including ulcerative colitis, are autoimmune diseases that lead to severe inflammation of the colon, weight loss, and diarrhea (Guan, 2019; Hardy, 1949; Warren & Sommers, 1949). In Chapter 4, we explored the role of CDP-choline derived PC in the colon. In our $CT\alpha^{IKO}$ mice, the Cre-Lox system also affects the IECs of the colon, though to a lesser degree. Despite isolated IECs from colons of $CT\alpha^{IKO}$ mice having just under 50% of CTα protein levels left, they still have a significant reduction in the PC:PE ratio. This alteration in phospholipid homeostasis was enough to cause our mice to develop spontaneous colitis – as seen by IEC injury and infiltration of lymphocytes and neutrophils along the length of the colon and the cecum (Figure 6.4). Interestingly, recent evidence shows that the colonic mucous collected from patients with ulcerative colitis have a significant reduction in PC levels (Braun et al., 2009; R. Ehehalt et al., 2004). While the role of PC in the development and progression of ulcerative colitis is unknown, patients with ulcerative colitis have improved disease management and quality of life after being treated with delayed-release PC medications (Karner et al., 2014; W. Stremmel et al., 2005; Wolfgang Stremmel, Ehehalt, Autschbach, & Karner, 2007).

Chapter 4 also aimed to determine the pathogenesis of spontaneous colitis in our mice. As transcriptomics and gene analysis from the small intestine (Chapter 3) have shown, reducing the PC:PE ratio in IECs led to ER stress, necroptosis, goblet cell loss, and bacterial stress. We also

observed these changes in the colonic IECs of CTα^{IKO} mice which explains the development of spontaneous colitis. Electron microscopy of IECs in CTα^{IKO} mice showed dilated and distended ERs. Additionally, CTα^{IKO} mice had increased protein levels involved in all arms of the unfolded protein response which were not attenuated by PBA treatment. PBA is a chemical chaperone that reduces the incidence of misfolded proteins indicating that the activation of the unfolded protein response is not protein misfolding, strengthening the argument that the ER stress is caused by a reduced PC:PE ratio. Colonic IECs also have an increased incidence of alternative forms of programmed cell death, including necroptosis and pyroptosis that lead to a significant reduction in colonic goblet cells. Consequently, CTα^{IKO} mice have a reduced thickness of the mucosal layer. Interestingly, patients with ulcerative colitis have damaged goblet cells, which leads to altered integrity of the mucosal layer (B H Smith, 1967; Gersemann et al., 2009). Additionally, the thickness of the mucosal layer in the colons of patients with ulcerative colitis is inversely correlated to the severity of their colonic inflammation (Strugala, Dettmar, & Pearson, 2008). Finally, the development of spontaneous colitis in CTα^{IKO} mice was not prevented by increasing the supply of dietary PC or treatment with antibiotics (Figure 6.4).

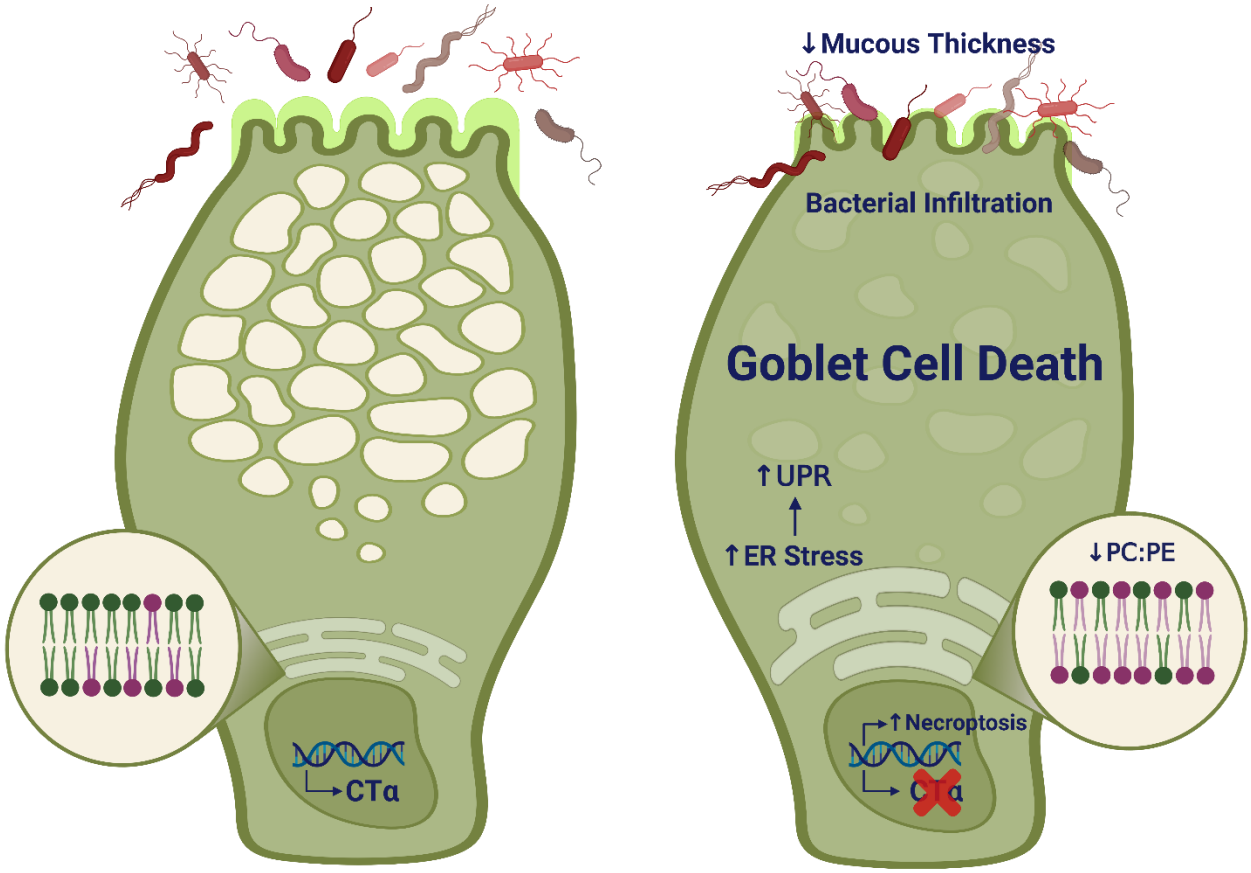

Figure 6.4: The mechanism of spontaneous colitis development in CTα^{IKO} mice. When CTα is knocked out of the intestinal epithelial cells in the colon it leads to a reduction in the availability of cellular PC and subsequently an altered PC:PE ratio. A reduced PC:PE ER membrane ratio induces ER stress including the unfolded protein response (UPR). The UPR is a known inducer of necroptosis, an alternative form of programmed cell death. In CTα^{IKO} mice, goblet cells were especially susceptible to necroptosis leading to a reduction in the thickness of the mucous membrane. A reduction in the mucous membrane allowed for bacterial translocation into intestinal epithelial cells and an induction of the inflammatory response leading to the development of spontaneous colitis.

The gallbladder has an important role in enterohepatic circulation as the storage organ for bile, and for hormonal regulation of biliary release into the small intestine upon dietary stimuli (Krishnamurthy & Brown, 2002; Lanzini, Jazrawi, & Northfield, 1987; Helen H. Wang et al., 2010). In Chapter 5 we discussed how alterations in intestinal *de novo* PC synthesis affected function of the gallbladders as CTα^{IKO} mice have increased gallbladder size in the postprandial state. The research performed is in conflict with previously published data showing that CTα^{IKO} mice whose gallbladders were cannulated have increased bile flow (Kennelly et al., 2018). CTα^{IKO} mice were also found to have increased secretion of bile acids, PC, and cholesterol. The conclusions drawn were that a reduction in small intestinal PC levels altered enterohepatic circulation leading to increased bile flow as a compensatory mechanism to increase PC availability to the small intestine (Kennelly et al., 2018). Surprisingly, we determined in Chapter 3 that feeding CTα^{IKO} mice a diet supplemented with PC improved IEC total PC levels and improved lipid metabolism. The impact of dietary PC was surprising given that CTα^{IKO} mice were initially thought to have increased small intestinal PC availability through biliary secretion.

Interestingly, when the gallbladders of CTα^{IKO} mice were cannulated, that diminished the functional role of the gallbladder by bypassing hormonal regulations. CTα^{IKO} mice have reduced duodenal mRNA expression of *Cck*, as well as reduced circulating plasma CCK levels. The altered CCK hormonal regulation likely accounts for the enlarged gallbladders. CCK knockout mice have significantly reduced biliary secretion and enlarged gallbladders in the postprandial state, as seen in our mice (H. H. Wang, Liu, Portincasa, Tso, & Wang, 2016). To improve gallbladder contractility in CTα^{IKO} mice, we injected them with a CCK analogue. CCK-injected CTα^{IKO} mice did not have improved lipid absorption but surprisingly had improved weight gain compared to saline-injected mice. Chapter 5 introduces a novel role of intestinal *de novo* PC synthesis in the

regulation of CCK levels and subsequently gallbladder function. Additionally, the data reiterates how complex a role intestinal PC synthesis has on maintaining communication between the gut-liver axis.

6.2 Future directions

Despite the research performed in this thesis, as well many years of research into the importance and function of PC in our health, there still remains many unknown factors. In agreement with that statement, future directions for this research are extensive. With all the attempted treatments in CTα^{IKO} mice (including PC supplementation, antibiotic treatment, bile acid supplementation, and CCK injections) none were able to fully resolve the changes in lipid absorption and metabolism. Next steps for this research include attempting all the previous treatments at one once to see if they are all needed for proper lipid absorption. Additionally, the use of external pancreatic enzymes should be considered since CCK also regulates the release of pancreatic enzymes into the lumen of the small intestine. With the reduction in CCK, CTα^{IKO} mice may not be receiving pancreatic enzymes along with bile. Another area of this research that has not been answered is the cause of weight loss in CTα^{IKO} mice. Some treatments given to CTα^{IKO} mice have improved weight loss without improving lipid absorption and vice versa. Antibiotics are the only treatment that have improved some aspects of both weight loss and lipid malabsorption in CTα^{IKO} mice. Studies into the mitochondria in CTα^{IKO} mice should be considered in an attempt to explain these findings.

In Chapter 3 we tried to determine the cause of the increased secretion of GLP-1 in CTα^{IKO} mice. Unfortunately, we were only able to determine that the ileal brake – a known inducer to GLP-1 secretion – is not the cause. Future work should be done to determine how GLP-1 is

increased as it may be very beneficial to those who are overweight or those suffering from type 2 diabetes. GLP-1 analogues are used as very useful treatments for these conditions. Finally, chapter 4 determined that reduced colonic CDP-choline derived PC leads to the development of spontaneous colitis. Future work should look into delineating the role of PC in the development of colitis. Experiments should include inducing colitis in mice with normal CDP-choline pathways and determining whether there are alterations in PC regulation. Additionally, experiments should include treating $CT\alpha^{IKO}$ mice with traditional colitis medications and determining if that improves PC synthetic pathway, ER stress, or goblet cell health.

In conclusion, CDP-derived PC has an intricate role in the maintenance of cellular, organ, and systemic homeostasis. CDP-derived PC has a variety of important functions including regulating lipid metabolism, maintaining physiologic hormonal levels, and the prevention of disease states including NAFLD and colitis. In particular, this research shows the importance of PC in maintaining hepatic and intestinal homeostasis. Additionally, this research also highlights an important role for PC in the communication throughout gut-liver axis and shows how disrupting one small portion can have a great effect on the entire system.

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