De Novo Phosphatidylcholine Synthesis in Intestinal Lipid Metabolism and Disease

by

John Paul Kennelly

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Department of Agricultural, Food and Nutritional Science

University of Alberta

Abstract

Phosphatidylcholine (PC), the most abundant phospholipid in eukaryotic cells, is an important component of cellular membranes and lipoprotein particles. The enzyme CTP: phosphocholine cytidylyltransferase (CT) regulates *de novo* PC synthesis in response to changes in membrane lipid composition in all nucleated mammalian cells. The aim of this thesis was to determine the role that CTα plays in metabolic function and immune function in the murine intestinal epithelium.

Mice with intestinal epithelial cell-specific deletion of CTα ($CT\alpha^{IKO}$ mice) were generated. When fed a chow diet, $CT\alpha^{IKO}$ mice showed normal lipid absorption after an oil gavage despite a ~30% decrease in small intestinal PC concentrations relative to control mice. These data suggest that biliary PC can fully support chylomicron output under these conditions. However, when acutely fed a high-fat diet, $CT\alpha^{IKO}$ mice showed impaired intestinal fatty acid and cholesterol uptake from the intestinal lumen into enterocytes, resulting in lower postprandial plasma triglyceride concentrations. Impaired intestinal fatty acid uptake in $CT\alpha^{IKO}$ mice was linked to disruption of intestinal membrane lipid transporters (*Cd36*, *Slc27a4* and *Npc1l1*) and higher postprandial plasma Glucagon-like Peptide 1 and Peptide YY. Unexpectedly, there was a shift in expression of bile acid transporters to the proximal small intestine of $CT\alpha^{IKO}$ mice, which was associated with enhanced biliary bile acid, PC and cholesterol output relative to control mice.

Gene expression profiling of small intestinal epithelial cells showed induction of transcripts linked to cellular proliferation and inflammation in $CT\alpha^{IKO}$ mice relative to control mice. Colonic inflammation after loss of intestinal epithelial cell CTα was linked to increased intestinal permeability (as assessed by Fluorescein Isothiocyanate-Dextran gavage), invasion of the intestinal epithelium by microbes, and enhanced inflammatory cytokine secretion. Impaired

intestinal barrier function in CTα^{IKO} mice was mechanistically linked to induction of endoplasmic reticulum stress and depletion of goblet cells. Antibiotics and 4-phenylbutyric acid both partially ameliorated inflammatory cytokine secretion in CTα^{IKO} mice, suggesting that microbes and endoplasmic reticulum stress are key drivers of the inflammatory phenotype in CTα^{IKO} mice. In further support of a role for *de novo* PC synthesis in colonic barrier function, feeding C57BL/6J mice a choline-deficient diet increased their susceptibility to *Citrobacter rodentium*-induced colitis relative to mice fed sufficient dietary choline.

In conclusion, *de novo* PC synthesis in the small intestinal epithelium is required for dietary lipid absorption under certain dietary conditions, and the re-acylation of biliary lyso-PC cannot compensate for loss of CTα under these conditions. Furthermore, CTα activity prevents invasion of the intestinal epithelium by microbes. Accordingly, disruption of CTα in intestinal epithelial cells induces spontaneous colitis in mice. Finally, an adequate supply of dietary choline, the essential nutritional substrate for CTα, is an important factor in protecting the colon from *Citrobacter rodentium* -induced inflammation.

Preface

This thesis is original work by John Paul Kennelly. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta's Institutional Animal Care Committee and is listed as '*Dietary determinants of metabolic disorders*' with protocol number AUP00000175, July 2009. The contributions made by the candidate, John P. Kennelly, and the co-authors of these studies, are described below.

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List of abbreviations

AdoMet - S-adenosylmethionine
AA – amino acid
Apo – apolipoprotein
ATP - adenosine triphosphate
CD36 - cluster of differentiation 36
CDP - cytidine diphosphate
CEPT - CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase
CK - choline kinase
CPT - CDP-choline:1,2-diacylglycerol cholinephosphotransferase
CT - CTP: phosphocholine cytidylyltransferase
CTP - cytidine triphosphate
DAVID – Database for Annotation, Visualization and Integrated Discovery
DG – diacylglycerol
EK - ethanolamine kinase
ET - CTP:phosphoethanolamine cytidylyltransferase
ER – endoplasmic reticulum
FA – fatty acid
FATP4 – fatty acid transport protein 4
FABP6 – fatty acid binding protein 6
FGF21 - Fibroblast-like growth factor 21
FPLC - fast protein liquid chromatography
GLP-1 - glucagon-like peptide 1
H&E - hematoxylin and eosin
HFD - high fat diet
IEC – intestinal epithelial cell
LPCAT - lyso-PC acyltransferase
MAM – mitochondria-associated membranes
MTP- microsomal triacylglycerol transfer protein
TG – triglyceride
PC - phosphatidylcholine
PE – phosphatidylethanolamine
PEMT - phosphatidylethanolamine N-methyltransferase
PS – phosphatidylserine
PSD - phosphatidylserine decarboxylase
PYY - peptide tyrosine tyrosine
SREBP - sterol regulatory element-binding proteins
TG – triglyceride
UC – ulcerative colitis
VLDL – very low-density lipoprotein

CHAPTER 1: Introduction and literature review

A modified version of this chapter has been published as van der Veen, J. N., J. P. Kennelly, S. Wan, J. E. Vance, D. E. Vance, and R. L. Jacobs. 2017. *The critical role of phosphatidylcholine and phosphatidylethanolamine metabolism in health and disease*. Biochim Biophys Acta 1859: 1558-1572.

1.1 Phospholipid metabolism

1.1.1 Overview of phospholipids

Phosphatidylcholine (PC) is the most abundant phospholipid of all mammalian cell types and subcellular organelles. In general, PC comprises 40–50% of total cellular phospholipids, although different cell types, individual organelles and even the two leaflets of organelle membranes contain distinct phospholipid compositions (Verkleij et al., 1973). The second most abundant phospholipid in mammalian membranes is phosphatidylethanolamine (PE), which is enriched in mitochondrial inner membranes (~40% of total phospholipids) compared to other organelles (15–25% of total phospholipids). PC and PE can contain acyl-, ether-, or vinyl-ether bonds at the *sn*-1 position and are thus sub-classified into diacyl, alkylacyl or alkenylacyl phospholipids, respectively (Yamashita et al., 2014). Most PC and PE (~95–100%) in rat and human liver is diacylated (Diagne et al., 1984). Furthermore, an enormous diversity of PC and PE molecular species is present in mammalian cells since the acyl-chain constituents of PC and PE can be remodeled by the action of phospholipases and lysophospholipid acyltransferases [(LANDS, 1958, Hishikawa et al., 2008, Yamashita et al., 1997) reviewed in (Hishikawa et al., 2014)]. In the 1950s, Eugene Kennedy and co-workers performed groundbreaking research that established the general outline of many phospholipid biosynthetic pathways (Kennedy and Weiss,

1956). A key, unexpected, finding was that a substrate for the biosynthesis of PC and PE was CTP, rather than ATP (Kennedy and Weiss, 1956). This was the first report that high-energy compounds, other than ATP, could be used for activation of metabolites. It is unlikely that Kennedy and colleagues would have predicted the broad impact that alterations in PC and PE metabolism would have on disease processes.

The overall aim of this thesis is to investigate the physiological role of *de novo* PC synthesis in dietary lipid uptake and intestinal barrier function. Our results show that *de novo* PC synthesis is involved in maintaining many important aspects of intestinal physiology including lipid uptake, normal enterohepatic circulation of bile, enteroendocrine hormone secretion, and intestinal immune homeostasis by mechanisms that involve both the small and large intestine. This chapter will first introduce literature on the molecular and cell biology of phospholipids, and current understanding of the role of phospholipids in mammalian physiology. This chapter will then introduce relevant concepts in intestinal physiology, intestinal lipid metabolism, and intestinal barrier function.

1.1.2 PC biosynthesis by the CDP-choline pathway

In all nucleated mammalian cells PC is synthesized by the CDP-choline pathway (**Figure 1.1**), also called the Kennedy pathway (Kennedy and Weiss, 1956). Choline enters the cell via three classes of choline transporters: the high-affinity transporter (CHT1), the intermediate-affinity transporters (CTL family) and the low-affinity organic cation transporters (OCT family) [(Meyer et al., 1982, Hegazy and Schwenk, 1984) and reviewed in (Traiffort et al., 2013)]. Upon entering the cell, choline is rapidly phosphorylated by ATP to phosphocholine via the cytosolic enzyme choline kinase (CK) encoded by two distinct genes *Chka* and *Chkb* from which CKα and CKβ, respectively, are derived [(Wittenberg and Kornberg, 1953, Tadokoro et al., 1985) and reviewed

in (Aoyama et al., 2004)]. The second reaction of the CDP-choline pathway for PC synthesis is the conversion of CTP and phosphocholine to CDP-choline via the enzyme CTP:phosphocholine cytidylyltransferase (CT) (**Figure 1.1**). Two isoforms of CT have been identified in mice - CTα, which is encoded by the *Pcyt1a* gene, and CTβ, which is the product of the *Pcyt1b* gene (Lykidis et al., 1998, Kalmar et al., 1990). CTα is ubiquitously expressed in mammalian tissues whereas CTβ is most highly expressed in the brain (Lykidis et al., 1999, Karim et al., 2003). CTα contains a nuclear localization signal and resides primarily in the nucleus (Wang et al., 1993b, Northwood et al., 1999, Gehrig and Ridgway, 2011, Aitchison et al., 2015, Wang et al., 1993a). In contrast, CTβ lacks a nuclear localization signal and, consequently, is exclusively extra-nuclear. Both CT isoforms are amphipathic proteins that can associate reversibly with membranes, particularly those enriched in diacylglycerol (DG) and fatty acids; reviewed in (Cornell and Northwood, 2000, Cornell and Ridgway, 2015). In addition, the association of CTα with membranes results in dephosphorylation of the protein thereby increasing its enzymatic activity (Houweling et al., 1994, Chong et al., 2014, Pelech and Vance, 1984). Thus, upon translocation to membranes (Pelech et al., 1983, Cornell and Vance, 1987, Johnson et al., 2003), CTα becomes activated [reviewed in (Cornell and Northwood, 2000, Cornell and Ridgway, 2015, Pelech and Vance, 1984)]. The reaction catalyzed by CT is, under most metabolic conditions, the rate-limiting reaction for PC synthesis via the CDP-choline pathway (Choy et al., 1979). The final reaction in the CDP-choline pathway is catalyzed by the integral membrane protein CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) and the dual-specificity protein, CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase (CEPT) (Henneberry and McMaster, 1999, Henneberry et al., 2000, Van Golde et al., 1971). These proteins are tightly embedded in the

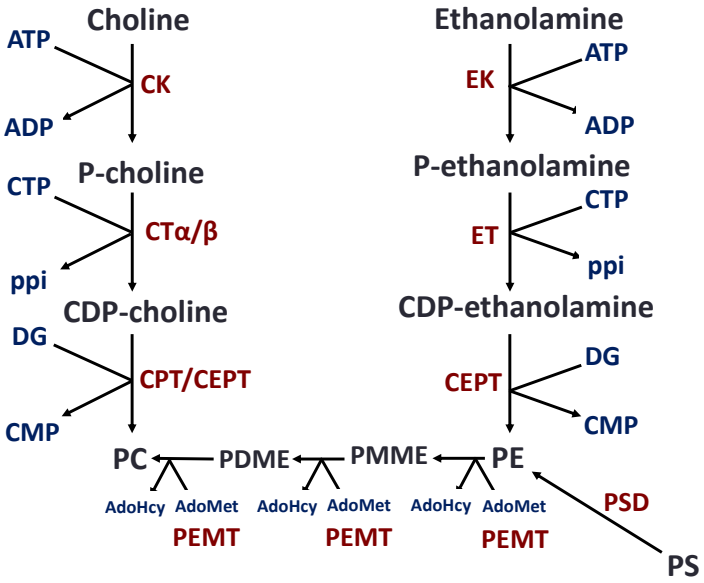

Figure 1.1 Biosynthetic pathways for phosphatidylcholine (PC) and phosphatidylethanolamine (PE) in mammalian cells. In the CDP-choline pathway, choline enters the cell and is rapidly phosphorylated to phosphocholine by the cytosolic enzyme choline kinase (CK). In the rate-limiting step of the pathway, phosphocholine is converted to CDP-choline by CTP:phosphocholine cytidylyltransferase (CT), an amphitropic enzyme that is activated when bound to membranes. In the final step of the CDP-choline pathway, phosphocholine is transferred from CDP-choline to diacylglycerol (DG) by the integral ER membrane proteins, CDP-choline:1,2-diacylglycerol cholinephosphotransferase (CPT) or CDP-choline:1,2-diacylglycerol choline/ethanolamine phosphotransferase (CEPT), to produce PC. In the other PC biosynthetic pathway, PE is converted to PC by three successive methylation reactions catalyzed by phosphatidylethanolamine N-methyltransferase (PEMT) using S-adenosylmethionine as the methyl-group donor. PEMT activity is only quantitatively important in hepatocytes of mammals. PE is made by two spatially-separated biosynthetic pathways. In the CDP-ethanolamine pathway, which parallels the CDP-choline pathway for PC synthesis, ethanolamine is phosphorylated to phosphoethanolamine by the cytosolic enzyme ethanolamine kinase (EK). Another cytosolic protein, CTP:phosphoethanolamine cytidylyltransferase (ET) converts phosphoethanolamine and CTP to CDP-ethanolamine. The final step in the pathway is catalyzed by the ER integral membrane CEPT which transfers phosphoethanolamine to DG to generate PE in the ER. The alternative pathway for PE synthesis, the phosphatidylserine (PS) decarboxylase (PSD) pathway occurs only in mitochondrial inner membranes. PS is imported from its site of synthesis in the ER/MAM to mitochondrial inner membranes where PSD converts PS to PE.

endoplasmic reticulum (ER) membrane (Van Golde et al., 1971, Vance and Vance, 1988, Henneberry et al., 2002) and transfer phosphocholine from CDP-choline to DG thereby generating PC; some CPT activity also resides in Golgi membranes (Henneberry et al., 2002)(**Figure 1.1**).

1.1.3 PC biosynthesis by phosphatidylethanolamine N-methyltransferase

In addition to the CDP-choline pathway for PC synthesis, which is ubiquitously expressed in all nucleated mammalian cells, the liver also utilizes an additional pathway for PC synthesis [(Bremer and Greenberg, 1959, Bremer et al., 1960); reviewed in (Vance, 2014a)]. In this pathway, PE is methylated to PC via three sequential methylation reactions in which S-adenosylmethionine (AdoMet) is the methyl group donor (Bremer et al., 1960, Bremer and Greenberg, 1961)(**Figure 1.1**). The enzyme phosphatidylethanolamine N-methyltransferase (PEMT) catalyzes all three methylation reactions [(Bremer and Greenberg, 1959, Bremer et al., 1960, Bremer and Greenberg, 1961) reviewed in (Vance, 2014a, Vance, 2013)]. Although the liver is the only mammalian tissue that contains significant PEMT protein or activity (Bremer and Greenberg, 1961, Ridgway and Vance, 1988), a small amount of PEMT activity (less than 1% of that in liver) has been reported in adipocytes during differentiation (Cole and Vance, 2010, Hörl et al., 2011). In rodents, approximately 30% of PC biosynthesized in the liver is derived from the PEMT pathway with the remaining 70% of PC being generated by the CDP-choline pathway (DeLong et al., 1999). PEMT is a small (~20 kDa) integral ER membrane protein (Ridgway and Vance, 1987) that is highly enriched in specialized ER-mitochondria membrane contact sites called “mitochondria-associated membranes” (MAM). MAM consist of a specific domain of the ER that transiently forms contact sites with mitochondrial outer membranes, (Vance, 1990, Cui et al., 1993) and have been implicated in the import of some phospholipids, particularly phosphatidylserine (PS), into mitochondria (Cui et al., 1993, Vance, 2014b).

1.1.4 Phospholipid remodeling by Lands' cycle

In most mammalian cells under steady-state conditions, phospholipids contain a saturated fatty acid at the *sn*-1 position and an unsaturated fatty acid at the *sn*-2 position (Hanahan et al., 1960). This divergence in the acyl chain content of the *sn*-1 and *sn*-2 position of phospholipids is primarily achieved through a remodeling process that occurs after *de novo* synthesis (LANDS, 1958, Lands, 1960). During the phospholipid remodeling process, termed Land's Cycle (**Figure 1.2**), phospholipase enzymes hydrolyze a fatty acid from the *sn*-2 position of the phospholipid before lyso-phospholipid acyltransferase enzymes catalyze the esterification of a fatty acyl CoA onto the vacant *sn*-2 position (King, 1931, Epstein and Shapiro, 1959, LANDS, 1958, Lands, 1960) (**Figure 1.2**). Land's cycle ensures diversity of phospholipid species in cells and facilitates tissue-specific phospholipid requirements (e.g. a high proportion dipalmitoyl-PC in lung)(Huang, 2001, Harayama et al., 2014). Lyso-PC acyltransferase 3 (LPCAT3) is the predominant LPCAT in the liver and intestine and plays an important role in lipoprotein metabolism (discussed below).

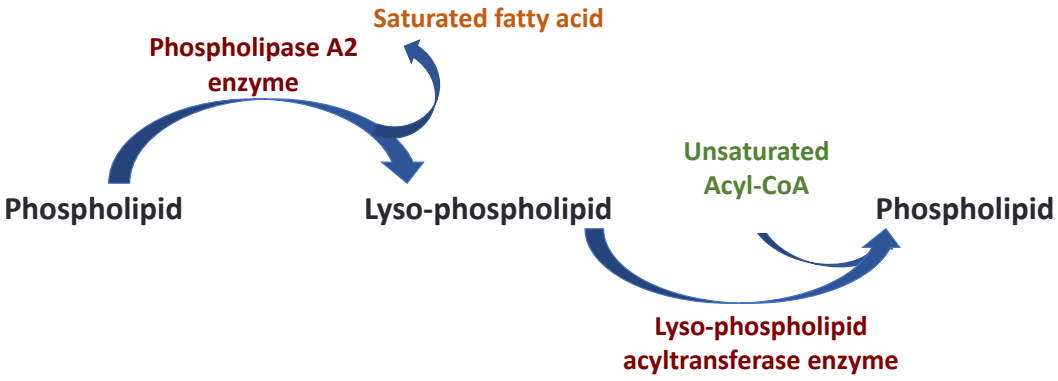

Figure 1.2 Lands' cycle of phospholipid remodeling. After *de novo* synthesis, phospholipids can be remodeled by Land's cycle. During this process, a phospholipase enzyme hydrolyzes a fatty acid from the *sn*-2 position of the phospholipid before a lyso-phospholipid acyltransferase enzyme catalyzes the esterification of a fatty acyl CoA onto the vacant *sn*-2 position of the phospholipid. Lands' cycle ensures that phospholipids in most mammalian cell types typically contain a saturated fatty acid at the *sn*-1 position and an unsaturated fatty acid at the *sn*-2 position.

1.1.5 PE biosynthesis

The two major pathways utilized by mammalian cells for the biosynthesis of PE are the CDP-ethanolamine pathway (Kennedy and Weiss, 1956) and the phosphatidylserine decarboxylase (PSD) pathway (Percy et al., 1983, Zborowski et al., 1983, Bremer et al., 1960)(**Figure 1.1**). The PE derived from these two pathways is synthesized in two spatially separated organelles: the ER and mitochondria, respectively. Small amounts of PE can also be synthesized by a base-exchange reaction in the ER, catalyzed by phosphatidylserine synthase-2, in which the serine residue of PS is exchanged for ethanolamine (Sundler et al., 1974, Bjerve, 1984). The reactions of the CDP-ethanolamine pathway for PE synthesis parallel those of the CDP-choline pathway for PC synthesis (Kennedy and Weiss, 1956). Ethanolamine is imported into the cell via transporters that are incompletely defined. The ethanolamine is subsequently phosphorylated by two cytosolic, ethanolamine-specific kinases that are particularly abundant in the liver and reproductive tissues (Lykidis et al., 2001, Tian et al., 2006, Gustin et al., 2008)

(**Figure 1.1**). The second reaction of the CDP-ethanolamine pathway is catalyzed by the cytosolic enzyme CTP:phosphoethanolamine cytidylyltransferase (ET), which is encoded by the *Pcyt2* gene (Nakashima et al., 1997, Poloumienko et al., 2004)(**Figure 1.1**). This enzyme converts CTP and phosphoethanolamine to CDP-ethanolamine and pyrophosphate and is normally the rate-limiting enzyme of the pathway (Sundler and Akesson, 1975, Sundler, 1975, Tijburg et al., 1987). The ET protein contains two similar catalytic motifs, both of which are required for activity (Nakashima et al., 1997, Tie and Bakovic, 2007); however, unlike CT, ET activity is not regulated by reversible binding to membranes. The final step of this pathway for PE synthesis is mediated by CEPT and, alternatively, by CDP-ethanolamine:1,2-ethanolaminephosphotransferase (Henneberry and McMaster, 1999, Horibata and Hirabayashi, 2007)(**Figure 1.1**). These enzymes are integral membrane proteins of the ER and convert CDP-ethanolamine and DG to PE (**Figure 1.1**). The other major pathway for PE synthesis in mammalian cells is the PSD pathway that operates exclusively on the outer aspect of mitochondrial inner membranes (Percy et al., 1983, Zborowski et al., 1983). The substrate for the decarboxylase is PS that is made by two PS synthases, PSS1 and PSS2, which are restricted to ER membranes (HUBSCHER et al., 1959, Suzuki and Kanfer, 1985, Kuge et al., 1997). These two PS synthases are highly enriched in the MAM (Stone and Vance, 2000). The translocation of PS from the ER/MAM to the site of PSD in mitochondrial inner membranes is ATP-dependent and is the rate-limiting step for PE synthesis via this route (Shiao et al., 1995, Voelker, 1989). PS-derived PE can be rapidly exported, by unknown mechanisms, from mitochondria to other cellular organelles, such as the ER and plasma membrane (Shiao et al., 1995, Vance et al., 1991, Kainu et al., 2013). However, the PE that is present in mitochondrial membranes is preferentially derived in situ from PSD rather than from the CDP-ethanolamine pathway in the ER (Shiao et al., 1995). Thus, the pools of PE made by the two spatially separated

pathways (in the ER from CDP-ethanolamine and in mitochondria from PSD) are mainly compartmentalized. This conclusion is supported by experiments in knockout mice in which genetic elimination of either PE biosynthetic pathway is embryonically lethal despite normal, or enhanced, operation of the other pathway (Steenbergen et al., 2005, Fullerton et al., 2007, Leonardi et al., 2009).

1.2 The subcellular roles of PC and PE synthesis

1.2.1 Phospholipids control *de novo* lipogenesis via regulation of sterol regulatory element-binding proteins (SREBPs)

The SREBPs are master regulators of *de novo* lipid synthesis in mammalian cells [(Rajavashisth et al., 1989, Briggs et al., 1993) and reviewed in (Brown and Goldstein, 1997)]. SREBP-1a and -1c preferentially regulate the expression of genes involved in fatty acid, phospholipid and TG synthesis, whereas SREBP-2 preferentially regulates expression of genes involved in cholesterol synthesis (Horton et al., 2002). The processing of SREBP-2 into its mature, active form is regulated by cholesterol levels in the ER [(Wang et al., 1994) and reviewed in [(Horton et al., 2002, Brown and Goldstein, 1997)]. SREBP-1 processing is regulated by insulin (Browning and Horton, 2004) and there is also evidence that ER phospholipid composition influences SREBP-1 processing (Dobrosotskaya et al., 2002, Seegmiller et al., 2002, Lim et al., 2011, Walker et al., 2011, Rong et al., 2017)(**Figure 1.3**). In Drosophila melanogaster, PE is the most abundant phospholipid in membranes (50% of total phospholipids). In this organism, processing of SREBP is inhibited by PE in a feedback manner. For example, when the level of PE was increased in Drosophila S2 cells, maturation and activation of SREBP-1 were inhibited. Furthermore, low PE levels in hearts of the Drosophila easily shocked mutants which lacked ethanolamine kinase, stimulated the SREBP pathway (Lim et al., 2011). Thus, PE appears to be a

major regulator of the SREBP pathway in Drosophila. Regulation of SREBPs by phospholipids is not restricted to Drosophila. For example, in both Caenorhabditis elegans and mammalian cells (in which PC is the major phospholipid), PC inhibited SREBP-1 processing (Walker et al., 2011). In C. elegans, reducing PC synthesis by inhibition of the CDP-choline pathway, the PEMT pathway or the production of AdoMet, increased the processing and maturation of SBP-1 (the C. elegans ortholog of SREBP) and increased expression of its target genes. Similarly, when PC synthesis was attenuated in human hepatoma cells (by small interfering RNAs directed against one of CTα, CEPT, or PEMT) mature SREBP-1 accumulated in the nucleus (Walker et al., 2011). Moreover, in mouse livers that lacked CTα, PC levels were reduced leading to increased maturation of SREBP-1 and increased expression of lipogenic genes (Walker et al., 2011). The mechanism by which low PC levels activate SREBP-1 involves inactivation of ADP ribosylation factor-1 (Smulan et al., 2016), which mis-localizes the site 1 protease and site 2 protease to the ER from the Golgi, thereby promoting ectopic proteolytic cleavage and activation of SREBP-1c. Disruption of Golgi function could also affect important cellular processes including Golgi-to-plasma membrane secretory pathways; however, these processes were not investigated. Additionally, lipin1, a phosphatidic acid phosphatase that generates DG, has been implicated in the activation of SREBP-1 in response to low PC levels (Smulan et al., 2016). Consequently, depletion of lipin1 restored the activation of SREBP-1 caused by low levels of PC. Thus, the lipogenic program mediated by the SREBPs appears to be activated when the balance among PC, DG and phosphatidic acid in the ER is disturbed (Smulan et al., 2016). The PC molecular species composition of the ER in hepatocytes also influences SREBP-1c processing in mice (Rong et al., 2017)(**Figure 1.3**). Liver X receptor (LXR)-mediated induction of LPCAT3 promoted SREBP-1c processing by promoting production of PC containing polyunsaturated fatty acids (Rong et al.,

2017). Furthermore, delivery of exogenous polyunsaturated PC species to the ER promoted SREBP-1c processing. Conversely, loss of LPCAT3 reduced the maturation of SREBP-1c (Rong et al., 2017). Thus, PC acyl chain composition, in addition to PC mass, influences SREBP-1c processing in the mammalian liver (**Figure 1.3**).

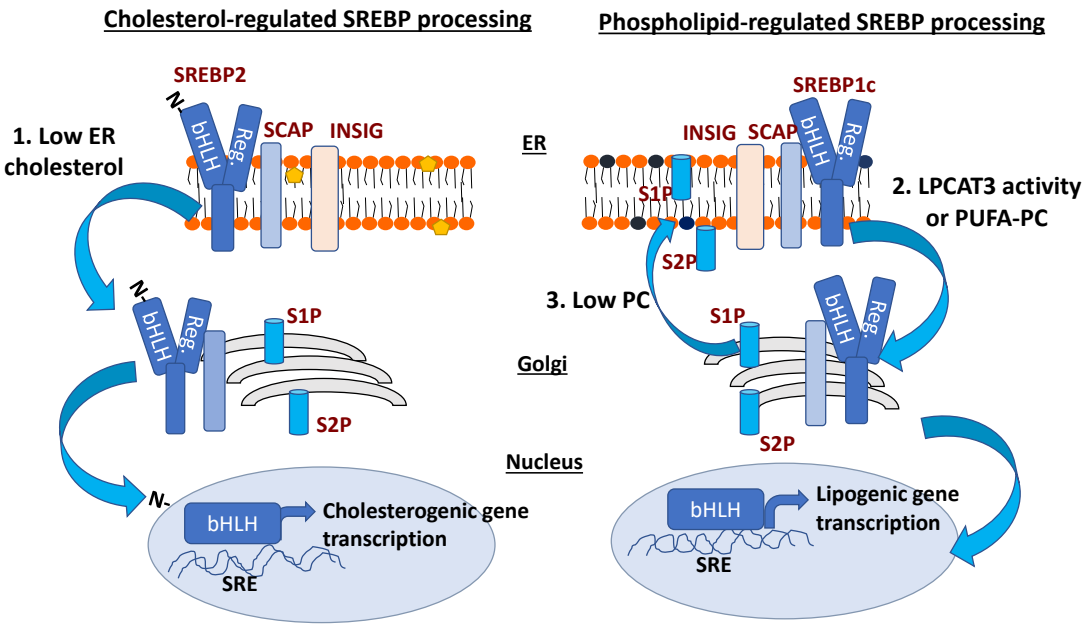

Figure 1.3. Cholesterol-regulated SREBP processing compared to phospholipid-regulated SREBP processing. **Cholesterol-regulated SREBP processing:** When ER cholesterol levels are high, Insulin-induced gene-1 and -2 (INSIG-1 and -2) bind to SREBP cleavage-activating protein (SCAP) to prevent SREBP from traveling to the Golgi for processing. When ER cholesterol levels drop below a threshold of ~5% total (molar) ER lipids (Radhakrishnan, Cell Metabolism, 2008), SCAP escorts SREBP to the Golgi by COPII-mediated vesicle transport. In the Golgi, Site-1-Protease (S1P) and Site-2-Protease (S2P) free the basic-helix-loop-helix leucine zipper (bHLH) active transcription factor domain of SREBP from the regulatory (Reg.) domain, allowing it to enter the nucleus to promote the transcription of cholesterogenic genes. **Phospholipid-regulated SREBP processing:** Changes to the phospholipid composition of the ER influences SREBP-1c processing. Liver X receptor (LXR)-mediated induction of LPCAT3 promotes SREBP-1c processing by increasing the abundance of PC species containing polyunsaturated fatty acids. Furthermore, reducing the PC content of the ER mis-localizes S1P and S2P from the Golgi to the ER, inducing constitutive SREBP-1c processing. Diagram adapted from Walker et al., Cell, 2011.

1.2.2 Phospholipids in mitochondria

Mitochondrial dysfunction has been implicated in the development of cardiovascular disease (Ren et al., 2010), type 2 diabetes (Supale et al., 2012), neurodegenerative diseases (Johri and Beal, 2012) and cancer progression (Baysal et al., 2000). The majority of ATP in mammalian cells is generated in mitochondria by oxidative phosphorylation *via* multimeric protein complexes (complexes I to IV) of the electron transport chain [reviewed in (Dudkina et al., 2011)]. Mitochondrial lipids define the physical properties of mitochondrial membranes and might, therefore, be expected to regulate the activities of proteins of the electron transport chain as well as ATP production [(Baker et al., 2016); reviewed in (Calzada et al., 2016)]. Indeed, a role for mitochondrial phospholipids in multiple key mitochondrial functions including programmed cell death (Choi et al., 2007), autophagy (Fadok et al., 2001, Hailey et al., 2010) and mitochondrial fusion (Steenbergen et al., 2005, Tasseva et al., 2013) has been proposed. For example, a reduced mitochondrial content of the non-bilayer-forming phospholipid cardiolipin (which normally comprises ~10% of mitochondrial phospholipids) disrupts the super-complexes of the respiratory chain that are embedded in the mitochondrial inner membrane (Pfeiffer et al., 2003, Zhang et al., 2005). Moreover, reduction in the mass of cardiolipin and specific cardiolipin molecular species underlies the cardiomyopathy in Barth syndrome [(Vreken et al., 2000, McKenzie et al., 2006); reviewed in (Hauff and Hatch, 2006)]. Although mitochondrial membranes consist of a wide variety of different lipids, the two most abundant phospholipids of mitochondria of mammalian cells are PC and PE, with PE being highly enriched in mitochondrial inner membranes (~40% of total phospholipids) compared to other organelle membranes (15–25% of total phospholipids). As discussed above, the majority of mitochondrial PE in mammalian cells is made in situ in mitochondria via PSD, with only a small fraction of mitochondrial PE being imported from the

ER (Shiao et al., 1995, Kainu et al., 2012, Birner et al., 2001). The importance of the mitochondrial synthesis of PE was highlighted when PE production via the mitochondrial enzyme PSD was globally eliminated in mice (Steenbergen et al., 2005). The *Pisd*$^{-/-}$ mice died during embryonic development (around day e9.5) and the embryos exhibited profound defects in mitochondrial morphology, including extensive mitochondrial fragmentation, even when PE production in the ER by the CDP-ethanolamine pathway was active. Moreover, whole-body elimination of the CDP-ethanolamine pathway (by genetic disruption of the *Pcyt2* gene encoding ET) was also embryonic lethal (Fullerton et al., 2007, Leonardi et al., 2009). Consequently, both major pathways for PE biosynthesis are required for viability/development of mice. Although the complete elimination of PSD activity caused embryonic lethality, heterozygous *Pisd*$^{+/-}$ mice with 50% reduction of PSD activity did not exhibit any obviously deleterious phenotype (Steenbergen et al., 2005). Therefore, the cause of lethality in the *Pisd*$^{-/-}$ mice was investigated in cultured Chinese hamster ovary cells in which PSD expression was reduced by ~85% using RNA interference (Tasseva et al., 2013). When the PE content of mitochondria, but not of whole cells, was decreased by ~20%, the PC/PE molar ratio of mitochondria was correspondingly increased, and cell survival and growth were severely impaired. In addition, oxygen consumption, cellular ATP levels and the rate of ATP production were markedly reduced, consistent with defects in complexes I and IV of the electron transport chain. Furthermore, mitochondria in the PSD-deficient cells were extensively fragmented and mitochondrial ultrastructure was grossly aberrant (Tasseva et al., 2013). Thus, even a modest decrease in mitochondrial content of the non-bilayer-forming phospholipid PE (corresponding to ~30% increase in the PC/PE molar ratio) profoundly impaired mitochondrial dynamics and functions. In contrast to the increased molar ratio of PC/PE in mitochondria of PSD-deficient cells (Tasseva et al., 2013), elimination of PEMT in mice decreased the molar ratio of PC/PE in liver

by 35% (to 1.17 from 1.81); in livers of *Pemt*$^{-/-}$ mice, the amount of mitochondrial PC was 19.4% lower than in *Pemt*$^{+/+}$ mice, whereas the level of PE (a substrate for PEMT) was 18.6% higher (van der Veen et al., 2014). In addition, the mitochondria in livers of mice lacking PEMT were smaller and more elongated than those of *Pemt*$^{+/+}$ mice. Interestingly, PEMT deficiency greatly attenuated the rate of hepatic glucose production from pyruvate (van der Veen et al., 2014). In addition, the lack of PEMT, and the corresponding increase in mitochondrial PE content, as well as the decrease in the PC/PE molar ratio, stimulated mitochondrial respiration and activities of proteins of the electron transport chain. Furthermore, the level of ATP in hepatocytes from *Pemt*$^{-/-}$ mice was double that in *Pemt*$^{+/+}$ hepatocytes. Indeed, a clear positive correlation was observed between the amount of mitochondrial PE and the ATP content of the hepatocytes (van der Veen et al., 2014). Thus, these experiments demonstrate that the mitochondrial PE content and/or the molar ratio of PC/PE can modulate key mitochondrial functions such as energy production.

1.2.3 Phospholipids and lipid droplet formation

Excess intracellular fatty acids are incorporated into TGs and stored in cytosolic lipid droplets to prevent lipotoxicity (Listenberger et al., 2003)(**Figure 1.4**). The neutral lipids in the lipid droplet core are surrounded by a monolayer of phospholipids [reviewed in (Pol et al., 2014)]. Inhibition of PC biosynthesis during conditions that promote TG storage increases the size of the lipid droplets because the ratio of surface area (amount of phospholipid) to volume (TG) of larger droplets is less than that of smaller droplets (Aitchison et al., 2015, Guo et al., 2008, Krahmer et al., 2011). Additionally, an increase in the relative amount of PE on the surface of lipid droplets can promote fusion of smaller droplets into larger ones (Krahmer et al., 2011).

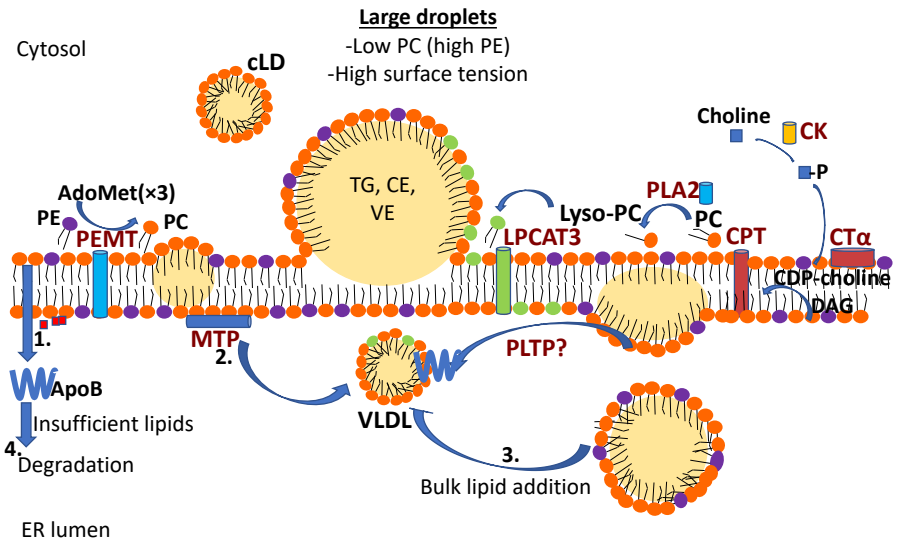

Figure 1.4. PC and the formation of cytosolic lipid droplets and VLDL particles. Excess intracellular fatty acids are funneled into TGs in the ER membrane before budding from the ER to form cytosolic lipid droplets. These droplets also store cholesterol esters (CE) and fat-soluble vitamin esters (VE). The size of cytosolic lipid droplets depends on factors including the relative abundance of PC on the surface of the ER and/or on the droplet. Adequate PC supply and/or low ER surface tension allows budding of small lipid droplets in mammalian cells (Ben M'barek et al., Dev Cell, 2017). Conversely, an increase in the relative amount of PE on the surface of lipid droplets can promote fusion of smaller droplets into larger ones**.** To form VLDL particles in the lumen of the ER, ApoB is first translated by ER-bound ribosomes and translocated into the lumen of the ER (**Step 1**). Microsomal triglyceride transfer protein (MTP) transfers lipids from the ER to the nascent ApoB, forming a nascent particle (**Step 2**). There is evidence that phospholipid transfer protein (PLTP) transfers phospholipids to ApoB particles in the ER lumen (Manchekar et al. JLR, 2015). The nascent ApoB particle may be further lipidated, possibly by merging with an ApoB-free droplet in the ER lumen (**Step 3.)**. If there are insufficient lipids available for the lipidation of ApoB, there will be impaired translocation or misfolding of ApoB, resulting in its degradation by ER-associated co-translational degradation and the ubiquitin-proteasome pathway (**Step 4). Some PC for lipid droplet and VLDL** formation is provided by PEMT, which converts PE to PC by three successive methylation reactions using S-adenosylmethionine (AdoMet) as the methyl-group donor. Alternatively, dietary choline is converted to PC by the CDP-choline pathway. PC on the surface of the ER can be remodeled by Lands' cycle to alter ER membrane fluidity and lipid loading to droplets.

Furthermore, addition of PC to the surface of expanding lipid droplets reduced the relative abundance of PE on the droplet and thereby prevented droplet coalescence (Krahmer et al., 2011). Krahmer et al. showed that CT is recruited to lipid droplets upon lipid loading of Drosophila Schneider 2 (S2) cells (Krahmer et al., 2011). The targeting of CT to lipid droplets increased cellular PC levels and promoted the formation of lipid droplets and the storage of TG. Nevertheless, since the final step of PC synthesis via the CDP-choline pathway is catalyzed by the ER integral membrane proteins CPT/CEPT, PC must be made on the ER membrane bilayer, not on the monolayer surrounding the lipid droplets. Adipocytes are specialized cells that store excess energy as TG in lipid droplets. During differentiation of 3T3-L1 cells into adipocytes, the requirement for PC is increased and CTα expression is strongly stimulated to facilitate the expansion of lipid droplets (Aitchison et al., 2015). In mammalian 3T3-L1 cells, however, unlike S2 insect cells, CTα does not associate with lipid droplets, but shuttles from the nucleoplasm to the nuclear envelope and cytoplasmic membranes (Aitchison et al., 2015). The difference between the subcellular localization of CT in insect and mammalian cells might be due to the large difference in relative abundance of PC and PE in the two cell types. In insect cells the cellular PC/PE molar ratio is 1/3 whereas in mammalian cells the PC/PE ratio is 3. Interestingly, during differentiation of 3T3-L1 fibroblasts into adipocytes, a process that involves storage of TG in lipid droplets, PEMT expression is strongly induced (Cole and Vance, 2010). In concert with increased PEMT expression during the differentiation period, levels of mRNAs encoding PS synthase-1 and PSD were increased, suggesting a tight regulation of phospholipid biosynthetic pathways during adipocyte differentiation and lipid droplet expansion (Hörl et al., 2011). As was the case for CTα in insect cells, a small portion of PEMT was localized to a region of the ER close to the periphery of lipid droplets, possibly as a mechanism for promoting localized PC synthesis for lipid droplet

formation (Hörl et al., 2011). Similarly, since PEMT contains four putative transmembrane domains, integration of PEMT into a lipid bilayer, rather than association with the monolayer surface of the droplet, would be required for enzymatic activity (Shields et al., 2003). Nevertheless, this juxtaposition of PEMT with lipid droplets seems to be important for both the formation and stability of lipid droplets in adipocytes, despite the very small amount of PEMT in adipocytes. For example, knock-down of PEMT in 3T3-L1 adipocytes increased basal hydrolysis of TG (Hörl et al., 2011). Moreover, adipocytes from *Pemt*$^{-/-}$ mice appeared to be resistant to diet-induced hypertrophy: when *Pemt*$^{-/-}$ mice were fed a high-fat diet, their adipocytes were smaller in diameter than those of *Pemt*$^{+/+}$ mice, although factors extrinsic to the adipocyte likely contribute to this effect (Jacobs et al., 2010). However, PEMT deficiency in mice did not impair adipocyte differentiation or lipolysis, but marginally reduced de novo lipogenesis in white adipose tissue (Gao et al., 2015). Intriguingly, a recent study showed that single nucleotide polymorphisms in the *PEMT* gene correlate with the percentage of body fat mass in humans (Sharma et al., 2013). High levels of *PEMT* and *PCYT1A* mRNA in adipose tissue were positively correlated with fat mass and waist-hip ratio, underscoring a role for PC biosynthesis in lipid droplet formation and/or adipocyte differentiation.

1.2.4 Phospholipids and very-low density lipoprotein (VLDL) secretion

Lipoprotein particles consist of a monolayer of phospholipids and cholesterol surrounding a neutral lipid core of triacylglycerols (TG), cholesteryl esters and vitamin esters. Other lipid species such as DG and sphingolipids can be present at relatively low concentrations on lipoproteins (Ståhlman et al., 2012). In humans, the assembly of TG-rich VLDL particles in the liver requires apolipoprotein (Apo) B100, whereas assembly of the TG-rich chylomicrons in the intestine depends on ApoB48. In addition to ApoB, PC is required for assembly and secretion of

VLDLs (Yao and Vance, 1988). It has been estimated that PC comprises 60–80% of the phospholipids on the surface of ApoB-containing lipoproteins (Skipski et al., 1967, Agren et al., 2005).

In the liver, assembly of a VLDL particle starts when ApoB is co-translationally translocated into the ER lumen where it is associates with phospholipids, cholesterol, cholesteryl esters and TG (Alexander et al., 1976, Pullinger et al., 1989, Dixon et al., 1991, Jamil et al., 1995) (**Figure 1.4**). Microsomal triacylglycerol transfer protein (MTP) facilitates the translocation of ApoB into the ER lumen by loading it with lipids derived from membranes of the ER (Wetterau et al., 1992, Benoist and Grand-Perret, 1997, Gordon et al., 1996). Although MTP prefers binding TG and cholesteryl esters, it also has phospholipid transfer activity (Jamil et al., 1995). Another ER luminal protein, the phospholipid transfer protein, is also involved in transferring phospholipids to ApoB in the early stages of lipoprotein assembly (**Figure 1.4**). The nascent TG-poor, ApoB-containing particle is further lipidated, likely by merging with an ApoB-free particle in the ER lumen (Alexander et al., 1976, Borén et al., 1994, Gordon et al., 1996) (**Figure 1.4**). Insufficient availability of lipids for the initial lipidation of ApoB100 leads to diminished translocation or misfolding of ApoB100, resulting in degradation of ApoB100 via ER-associated co-translational degradation and the ubiquitin-proteasome pathway (Benoist and Grand-Perret, 1997, Fisher et al., 1997) (**Figure 1.4**). Interestingly, poorly-lipidated intestine-derived ApoB48 does not undergo post-translational degradation to the same extent as poorly lipidated ApoB100 and is instead secreted as a TG-poor ApoB48-contaning particle (Liao and Chan, 2000). Low PC levels, or a low PC/PE molar ratio, in the ER membrane causes degradation of the nascent VLDL (Verkade et al., 1993). Thus, the availability and transfer of lipids to ApoB is crucial for successful secretion of VLDL.

PC was first linked to hepatic lipid accumulation in the 1930s when Best and Huntsman showed that rats supplemented with dietary choline or PC had lower liver fat content compared to control rats receiving no additional choline source (Best and Huntsman, 1932, Best et al., 1932). Subsequently, a choline-deficient diet was shown to induce hepatic steatosis in humans (Zeisel et al., 1991, Buchman et al., 1995), at least partly due to impaired VLDL secretion (Yao and Vance, 1990). Mechanistic studies in rat hepatocytes incubated with methionine- and/or choline-deficient medium, which limited substrate availability for both PC biosynthetic pathways, impaired the secretion of ApoB and lipids associated with VLDL (Yao and Vance, 1990, Verkade et al., 1993, Fast and Vance, 1995). The choline-deficient medium resulted in the production of abnormal, PC-deficient VLDL particles that were degraded intracellularly (Verkade et al., 1993). More recently, the consequence of reduced PC biosynthesis on VLDL secretion was directly investigated in mouse models that lacked either PEMT or CTα specifically in the liver. The fasting concentrations of TG and ApoB100 in plasma of liver-specific CTα knockout mice were 50% lower than in control mice due to impaired VLDL secretion (Jacobs et al., 2004). Similarly, hepatocytes from mice lacking PEMT secreted 50% and 70% less TG and ApoB100, respectively, than did wild-type hepatocytes (Noga et al., 2002). In addition, plasma from fasted *Pemt*$^{-/-}$ mice fed a high fat/high cholesterol diet for 3 weeks contained less TG and ApoB100 compared to control mice, due to reduced VLDL secretion (Noga and Vance, 2003). Moreover, the rate of secretion of VLDL-TG was 70% lower in *Pemt*$^{-/-}$ mice fed a high-fat diet than in *Pemt*$^{+/+}$ mice fed the same diet (van der Veen et al., 2016) Together, these studies clearly show that hepatic PC biosynthesis by both pathways for PC biosynthesis is independently required for normal VLDL secretion. Conversely, increasing hepatic PC biosynthesis stimulates the production of VLDL particles (Martinez-Una et al., 2013). Mice lacking glycine N-methyltransferase (GNMT) have 50-fold elevated hepatic

AdoMet levels (Martinez-Una et al., 2013), leading to increased flux through the PEMT pathway and increased VLDL secretion (Martinez-Una et al., 2013, Martinez-Una et al., 2015).

1.2.5 Phospholipid remodeling and VLDL secretion

In addition to *de novo* PC synthesis, PC remodeling in hepatocytes influences VLDL secretion (**Figure 1.4**). The enzyme LPCAT3 can remodel PC so that polyunsaturated fatty acids, mainly arachidonate and linolenate, are incorporated into the *sn*-2 position (Hishikawa et al., 2008, Zhao et al., 2008b). Deletion of hepatic LPCAT3 lowers the amount of arachidonoyl acyl-chains in PC and impairs VLDL lipidation and secretion, resulting in hepatic TG accumulation (Rong et al., 2015, Hashidate-Yoshida et al., 2015). Phospholipids containing polyunsaturated fatty acyl-chains increase membrane fluidity relative to phospholipids containing saturated fatty acids (Rawicz et al., 2000). Therefore, incorporation of polyunsaturated fatty acids into membranes might be necessary to establish a suitable ER membrane microenvironment for the transfer of lipids from the ER membrane to ApoB (Rong et al., 2015, Hashidate-Yoshida et al., 2015). These observations indicate that the acyl-chain composition of hepatic PC can modulate VLDL secretion.

1.3 The physiological roles of PC and PE synthesis in mammals

Phospholipid biosynthetic enzyme activity and phospholipid acyl chain composition vary among tissues, reflecting tissue-specific phospholipid requirements (Harayama et al., 2014, Hanahan et al., 1960). For example, saturated dipalmitoyl-PC is a major component of pulmonary surfactant, which reduces lung alveolar surface tension and prevents alveolar collapse upon expiration, particularly in new-born infant mice (Tian et al., 2007, Bridges et al., 2010). Reducing levels of dipalmitoyl PC in lung surfactant by deletion of the PC remodeling enzyme LPCAT1 caused respiratory failure in neonatal mice, suggesting that there is a specific requirement for dipalmitoyl PC for lung function (Bridges et al., 2010). Other mouse models have been generated to determine the function of PC in various cell types. For example, an inability to synthesize PC by the CDP-choline pathway rendered macrophages susceptible to cholesterol-induced death (Zhang et al., 2000). Global deletion of CTβ resulted in gonadal dysfunction and reduced reproductive capacity in mice (Jackowski et al., 2004). In addition, neuronal branching in CTβ-deficient mice was impaired (Strakova et al., 2011). In the following section, the role of phospholipids in relevant aspects of liver and muscle physiology will be highlighted.

1.3.1 Phospholipids in the liver

Mice with liver-specific deletion of CTα have mild steatosis and lower fasting plasma TG concentrations compared to floxed control mice when fed a chow diet (Jacobs et al., 2004). However, when fed a high fat diet, liver-specific CTα knockout mice experience massive liver fat accumulation that progresses to non-alcoholic steatohepatitis (Ling et al., 2012, Niebergall et al., 2011). Hepatic TG accumulation in liver-specific CTα knockout mice fed a high fat diet is at least partly due to impaired VLDL secretion (Jacobs et al., 2004), although constitutive activation of SREBP-1c or conversion of excess DG to TG might also contribute (Walker et al., 2011). Two

patients with bi-allelic loss-of-function mutations in *PCYT1A,* the gene encoding CTα, have been identified (Payne et al., 2014). In these patients, PC synthesis by the CDP-choline pathway was reduced and was associated with severe fatty liver (Payne et al., 2014), consistent with findings from liver-specific CTα knockout mice.

Like liver-specific CTα knockout mice, PEMT knockout mice develop steatosis that progresses to severe non-alcoholic steatohepatitis when fed a high fat diet (van der Veen et al., 2016, Jacobs et al., 2010, Ling et al., 2012). Therefore, both pathways for hepatic PC synthesis are independently required to prevent non-alcoholic steatohepatitis in the setting of a high fat diet. When PEMT knockout mice are fed a choline-deficient diet (which reduces PC production *via* the CDP-choline pathway), hepatic PC concentrations are reduced by ~50% and liver failure occurs within 3 days (Li et al., 2006). A large proportion of hepatic PC is lost during biliary secretion via the multiple drug-resistant protein 2 (MDR2). Therefore, it was hypothesized that reducing biliary PC secretion might prevent liver failure in PEMT knockout mice fed a choline-deficient diet (Li et al., 2006). In contrast to PEMT knockout mice, PEMT/MDR2 double-knockout mice fed a choline-deficient diet did not suffer liver failure and survived beyond 3 months (Li et al., 2006). These data show that biliary PC secretion makes a major contribution to hepatic PC homeostasis.

1.3.2 Phospholipids in skeletal muscle

Skeletal muscle is the major contributor to insulin-stimulated glucose disposal, making it an important player in whole body energy homeostasis (O'Neill et al., 2013). Studies using mice with skeletal muscle-specific disruption of genes that encode enzymes involved in phospholipid synthesis have recently helped to elucidate some of the mechanisms by which changes in skeletal muscle phospholipids influence insulin sensitivity. It is likely that unbalanced synthesis of muscle PC and PE influences muscle insulin sensitivity by disrupting cellular calcium homeostasis (Funai

et al., 2013, Funai et al., 2016). The molar ratio of PC/PE in the sarcoplasmic reticulum influences the activity of SERCA, a key player in skeletal muscle contraction (Gustavsson et al., 2011). Mice with muscle-specific deletion of CEPT1, the enzyme that catalyzes the terminal reactions in the CDP-choline and CDP-ethanolamine pathways for PC and PE synthesis, respectively, contained lower sarcoplasmic reticulum concentrations of PE (but not PC) in muscle, thereby increasing the PC/PE ratio by approximately 60% (Funai et al., 2016). Similarly, inhibition of fatty acid synthase in skeletal muscle reduced sarcoplasmic reticulum PE levels and increased the sarcoplasmic reticulum PC/PE molar ratio by approximately 60% (Funai et al., 2013). Increased sarcoplasmic reticulum PC/PE ratio in each of these models was associated with impaired SERCA-mediated calcium uptake, and muscle weakness (Funai et al., 2013, Funai et al., 2016).

1.4 Overview of the intestinal epithelium

The mammalian small intestine must balance nutrient uptake with the ability to prevent passage of luminal antigens to host tissues. These diverse requirements are fulfilled by four different intestinal epithelial cell types (**Figure 1.5**): enterocytes (termed colonocytes in the colon), enteroendocrine cells, goblet cells, and Paneth cells. Most intestinal epithelial cells turn over every 3-5 days owing to the harsh intestinal environment, making it important to tightly regulate intestinal stem cell proliferation and differentiation to prevent cancer development (Potten et al., 1992). The submucosal crypts contain a bank of leucine-rich repeat-containing G-protein coupled receptor 5 (LGR5+) proliferative stem cells that divide asymmetrically to produce one daughter cell (which will differentiate into one of the four intestinal epithelial cell types) and one stem cell (which remains undifferentiated and multipotent) (Barker et al., 2007). Daughter cells undergo multiple rounds of cell division while migrating up the villi, so that the tips of most villi contain mature post-mitotic intestinal epithelial cells (Potten et al., 1992, Katz et al., 2002). The

homeodomain transcription factor caudal type homeobox 2 (CDX2) is critical for intestinal development and maintenance of intestinal identity (Silberg et al., 2002, Liu et al., 2007, Gao et al., 2009). Loss of CDX2 in adult mice causes failure of intestinal epithelial cells to differentiate (Gao et al., 2009), and ectopic expression of CDX2 in the stomach or esophagus activates an intestine-specific gene expression program (Silberg et al., 2002, Liu et al., 2007). CDX2 partners with the transcription factors GATA Binding Protein 4 to control crypt cell proliferation, and with Hepatocyte nuclear factor 4-α to maintain normal intestinal lipid absorption (San Roman et al., 2015).

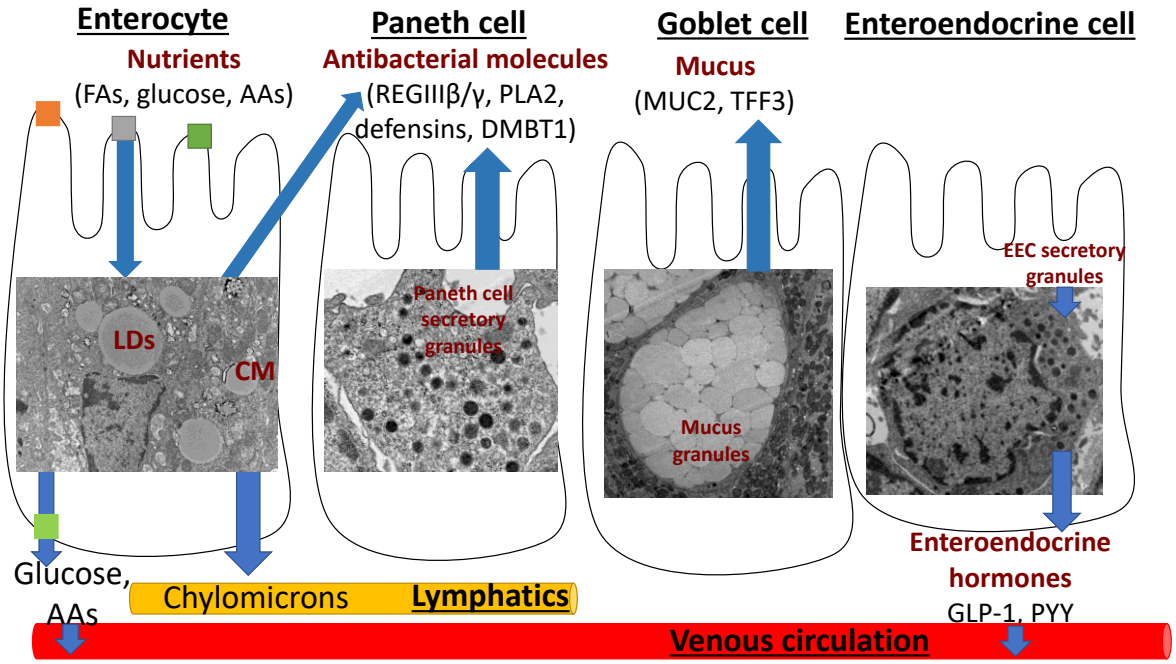

Figure 1.5. Intestinal epithelial cell types and their major functions. **Enterocytes** comprise ~80% of intestinal epithelial cells and primarily function to absorb nutrients (fatty acids (FAs), glucose and amino acids (AA)) from the intestinal lumen. The uptake of amino acids and glucose is mediated by transporters, while fatty acid uptake might be transport-mediated under certain conditions but appears to occur primarily by passive diffusion. Glucose and amino acids are transported into venous circulation. Fatty acids can be transiently stored as TGs in lipid droplets before being transferred to chylomicrons for secretion into the lymphatics. In addition to absorbing nutrients, enterocytes can secrete a variety of antimicrobial molecules. Microbes in the intestinal lumen interact with **Paneth cells** to promote the secretion of antimicrobial peptides including lysozyme, REGIIIγ REGIIIβ, phospholipase A2 (PLA2), defensins, and deleted in malignant brain tumors 1 (DMBT1). **Goblet cells** contain large, cytoplasmic mucus granules which are secreted into the intestinal lumen to form a protective mucus barrier. **Enteroendocrine cells** secrete various hormones including glucagon-like peptide 1 (GLP-1) and peptide tyrosine-tyrosine (PYY) in response to luminal nutrients to assist with nutrient disposal, gut motility and appetite. The electron micrographs in this diagram are from mice on a C57B6/J background and were obtained by J.P.K.

Cell fate decisions in the intestinal epithelium are coordinated by the transcription factors Neurogenic locus notch homolog protein 1 (Notch1) and Atonal homolog 1 (Atoh1; also known as Math1), and their downstream effectors (Fre et al., 2005, Yang et al., 2001). Notch1 signaling

drives epithelial cells towards absorptive enterocytes, and inhibition of Notch1 results in most intestinal epithelial cells differentiating into secretory goblet cells (Fre et al., 2005, Jensen et al., 2000, van Es et al., 2005). Notch1 positive cells typically also contain Hes family bHLH transcription factor 1 (Hes1), a transcriptional repressor that prevents commitment to the secretory cell lineage (Jensen et al., 2000). Conversely, progenitors in which the transcription factor Atoh1 is induced are committed to the secretory cell lineage which includes goblet cells, Paneth cells and enteroendocrine cells (Yang et al., 2001). The transcriptional repressor growth factor independent 1 transcriptional repressor (Gfi1) acts downstream of Atoh1 to assist the differentiation and development of goblet cells and Paneth cells (Shroyer et al., 2005). The transcription factors kruppel like factor 4 (Klf4) and SAM pointed domain-containing Ets transcription factor (Spdef) function downstream of Gfi1 to control the development and maturation of goblet cells (Katz et al., 2002, Noah et al., 2010, Gregorieff et al., 2009).

1.4.1 Intestinal epithelial cell types

1.4.1.1 Enterocytes and colonocytes

Enterocytes account for ~80% of total intestinal epithelial cells (Chang and Leblond, 1971, Cheng and Leblond, 1974). They are highly polarized columnar cells with an apical membrane containing microvilli to increase the absorptive area and a basolateral membrane across which nutrients diffuse or are actively transported before entering the blood or lymphatics (**Figure 1.5**). The major function of enterocytes is to absorb nutrients and to secrete various factors that protect the intestinal epithelium against damage by luminal antigens. Enterocytes in the proximal small intestine and distal small intestine have distinct transcriptional signatures. For example, genes involved in lipid and carbohydrate absorption are more abundantly expressed in the duodenum and jejunum while genes involved in bile acid uptake are more abundantly expressed in the ileum.

Colonocytes specialize in the absorption of water, short-chain fatty acids and sodium (Chang and Leblond, 1971).

1.4.1.2 Enteroendocrine cells

Enteroendocrine cells comprise ~1% of total cells in the intestinal epithelium and secrete a variety of hormones that regulate many physiological processes including nutrient disposal, gut motility and appetite [(Kreymann et al., 1987, Turton et al., 1996) and reviewed in (Baggio and Drucker, 2007)] (**Figure 1.5**). Types of enteroendocrine cells in the small intestine include L, D, I, K and enterochromaffin cells. The ability of gut-derived factors to enhance glucose-stimulated insulin secretion is termed the incretin effect (Labarre, 1932). Glucagon-like peptide-1 (GLP-1), a product of the proglucagon gene, is secreted from enteroendocrine L cells located predominantly in the ileum and colon in response to nutrients (Mojsov et al., 1987, Kreymann et al., 1987) The major physiological role of GLP-1 is to promote glucose-stimulated insulin secretion (Kreymann et al., 1987, Mojsov et al., 1987). Peptide tyrosine tyrosine (PYY) is co-secreted with GLP-1 from L cells. Like GLP-1, circulating PYY concentrations rise 15 minutes after a meal due to neural responses (mediated by the vagus nerve), peak at 90 minutes as nutrients directly interact with L cells, and remain elevated compared to the fasting state for up to 6 hours (Adrian et al., 1985). PYY3-36, the major form found in circulation following a meal, influences appetite by directly interacting with neural circuits that regulate appetite, and can also influence gut motility (Batterham et al., 2002, Batterham et al., 2003). Glucagon-like peptide-2 (GLP-2) is secreted in equimolar concentrations to GLP-1 from L cells and functions to promote intestinal lipid uptake (Hsieh et al., 2009). Importantly, fatty acids can directly interact with G-protein coupled receptors on L cells to promote hormone secretion (Hirasawa et al., 2005). Therefore, humans and mice that experience fat malabsorption in the proximal small intestine typically have enhanced postprandial

secretion of enteroendocrine hormones as higher concentrations of fatty acids interact with L cells of the distal intestine (Damci et al., 2004, Ables et al., 2012, Wang et al., 2016, Yen et al., 2009). D cells, located predominantly in the stomach and small intestine, secrete somatostatin to reduce gastric acid secretion from parietal cells through inhibition of histamine and gastrin release (Hökfelt et al., 1976, Larsson et al., 1979). Enterochromaffin cells primarily secrete 5-hydroxytryptamine, a promoter of intestinal motility (BULBRING and CREMA, 1959). K cells are the major source of circulating glucose-dependent insulinotropic polypeptide (GIP), the first incretin hormone identified, which promotes glucose-stimulated insulin secretion (Brown et al., 1975, Lauritsen et al., 1980). I cells secrete cholecystokinin (CCK) to slow gastric emptying and induce satiety (Whited et al., 2006).

1.4.1.3 Paneth cells

Paneth cells lie at the base of the crypts and play an important role in innate immunity by secreting a variety of antimicrobial peptides (Vaishnava et al., 2008)(**Figure 1.5**). Paneth cells (which are easily identified by histology due to their large eosinophilic granules) have a slow turnover rate of approximately 20 days compared to other epithelial cell types which turnover every 3-5 days (Cheng and Leblond, 1974). Commensal microbes have been shown to interact with the myeloid differentiation primary response 88 (Myd88) receptor on Paneth cells to control the secretion of antibacterial molecules including lysozyme, REGIIIγ, phospholipase A2, defensins and DMBT1 (Deleted in malignant brain tumors 1) (Ayabe et al., 2000, Hooper et al., 2003, Cash et al., 2006, Vaishnava et al., 2008). Due to their role in shaping the gut flora, there has been increasing interest in Paneth cells in the context of inflammatory bowel diseases (Bevins and Salzman, 2011).

1.4.1.4 Goblet cells

Goblet cells comprise about 5% of epithelial cells in the small intestine and about 16% in the colon (Chang and Leblond, 1971, Cheng and Leblond, 1974). Goblet cells can be readily identified by histology due to their large, cytoplasmic mucus granules (**Figure 1.5**). These mucus granules are secreted into the intestinal lumen to form a polymeric gel-like mucus layer that ranges from 50-500 µm (Johansson and Hansson, 2016). Goblet cells produce Mucin 2 (MUC2), a heavily N-glycosylated polymeric protein that forms the scaffold for the components of mucus (Velcich et al., 2002). In the small intestine, goblet cells are more abundant in the crypts than on the villi. Furthermore, the mucus layer in the small intestine is thin and discontinuous relative to the colon, and so the small intestinal epithelium is more reliant on secreted antimicrobial peptides to protect against microbial damage than the colon (Johansson et al., 2008). The mucus layer in the colon consists of a sterile inner layer and a loose outer that limit microbial interaction with the epithelium (Johansson et al., 2008).

1.5 Overview of chylomicron formation and secretion

Most dietary lipids are in the form of TGs, with proportionally smaller contributions from cholesterol esters, vitamin esters and unesterified cholesterol (Abumrad and Davidson, 2012, Cordain et al., 2005). Dietary TGs are initially hydrolyzed to monoglycerides and fatty acids in the intestinal lumen by lipases before being taken up by the enterocyte by passive diffusion or by protein-mediated transport (discussed below). Fatty acids and monoglycerides are then transported to the ER where they are re-acylated in TG, packaged into chylomicron particles with ApoB48, and then secreted across the basolateral membrane into the lymphatics. Intestinal epithelial cells can derive PC from local *de novo* synthesis, diet, bile, or circulating lipoproteins (**Figure 1.6**). PC delivered to the intestinal lumen in the diet or in bile is first hydrolyzed to lyso-PC and fatty acids

by phospholipase A2 before crossing into enterocytes (**Figure 1.6**). This lyso-PC can be re-acylated into PC by LPCAT enzymes on the ER for use in lipid droplet and chylomicron formation (**Figure 1.6**). Alternatively, PC can be produced from dietary choline by the CDP-choline pathway and remodeled by LPCAT3 (**Figure 1.6**). The following section will the major steps involved in dietary lipid absorption including digestion of luminal lipids by lipases, passage of lipids from the intestinal lumen into enterocytes, chylomicron formation at the ER, and basolateral chylomicron secretion.

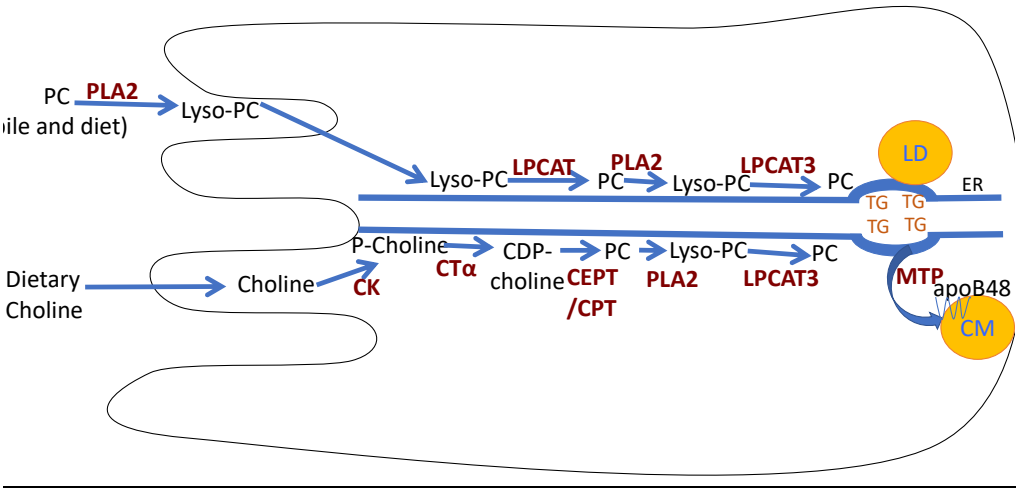

Figure 1.6. Intestinal epithelial cells derive PC from the CDP-choline pathway, from luminal sources (diet and bile) or from circulating lipoproteins. PC delivered to the intestinal lumen in the diet or in bile is first hydrolyzed to lyso-PC and fatty acids by phospholipase A2 before crossing into enterocytes. This lyso-PC can be re-acylated into PC by the LPCAT enzymes on the ER for use in lipid droplet and chylomicron formation. Alternatively, PC can be produced from dietary choline by the CDP-choline pathway. PC on ER membranes can be remodeled by Lands' cycle, which involves de-acylation by phospholipase A2 and subsequent re-acylation by LPCAT3 to increase the abundance of PC containing polyunsaturated fatty acids. There is a lack of literature on the mechanisms involved in intestinal PC uptake from circulating lipoproteins.

1.5.1 Dietary lipid digestion by lipases

Neutral lipid digestion and absorption is facilitated by the action of lipases (Blackfan and Wolbach, 1933, Ross, 1955)(**Figure 1.7**). Gastric lipase and lingual lipase (pre-duodenal lipases) are most active under acidic conditions (optimal pH 4-6) and make a relatively minor contribution to fat digestion (<20%) (Liao et al., 1984). Pre-duodenal lipases preferentially hydrolyze the *sn*-3 position of TGs and prefer short- and medium-chain TGs (Liao et al., 1984). The duodenal lipases (including pancreatic TG lipase, pancreatic TG lipase related protein 2, and cholesterol ester lipase) are activated at neutral pH (Giller et al., 1992, Lopez-Candales et al., 1993). Pancreatic TG lipase and pancreatic TG lipase related protein 2 require the co-factor co-lipase for activity and preferentially hydrolyze TG at the *sn*-1 and *sn*-3 position (Borgstrom and Erlanson, 1971, Giller et al., 1992, Paltauf et al., 1974). Pancreatic TG lipase is important for dietary TG and cholesterol absorption, especially in the setting of high fat/high cholesterol feeding (Figarella et al., 1980, Gilham et al., 2007). Pancreatic TG lipase related protein 2 is important for fat absorption during the perinatal period (Giller et al., 1992, Xiao et al., 2011). Cholesterol ester lipase is required for the hydrolysis and absorption of cholesterol esters but not for retinyl esters (Weng et al., 1999, Lopez-Candales et al., 1993). On the other hand, inactivating pancreatic TG lipase reduced retinyl ester absorption by ~45%, implicating pancreatic TG lipase in vitamin A absorption (Gilham et al., 2007).

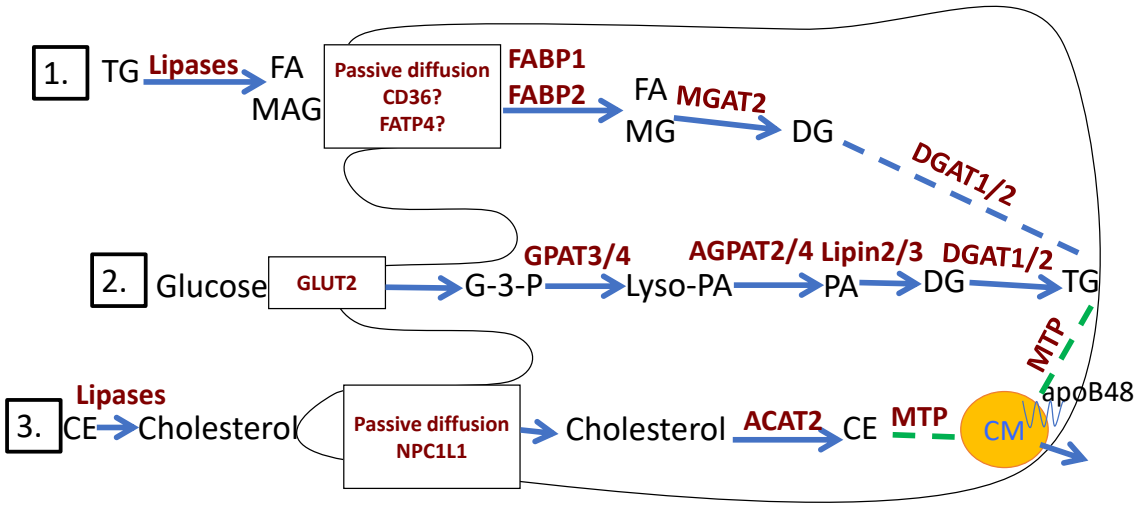

Figure 1.7. Intestinal lipid uptake and metabolism. Dietary TGs are initially hydrolyzed to monoglycerides and fatty acids in the intestinal lumen by lipases before being taken up by the enterocyte by passive diffusion or by protein-mediated transport (**Process 1**). Proteins that have been implicated in fatty acid uptake include CD36 and FATP4. These fatty acids are then transported to the ER by fatty acid binding protein 1 or 2 (FABP1 or FABP2) where they can be re-acylated into TGs by monoacylglycerol acyltransferase (MGAT) 2 and diacylglycerol acyltransferase (DGAT) 1 or 2. TG can also be formed in intestinal epithelial cells via glucose (**Process 2)**. This pathway involves metabolism of glucose to glycerol-3-phosphate (G-3-P). Glycerol-3-phosphate acyltransferase (GPAT) and 1-acyl glycerol-3-phosphate acyltransferase (AGPAT) enzymes sequentially esterify G-3-P with acyl-CoAs to form phosphatidic acid (PA). Lipin (also called phosphatidic acid phosphohydrolase) generates DG from PA before DGAT generates TG. Cholesterol esters (CE) in the intestinal lumen are hydrolyzed by lipases (**Process 3**). Cholesterol can then be taken up by the enterocyte by passive diffusion or by Niemann-Pick C1–like 1 (NPC111) before being esterified to cholesterol esters by acyl-CoA:cholesterol acyltransferase 2 (ACAT2). TG, CE and unesterified cholesterol can be transferred to ApoB48 by MTP to form chylomicrons.

1.5.2 Intestinal fatty acid uptake: A role for phospholipid remodeling

There has been a long-running debate whether intestinal fatty acid uptake occurs primarily by passive diffusion or protein-mediated transport (Tso et al., 2004, Abumrad and Davidson, 2012)(**Figure 1.7**). It was reported that fatty acid uptake is a saturable process and that competition

for uptake exists between fatty acid species, suggesting that the process might be carrier-mediated (Ho and Storch, 2001, Murota and Storch, 2005). Several apical membrane proteins that might mediate fatty acid uptake have been identified, including cluster of differentiation 36 (CD36) and fatty acid transport protein 4 (FATP4). However, deletion of either CD36 or FATP4 does not substantially impair fatty acid uptake from the intestinal lumen into enterocytes (Goudriaan et al., 2002, Nauli et al., 2006, Drover et al., 2005, Shim et al., 2009). Instead, CD36 might play a role in chylomicron formation after fatty acid uptake by enterocytes or in the clearance of chylomicrons from circulation (Drover et al., 2005).

Data from cell culture models which shows that fatty acid uptake is protease-resistant and temperature independent supports a role for passive diffusion (Chow and Hollander, 1979, Ling et al., 1989, Trotter et al., 1996). Strong *in vivo* support for a predominant role for passive diffusion comes from mice with intestine-specific deletion of the phospholipid remodeling enzyme LPCAT3 (Wang et al., 2016). These mice have lower concentrations of arachidonate- and linolenate-containing PC species in intestinal epithelial cells, thereby reducing membrane fluidity, fatty acid uptake and chylomicron secretion (Wang et al., 2016). Importantly, providing polyunsaturated PC to LPCAT3-deficient intestines increases fatty acid uptake at both 37 ˚C and 4 ˚C, strongly suggesting that membrane fluidity is a determinant of intestinal fatty acid uptake capacity (Wang et al., 2016).

Altering the phospholipid composition of intestinal epithelial cells by deletion of LPCAT3 has profound effects on whole-body energy metabolism. Impaired fatty acid uptake in the proximal small intestine has been shown to enhance release of GLP-1 and PYY because higher concentrations of fatty acids reach L-cells of the distal intestine (Ables et al., 2012, Cummings and Overduin, 2007). Accordingly, in adult mice lacking intestinal LPCAT3, postprandial GLP-1 and

PYY secretion is enhanced and food intake is markedly reduced when the mice are fed a high fat diet (containing 40%-60% of total kilocalories as fat), leading to rapid weight loss and morbidity within days of initiation of fat feeding (Wang et al., 2016). When the fat content of the diet is reduced to 30% of total kilocalories, a metabolically favorable phenotype was induced, with diminished diet-induced weight gain and lower levels of plasma lipids due to impaired intestinal fatty acid absorption (Wang et al., 2016). Interestingly, a different group linked impaired intestinal fatty acid uptake in LPCAT3-deficient mice to lower abundance of brush border CD36 and FATP4; however, they did not asses membrane biophysical properties in the mice (Li et al., 2015). Therefore, the phospholipid composition of the intestinal brush-border membrane influences long-chain fatty acid uptake and availability of lipids for assembly of TG-rich chylomicrons through effects on membrane biophysical properties and membrane protein stability (Li et al., 2015, Wang et al., 2016). The role of *de novo* PC synthesis in maintaining fatty acid uptake in the small intestine is poorly understood. However, rat dams that were fed a diet deficient in choline had a lower molar ratio of PC/PE in the small intestine relative to dams fed a choline-sufficient diet (da Silva et al., 2015). This alteration in intestinal phospholipid concentrations was associated with delayed chylomicron secretion after a fat challenge and lower fasting plasma lipids compared to controls (da Silva et al., 2015).

1.5.3 Intestinal cholesterol uptake

While intestinal fatty acid absorption is highly efficient (95-100%), intestinal cholesterol absorption is typically about 50% efficient and varies widely (20-80%) (Sudhop et al., 2002, Voshol et al., 2000). The membrane transporter Nieman Pick C1-like 1 (NPC1L1) plays a central role in intestinal cholesterol absorption (**Figure 1.7**). Deletion of the gene encoding NPC1L1 reduces cholesterol absorption by ~70% (Altmann et al., 2004), while administration of a NPC1L1

inhibitor to humans reduces cholesterol uptake by ~50% (Sudhop et al., 2002). Biliary cholesterol secretion is a well-established contributor to whole-body cholesterol balance. Recently, a pathway involving cholesterol excretion from venous circulation, through the enterocyte and into the intestinal lumen, termed trans-intestinal cholesterol efflux has been described in mice and humans (Kruit et al., 2005, Jakulj et al., 2016).

1.5.4 Intestinal fatty acid transport

After apical uptake, fatty acids are transported to the ER by fatty acid binding protein 1 and 2 (for example FABP1 and FABP2) where they are mainly re-acylated to TGs by the monoacylglycerol acyltransferase 2 (MGAT2) and diacylglycerol acyltransferase 1 or 2 (DGAT1 or DGAT2) enzymes at the ER (Thumser and Storch, 2000, Yen and Farese, 2003, Buhman et al., 2002)(**Figure 1.7**). Alternatively, DG could be funneled into phospholipid synthesis and used for the formation of lipid droplets and chylomicrons (**Figure 1.7**). Fatty acids are also taken into enterocytes through the basolateral membrane, and there is evidence that these fatty acids are preferentially shuttled into phospholipid synthesis (Storch et al., 2008).

1.5.5 Intestinal TG synthesis

The two pathways for TG formation in enterocytes are the MGAT2 pathway and the glycerol-3-phosphosphate (G-3-P) pathway, which converge on the production of DG (**Figure 1.7**). The MGAT pathway directly produces DG by the re-acylation of *sn*-2 monoacylglycerol (**Figure 1.7**). The glycerol-3-phosphosphate pathway produces DG by the sequential acylation of G-3-P (a product of glycolysis) by glycerol-3-phosphate acyltransferase (GPAT) and 1-acyl-glycerol-3-phosphate (AGPAT), and the removal of the phosphate group by phosphatidic acid phosphohydrolase (also called LIPIN) (**Figure 1.7**). In most tissues that synthesize TG, the G-3-P

pathway predominates; however, the re-acylation of monoacylglycerol predominates in the intestine when dietary fat content is high, accounting for approximately 80% of TG produced under these conditions (Kern and Borgström, 1965, Johnston et al., 1967, CLARK and HUBSCHER, 1961, CLARK and HUEBSCHER, 1960, Kayden et al., 1967, Kennedy, 1957)

Mice deficient in GPAT3 have impaired chylomicron secretion after a large bolus of olive oil, suggesting that the G-3-P pathway plays a role in chylomicron secretion in the setting of high dietary fat intake (Khatun et al., 2016). Intestine-specific deletion of MGAT2 does not reduce total dietary fat uptake (as indicated by normal fecal fat balance compared to control mice) but does shift fat absorption to the distal small intestine and delays entry of dietary TGs into circulation (Yen et al., 2009). A recent study suggested that ER-associated degradation (ERAD; a protein degradation system required for protein quality control) plays a role in dietary fat absorption by controlling the intestinal protein abundance of GPAT3, MGAT2 and DGAT2 (although as discussed below DGAT2 has not thus far been implicated in dietary fat absorption due to the perinatal death of the DGAT2-deficient mice, the low abundance of DGAT2 in the mouse intestine, and the absence of intestinal DGAT2 in the human intestine). Whole-body or intestine-specific deletion of Axin Interactor Dorsalization Associated (AIDA), which serves as a factor required for the activity of the ERAD-associated E3-ligase HMG-CoA reductase degradation protein 1 (HRD1), increased fat absorption and adiposity in mice (Luo et al., 2018).

DGAT1 is the major DGAT in the murine intestine, as DGAT1-deficient mice have 90% lower DGAT activity compared to control mice (Buhman et al., 2002). DGAT1 inhibition reduces diet-induced obesity, improves insulin sensitivity, and protects against fatty liver in mice DGAT1 (Ables et al., 2012, Cao et al., 2011, Zhao et al., 2008a, Smith et al., 2000). These metabolic benefits appear to be mediated by delayed dietary fat absorption and enhanced secretion of

enteroendocrine hormones following a meal (Liu et al., 2013). However, the favorable effects of DGAT1 inhibitors in humans (which include blunted intestinal TG secretion following a meal, delayed gastric emptying, enhanced GLP-1 secretion and improved insulin sensitivity) have been offset by severe gastrointestinal problems, precluding their widespread clinical use (Denison et al., 2014). Mice deficient in DGAT2 die during the perinatal period due to dehydration caused by skin abnormalities (Stone et al., 2004). There is no DGAT2 activity in the human intestine, and humans with a null mutation in DGAT1 experience severe diarrhea (Haas et al., 2012).

1.5.6 Chylomicron formation at the ER of enterocytes

Secretion of poorly lipidated ApoB48-contaning particles occurs in the fasting state (Guo et al., 2005). When dietary fat is consumed, both the neutral lipid content of ApoB48-contaning particles and the total number of particles secreted increases relative to the fasting state (Otokozawa et al., 2009). MTP transfers TG, cholesterol ester and phospholipids to ApoB48 in the ER lumen, forming a primordial ApoB-containing particle (Wetterau et al., 1992, Cartwright and Higgins, 2001). Loss of intestinal MTP or ApoB in mice or humans causes severe dietary fat malabsorption (Xie et al., 2006, Young et al., 1995). The primordial lipoprotein particle subsequently fuses with an ApoB-free lipid droplet in the ER lumen to form a pre-chylomicron (Cartwright and Higgins, 2001). ApoA-IV, an apolipoprotein that increases the lipid storage capacity of chylomicrons and clearance rates from circulation (but is not required for intestinal chylomicron secretion), also associates with the pre-chylomicron in the ER lumen (Kohan et al., 2013, Kohan et al., 2012).

The acyl chain composition of phospholipids in the ER of enterocytes, as in hepatocytes, might influence the assembly of TG-rich chylomicron particles (Rong et al., 2015, Hashidate-Yoshida et al., 2015). In contrast to adult intestine-specific LPCAT3-deficient mice, fatty acid

uptake is not impaired in global LPCAT3-deficient mice during the perinatal period (Hashidate-Yoshida et al., 2015, Rong et al., 2015). Instead, LPCAT3-deficient mice accumulate TG in enterocytes, suggesting that chylomicron secretion, rather than intestinal lipid uptake, is defective in the knockout mice. The reason for this developmental difference is not clear. However, based on experiments with global LPCAT3-deficient mice, Hashidate-Yoshida et al. suggested that appropriate ER membrane fluidity provided by LPCAT3 allows formation of TG-rich domains in the ER bilayer and an efficient transfer of lipids to nascent ApoB-containing chylomicron particles by MTP (Hashidate-Yoshida et al., 2015).

1.5.7 Chylomicron secretion into the lymphatics

Mature chylomicron particles are exocytosed across the basolateral membrane of the enterocyte into the lamina propria and enter the lymphatics through lacteals, specialized lymphatic capillaries located at the core of each intestinal villus. Vascular endothelial growth factor-C (VEGF-C) and Delta-like 4 (DLL4) are involved in formation of the lacteals, and deletion of the genes encoding VEGF-C and DLL4 reduces dietary lipid absorption and high fat diet-induced weight gain in mice (Nurmi et al., 2015, Bernier-Latmani et al., 2015). It has recently been shown that the passage of chylomicrons into lacteals is controlled by VEGF-A signaling (Zhang et al., 2018). In this model, the proteins Neuropilin-1 (NRP1) and Vascular endothelial growth factor receptor 1 (VEGFR1) act as double decoy receptors (located on blood endothelial cells neighboring lymphatic endothelial cells) which dampen VEGF-A signaling and prevent the closure of junctions between lymphatic endothelial cells (i.e. transition from button-like junctions to zipper-like junctions) to allow chylomicron passage into lacteals (Zhang et al., 2018). Accordingly, deletion of NRP1 and VEGFR1 results in closure of the lymphatics and protection from diet-induced obesity in mice (Zhang et al., 2018). After travelling through the mesenteric lymphatic

vessels, chylomicrons are drained into the thoracic duct before entering venous circulation at the left subclavian vein.

1.5.8 Lipid droplet formation in enterocytes

In addition to secreting chylomicrons, enterocytes store dietary lipids in cytosolic lipid droplets that act as a reservoir to sustain chylomicron secretion (Mattes, 2002). Cytosolic lipid droplets might be an important source of chylomicron lipids, as blocking lipid droplet mobilization in intestinal epithelial cells impairs intestinal lipid secretion (Xie et al., 2014). Current evidence suggests that, as TG is synthesized in the folds of the ER, a lens of neutral lipid forms and eventually buds off from the ER into the cytosol to form a cytosolic lipid droplet. This budding process may be mediated by changes to the surface tension on the ER, changes to the phospholipid content of the ER, or by proteins that have yet to be identified (Ben M'barek et al., 2017)(**Figure 1.4**). Neutral lipids of cytoplasmic lipid droplets can be mobilized by lipolysis or lipophagy, and both processes have been implicated in intestinal fat absorption. Mice with intestinal deletion of the lipid droplet-associated proteins, ATGL, CIDEB, PLIN2 or ABHD5, show changes to fat absorption (Frank et al., 2015, Obrowsky et al., 2013, Zhang et al., 2014, Xie et al., 2014). Intestine specific deletion of ATGL causes neutral lipid accumulation in enterocytes, suggesting that ATGL might play a role in liberating fatty acids from the cytosolic pool of enterocyte lipid droplets (Obrowsky et al., 2013). Interestingly, ATGL-deficient mice were also reported to have higher fecal fat content compared to control mice despite normal total fat absorption (Obrowsky et al., 2013). The reason for discrepancy in fat balance is not clear. Intestine-specific deletion of ABHD5 increases fecal fat content and reduces chylomicron secretion following an oral lipid bolus compared to control mice (Xie et al., 2014). Furthermore, deletion of CIDEB reduced the rate of intestinal TG secretion following an oral bolus of olive oil while overexpression of CIDEB in the

intestinal epithelial cell line Caco2 promoted TG secretion (Zhang et al., 2014). Both mice and humans deficient in lysosomal acid lipase (LAL) accumulate neutral lipids in enterocytes, implicating lipophagy in intestinal neutral lipid turnover (Du et al., 2001, Porto, 2014).

1.5.9 Nutritional and hormonal factors that influence chylomicron production

Dietary glucose and fructose intake promotes chylomicron secretion in humans (Xiao et al., 2013). Furthermore, high levels of free fatty acids in circulation promote chylomicron secretion (Duez et al., 2008). Chylomicron secretion is blunted by insulin in postprandial and hyper-insulinemic states (Pavlic et al., 2010). On the other hand, people with insulin resistance have enhanced intestinal TG and ApoB48 secretion relative to metabolically healthy individuals (Hogue et al., 2007, Duez et al., 2006, Shojaee-Moradie et al., 2013). Hyperglycemia induced by intravenous glucose infusions in healthy humans also stimulated chylomicron secretion, and so elevated circulating glucose concentrations in the setting of metabolic syndrome might contribute to hyperlipidemia (Xiao et al., 2016). GLP-1 receptor agonism (Xiao et al., 2012), and the DPP4 inhibitor sitagliptin (Xiao et al., 2014) reduce postprandial intestinal TG secretion in humans, which might be linked to delayed gastric emptying or enhanced postprandial insulin secretion.

1.5.10. Biliary secretions and lipid absorption

PC synthesized in the liver is secreted into bile by a PC-specific flippase, the multiple drug-resistant protein-2 (MDR2), along with bile acids and cholesterol (Voshol et al., 2000). Biliary PC is hydrolyzed by phospholipase A2 to lyso-PC and fatty acids before being absorbed across the intestinal brush border membrane (Voshol et al., 2000). $Mdr2^{-/-}$ mice, and rats with bile duct fistulas, have impaired intestinal TG secretion after a fat challenge, highlighting the importance of bile-derived PC for chylomicron secretion (Voshol et al., 2000, Morgan and Borgström, 1969). It

has been proposed that biliary lyso-PC is involved in chylomicron formation at the ER of enterocytes by activating protein kinase Cζ (Siddiqi and Mansbach, 2015). This kinase stimulates formation of pre-chylomicron transport vesicles that move from the ER to the Golgi prior to secretion of chylomicrons from the cell (Siddiqi and Mansbach, 2015). Therefore, liver-derived PC plays a role in intestinal fatty acid uptake and chylomicron secretion.

Primary bile acids are synthesized in the liver from cholesterol, stored in the gallbladder, and released into the intestinal lumen to solubilize lipids (Bloch et al., 1943, Kuipers et al., 2014). Primary bile acids are typically conjugated to glycine or taurine and can undergo modifications in the small intestine by microbes to become secondary bile acids (Huijghebaert and Hofmann, 1986). CCK-mediated gallbladder contraction in response to food intake releases millimolar concentrations of bile acids into the intestinal lumen where they help to form lipid micelles for the activation of intestinal lipases (Morgan and Borgström, 1969). In the absence of bile acids, fatty acid absorption is shifted from the proximal small intestine to the ileum (Morgan and Borgström, 1969). Bile acids are particularly important for cholesterol uptake, and patients with genetic defects that impair bile acid synthesis absorb only 20% of total cholesterol delivered to the intestine (Woollett et al., 2006). Conversely, delivery of cholic acid to the intestinal lumen enhances cholesterol uptake (Woollett et al., 2006, Woollett et al., 2004).

Bile acids in the distal intestine can interact with TGR5 on the apical surface of L cells to enhance the secretion of GLP-1 (Thomas et al., 2009). Bile acid uptake into enterocytes takes place in the ileum and is mediated by apical sodium-dependent bile acid transporter (ASBT; *Slc10a2*)(Lazaridis et al., 1997). The intracellular movement of bile acids in enterocytes is mediated by fatty acid transport protein 6 (FABP6) (Nakahara et al., 2005). Bile acids can bind the nuclear receptor farnesoid X receptor (FXR) in enterocytes to promote secretion of the

hormone fibroblast growth factor 19 (FGF19; termed Fgf15 in rodents) into portal circulation to mediate feedback inhibition of hepatic bile acid synthesis (Inagaki et al., 2005). FGF19 travels to the liver where it represses the rate limiting enzyme in the conversion of cholesterol to chenodeoxycholic acid and cholic acid, cholesterol 7-α-monooxygenase (CYP7A1), after interacting with the fibroblast growth factor receptor 4 (FGFR4) (Inagaki et al., 2005). In addition to FGF19, bile acids regulate feedback inhibition of bile acid synthesis via hepatic FXR (Makishima et al., 1999). When bile acids bind to FXR in the liver, the small heterodimer partner (SHP or NR0B2), a transcriptional repressor of CYP7A1, is induced (Holt et al., 2003). Some bile acids in portal circulation escape hepatic uptake, enter systemic circulation, and activate FXR or TGR5 signaling pathways (Watanabe et al., 2006).

1.6 The intestinal mucosal barrier in health and disease

Clearly, the intestine must balance the capacity to absorb nutrients with the ability to protect the intestinal epithelium from potentially harmful luminal contents. The intestinal epithelium consists of a layer of columnar epithelial cells which is covered by a viscous, lubricated mucus barrier that protects against microbes and debris present in the lumen [reviewed in (Johansson and Hansson, 2016)]. Most of the components of mucus are produced in goblet cells. Polymers of the heavily N-glycosylated mucin proteins (primarily MUC2 in the colon) form the basic mucus scaffold which also contains many antimicrobial molecules, trefoil factors and phospholipids which are derived from intestinal epithelial cells (Velcich et al., 2002, Mashimo et al., 1996, Braun et al., 2009). The mucus layer in the small intestine is a single, loosely adherent layer (making the small intestine more reliant on antimicrobial peptides than the large intestine) while the colonic mucus layer consists of an sterile inner layer with a loosely adherent outer layer (Johansson et al., 2008). Defects in mucosal barrier function (e.g. compromised mucus integrity

or impaired secretion of protective factors like Trefoil factor 3) are linked to the development of chronic intestinal inflammation (Mashimo et al., 1996, Van der Sluis et al., 2006).

Inflammatory bowel disease, comprising Chron's disease and ulcerative colitis (UC), are chronic inflammatory conditions of the gastrointestinal tract (Khor et al., 2011). UC is largely restricted to the colon and terminal ileum while Chron's disease can occur anywhere along the gastrointestinal tract (Khor et al., 2011). Furthermore, UC is characterized by apparent loss of goblet cell mucus granules upon histological assessment of the colonic epithelium, while this is not a feature of Chron's disease (McCormick et al., 1990, Pullan et al., 1994, Strugala et al., 2008). Environmental factors are thought to play a greater role in UC compared to Chron's disease, as monozygotic twins have a concordance rate of 10-15% for UC while concordance rates rise to 30-35% for Chron's disease (Spehlmann et al., 2008). Humans genetic studies have identified pathways involved in intestinal barrier function, microbial defense, innate immune response, epithelial restitution, autophagy, ER stress, and various metabolic pathways in the pathogenesis of IBD [reviewed in (Khor et al., 2011)].

The major mucus glycoprotein MUC2 is critical for intestinal barrier function (Van der Sluis et al., 2006, Velcich et al., 2002). Mice with a deletion of the gene that encodes MUC2 have impaired mucus integrity, increased microbial interaction with the intestinal epithelium, UC and colorectal tumors (Velcich et al., 2002, Van der Sluis et al., 2006). Folding of MUC2 at the ER of goblet cells is highly demanding and makes goblet cells more susceptible to protein misfolding-induced ER-stress than other intestinal epithelial cell types (Heazlewood et al., 2008). For example, missense mutations in the gene encoding MUC2 that increase protein misfolding have been shown to promote ER stress and colitis in mice and might also contribute to human UC (Heazlewood et al., 2008). Furthermore, loss of the ER proteins ER-to-nucleus signaling 2 (ERN2;

IRE1β) and anterior gradient protein 2 homologue (Agr2) result in impaired MUC2 secretion and increased susceptibility to colitis in mice (Park et al., 2009, Bertolotti et al., 2001). Additionally, impaired O-glycosylation of MUC2 cause spontaneous colitis in mice (An et al., 2007, Fu et al., 2011a). Patients with UC have a thinner mucus layer compared to controls without UC, and defective MUC2 O-glycosylation has been reported in some patients with UC (Fu et al., 2011a, Johansson et al., 2014).

1.6.1 Phospholipids and intestinal barrier function

PC is also present in the intestinal mucus layer, and patients with UC have lower levels of mucus PC relative to healthy controls (Ehehalt et al., 2004, Braun et al., 2009). Lower mucus layer hydrophobicity due to lower mucus PC content, and increased interaction of microbes with intestinal epithelial cells, might contribute to a chronically elevated inflammatory response in UC (Braun et al., 2009). Whether lower PC levels in the mucus layer of UC patients is the result of impaired PC synthesis and/or secretion, or increased catabolism of PC, has not yet been established. Nonetheless, providing UC patients with PC in a delayed-release capsule has shown clinical promise in alleviating the disease (Stremmel et al., 2005, Stremmel et al., 2007, Karner et al., 2014). A central aim of this thesis was to investigate the role of *de novo* PC in intestinal barrier function and immune homeostasis.

1.6.2 The *Citrobacter rodentium* model of colitis

Citrobacter rodentium is a murine attaching-effacing pathogen that is widely used to study how the mammalian immune system recognizes and eliminates pathogens from the colonic epithelium (Mundy et al., 2005, Brennan et al., 1965). Importantly, oral administration of *Citrobacter rodentium* to mice induces pathological features comparable to those seen in UC

(Mundy et al., 2005, Wlodarska et al., 2011, Wiles et al., 2004). Therefore, the *Citrobacter rodentium* model has been used to uncover several mechanisms by which environmental factors (including antibiotics and diet) and genetic factors that susceptibility to colonic damage (Wlodarska et al., 2011, Lebeis et al., 2007, Metidji et al., 2018). Interestingly, interaction of *Citrobacter rodentium* with intestinal epithelial cells activates SREBP-2 and cholesterol synthesis, demonstrating that bacterial interaction with the intestinal epithelium can alter intestinal lipid homeostasis (Berger et al., 2017). A key aim of this thesis was to investigate how dietary choline availability influences murine susceptibility to *Citrobacter rodentium*-induced colitis.

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CHAPTER 2: Research plan

2.1 Rationale

Phosphatidylcholine (PC) is the primary phospholipid in mammalian cell membranes (Keenan and Morré, 1970). The activity of the enzyme CTP: phosphocholine cytidylyltransferase (CT) is rate-limiting in the CDP-choline pathway for *de novo* PC synthesis in all nucleated mammalian cells under most conditions (Kennedy and Weiss, 1956, Sundler et al., 1972, Paddon and Vance, 1980)(Figure 1.1). Consistent with a critical role for membrane biogenesis during development, global deletion of *Pcyt1a* (the gene encoding CTα) is embryonically lethal in mice (Wang et al., 2005). Mouse models with tissue-specific deletion of CTα have revealed important roles for *de novo* PC synthesis in mammalian physiology and regulation of lipid homeostasis. In hepatocytes, PC produced b*y* the CDP-choline pathway and the PEMT pathway are independently required for VLDL secretion (Noga et al., 2002, Jacobs et al., 2004, Jacobs et al., 2008). Furthermore, disruption of hepatic PC homeostasis is linked to liver inflammation and fibrosis in humans and mice, reflecting the essential role of PC supply for membrane integrity (Li et al., 2006). In macrophages, impaired *de novo* PC synthesis increases susceptibility to cholesterol-induced death (Zhang et al., 2000). Humans with biallelic loss-of-function mutations in *Pcyt1a* have lipodystrophy, suggesting that *de novo* PC synthesis plays a role in adipose tissue lipid storage (Payne et al., 2014). Maintaining lipid homeostasis and membrane integrity is likely to be especially important in the intestinal epithelium, a monolayer of selectively permeable cells that controls nutrient uptake in the face of constant immunological challenges from various luminal antigens. However, the role of *de novo* PC synthesis in intestinal epithelial cells has not be investigated.

Dietary and biliary PC delivered to the small intestinal lumen is hydrolyzed to lyso-PC and unesterified fatty acids by phospholipase A2 (Nilsson, 1968, Parthasarathy et al., 1974). Mice with genetic ablation of ATP-binding cassette sub-family B member 4 are unable to secrete PC into bile and have impaired chylomicron output, suggesting that biliary PC is required for chylomicron formation (Voshol et al., 2000). Intestine-specific deletion of lyso-PC acyltransferase 3 in mice results in lower abundance of PC species containing polyunsaturated fatty acids in the intestinal epithelium, which is associated with impaired fatty acid uptake (Wang et al., 2016, Li et al., 2015). Taken together, these results show that biliary PC supply and phospholipid remodeling play important roles in dietary lipid absorption. Whether *de novo* PC synthesis plays a similarly important role in intestinal lipid metabolism remains unknown.

In addition to its role in nutrient absorption, the intestinal epithelium serves as a crucial barrier against antigens in the intestinal lumen. The mucosal barrier consists of a hydrated mucus layer, plasma membranes of epithelial cells, tight junctions between cells, and a variety of secreted antimicrobial molecules (Turner, 2009). Inflammatory bowel diseases arise when defects in the mucosal barrier allow antigens in the intestinal lumen to interact with the immune system underlying the intestinal epithelium (Khor et al., 2011). PC is abundant in both the mucus layer and membranes of the intestinal epithelium (Ehehalt et al., 2004). Patients with ulcerative colitis have lower PC concentrations in the mucus layer of their distal intestines relative to controls without ulcerative colitis (Braun et al., 2009, Ehehalt et al., 2004). Furthermore, PC delivery to the distal intestine of ulcerative colitis patients has been shown to reduce markers of disease severity (Stremmel et al., 2005, Stremmel et al., 2007, Karner et al., 2014). However, the role that *de novo* PC synthesis plays in mucosal barrier function *in vivo* has not been investigated.

2.2 Objectives and hypotheses

The overall objective of this research was to determine the role of *de novo* PC synthesis in intestinal lipid metabolism and mucosal barrier function. The specific aims of this research were:

1. To generate a mouse model with intestine-specific deletion of CTα using tamoxifen-inducible Cre-Lox recombination ($CT\alpha^{IKO}$ mice). We initially hypothesized that these mice would have impaired intestinal viability due to the purported role for PC in cellular proliferation.
2. To determine the role of *de novo* PC synthesis in intestinal TG uptake and secretion. We hypothesized that $CT\alpha^{IKO}$ mice would have impaired intestinal TG secretion due to inadequate PC supply for assembly of the lipoprotein phospholipid monolayer (Chapter 3).

Gene expression microarray analysis of intestinal epithelial cells isolated from control mice and $CT\alpha^{IKO}$ mice showed that loss of intestinal CTα induces a robust immune response. Based on these data, we generated the following aims and objectives:

3. To determine whether CTα activity is involved in intestinal barrier function and innate immune responses in the gut. We hypothesized that impaired intestinal *de novo* PC synthesis increases exposure of the intestinal epithelium to luminal antigens due to lower mucus PC concentrations and impaired intestinal barrier function. (Chapter 4).
4. To determine whether insufficient dietary choline, a key dietary substrate for *de novo* PC synthesis, increases susceptibility to colitis induced by *Citrobacter rodentium*, a murine attaching-effacing pathogen (Chapter 5).

2.3 Chapter format

Chapter 3 reports that loss of intestinal *de novo* PC synthesis impairs dietary lipid uptake in the setting of a high fat diet (HFD), but not a chow diet. Fat malabsorption after loss of intestinal CTα is linked to enhanced postprandial enteroendocrine hormone secretion and reduced adiposity. Furthermore, we report an unexpected crosstalk between the intestine and liver that that is modulated by impaired intestinal *de novo* PC synthesis: biliary bile acid and lipid secretion is enhanced in $CT\alpha^{IKO}$ mice due to augmented proximal intestine bile acid uptake and accelerated enterohepatic bile acid cycling. Our study shows that the intestine has a specific requirement for *de novo* PC synthesis and that the re-acylation of biliary lyso-PC is insufficient to maintain intestinal metabolic function in the setting of a HFD.

Chapter 4 reports that *de novo* PC synthesis in the intestinal epithelium protects against intestinal injury in mice. Loss of intestinal *de novo* PC synthesis results in rapid and spontaneous colitis with hyperproliferation of intestinal epithelial cells. Colitis development in $CT\alpha^{IKO}$ mice is linked to induction of endoplasmic reticulum (ER) stress in intestinal epithelial cells, impaired goblet cell maturation, and damage to the mucus barrier. Our results suggest that CTα activity in intestinal epithelial cells is important for maintaining gut barrier integrity.

Chapter 5 reports that mice fed insufficient dietary choline develop more severe colitis following *Citrobacter rodentium* infection compared with mice fed normal or high levels of choline. There was more severe loss of goblet cells and enhanced production of proinflammatory cytokines in the colons of mice fed insufficient dietary choline than mice fed adequate levels of choline. Our study shows that dietary choline supply is an important for maintaining goblet cell abundance in the setting of intestinal inflammation in mice.

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CHAPTER 3: Intestinal *De Novo* Phosphatidylcholine Synthesis is Required for Dietary Lipid Absorption and Metabolic Homeostasis

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3.1 Introduction

Phospholipids form the matrix of biological membranes, provide precursors for a variety of signaling molecules, and allow assembly and secretion of lipoproteins (van der Veen et al., 2017). Phosphatidylcholine (PC) is the primary phospholipid in eukaryotic cells (van der Veen et al., 2017). The amphitropic enzyme CTP: phosphocholine cytidylyltransferase (CT) regulates *de novo* PC synthesis in response to changes in membrane lipid composition in all nucleated mammalian cells (Cornell and Ridgway, 2015). Global deletion of CTα (encoded by *Pcyt1a*) is embryonic lethal in mice, reflecting the essential role of membrane biogenesis during development (Wang et al., 2005). Mice lacking CTα in the liver have impaired very-low-density lipoprotein (VLDL) secretion and accumulate neutral lipids in hepatocytes (Jacobs et al., 2004) despite the presence of a second route for hepatic PC synthesis *via* the methylation of phosphatidylethanolamine (PE) by phosphatidylethanolamine-N-methyltransferase (PEMT). Similarly, CTα cannot compensate for the loss of PEMT in generating PC for VLDL assembly (Noga et al., 2002).

Intestinal PC can be derived from the diet, bile, circulating lipoproteins, and from local *de novo* synthesis. Dietary and biliary PC is hydrolyzed in the intestinal lumen by phospholipase A2 to lyso-PC and fatty acids before being absorbed into enterocytes, delivered to the endoplasmic

reticulum, and re-acylated into PC by lyso-PC acyltransferase (LPCAT) enzymes (Nilsson, 1968, Parthasarathy et al., 1974). Mice lacking hepatic Abcb4, which is required for PC secretion into bile, have normal passage of fatty acids from the intestinal lumen into enterocytes but have impaired chylomicron assembly and secretion, highlighting the importance of biliary PC for chylomicron output (Voshol et al., 2000). Furthermore, loss of intestinal Lpcat3, which incorporates polyunsaturated fatty acids into PC, impairs dietary lipid absorption despite maintenance of total intestinal PC mass (Li et al., 2015, Wang et al., 2016). Conversely, increasing the delivery of PC to the intestinal lumen promotes chylomicron secretion (Tso et al., 1981). Therefore, while the re-acylation of lyso-PC derived from the intestinal lumen is important for chylomicron production, the precise contribution that intestinal *de novo* PC synthesis makes to this process is unknown.

In the present study we hypothesized that loss of intestinal CTα activity would cause TG accumulation in enterocytes due to insufficient supply of PC for chylomicron assembly. However, we unexpectedly found that loss of intestinal *de novo* PC synthesis impairs passage of fatty acids from the intestinal lumen into enterocytes in the setting of a high fat diet (HFD), but not a chow diet. Fat malabsorption is linked to the repression of genes involved in fatty acid uptake, enhanced postprandial enteroendocrine hormone secretion, and reduced adiposity. Furthermore, we show that loss of intestinal CTα promotes biliary bile acid and lipid secretion by accelerating enterohepatic bile acid cycling. Our data provide evidence of a specific requirement for intestinal *de novo* PC synthesis in the maintenance of intestinal metabolic functions and show that the re-acylation of lyso-PC from extra-intestinal sources is insufficient to maintain metabolic function during HFD feeding.

3.2 Materials and methods

3.2.1 Generation of mice with intestine-specific deletion of *Pcyt1a*

Mice carrying a floxed *Pcyt1a* locus (*Pcyt1a*$^{LoxP/LoxP}$) on a C57BL/6J background were crossed with mice expressing a tamoxifen-inducible Cre-recombinase transgene under the control of a villin promoter (villin-CreER^{T2}) (el Marjou et al., 2004) to generate *Pcyt1a*$^{LoxP/WT}$;villin-CreER^{T2} mice (with the capacity to induce a heterozygous deletion of intestinal CTα after tamoxifen treatment). Subsequent cross-breeding of *Pcyt1a*$^{LoxP/WT}$;villin-CreER^{T2} mice resulted in the generation of *Pcyt1a*$^{LoxP/LoxP}$;villin-CreER^{T2} mice (with the capacity to induce a homozygous deletion of intestinal CTα after tamoxifen treatment). Genomic DNA was extracted from tail biopsies with a DNeasy Blood and Tissue Kit (Qiagen, UK). PCR products were identified on a 3% agarose gel (floxed *Pcyt1a* band) or a 1.5% agarose gel (Villin Cre-inducible band). Cre was induced in 8-12-week-old male or female mice by intraperitoneal injection of tamoxifen (1mg/day in sunflower oil for 5 days; Sigma, St. Louis, MO), while tamoxifen-treated *Pcyt1a*$^{LoxP/LoxP}$ mice were used as controls. Mice were fed a chow diet (5001, Lab Diet, St. Louis, MO) or a semi-purified HFD containing 40% kilocalories as fat (Table 3.1). Mice were housed in a temperature-controlled room with 12-h light/dark cycle and free access to food and water. Samples were collected after a 10-h fast or after overnight fasting followed by 2-h re-feeding, unless otherwise stated. Small intestines were excised, flushed with phosphate buffered saline (PBS) containing protease inhibitor cocktail (Sigma, St. Louis, MO), and kept on ice. The small intestine was divided into 3 portions of length ratio 1:3:2 (corresponding to duodenum: jejunum: ileum) before jejunum sections were fixed in 10% neutral-buffered formalin for histology. Epithelial cells were collected in liquid nitrogen after segments were opened longitudinally. The University of Alberta's Institutional Animal Care Committee approved all animal procedures,

Table 3.1. Composition of high fat diet (per Kg diet)

Ingredients	
Casein[1] (g)	270
Corn Starch[1] (g)	170
Sucrose[2] (g)	195
Cellulose[1] (g)	80
AIN-93-VX Vitamin Mix[1] (g)	19
Bernhart-Tomarelli Mineral Mix[1] (g)	50
Calcium Phosphate Dibasic[2] (g)	3.4
myo-Inositiol[2] (g)	6.3
L-cysteine[2] (g)	1.8
Choline Bitartrate[2] (g)	4.2
Vegetable Oil[3] (g)	32
Corn Oil[4] (g)	10
Lard[5] (g)	155
DHAsco[6] (g)	1.5
Arasco[6] (g)	1.5
Fatty acid composition (g/100g of total fatty acids)[7]	
C14:0	1.10
C16:0	21.01
C16:1n-9	1.73
C18:0	12.28
C18:1n-9	37.05
18:2n-6	22.95
C20:0	0.33
C18:3n-3	1.64
C20:1	0.05
C20:2n-6	0.44
20:3n-6	0.03
C22:0	0.06
20:4n-6	0.31
22:6n-3	0.32
[1] Harlan Teklad (Indianapolis, IN, USA)	
[2] Sigma (St. Louis, MO, USA)	
[3]Crisco J.M. Smucker Company (Orrville, OH, USA)	
[4]Mazola ACH Food Companies Inc. (Oakbrook Terraces, IL, USA)	
[5]TenderFlake (Chicago, IL, USA)	
[6]DSM Nutritional Products Inc. (Heerlen, The Netherlands)	
[7]Determined by gas-liquid chromatography	

which were in accordance with guidelines of the Canadian Council on Animal Care.

3.2.3 Microscopy

Formalin-fixed, paraffin-embedded tissue slices (5µm thick) were stained with hematoxylin and eosin (H&E) and visualized with a light microscope. For electron microscopy, 2cm jejunal rings were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2). Sections were cryo-sectioned with an ultramicrotome (Ultracut E, Reichert-Jung) equipped with an FC4D attachment, and images were obtained using a Philips 410 transmission electron microscope.

3.2.3 mRNA isolation and quantification by PCR

Total RNA was isolated from intestinal scrapings or liver using Trizol (Invitrogen, CA, USA). RNA was reversed-transcribed using Superscript II (Invitrogen, CA, USA). Quantitative PCR was run on an Applied Biosystems StepOne Plus for 40 cycles using a Power SYBR Green PCR Master Mix (Applied Biosystems, MA, USA), in triplicate. Relative mRNA expression was normalized to cyclophilin. Quantitation was performed using the standard curves method. Primer sequences are listed in Table 3.2.

Table 3.2. qPCR primer sets used in Chapter 3

Gene	*Forward sequence*	*Reverse sequence*
Pcyt1a	GCT AAA GTC AAT TCG AGG AA	CAT AGG GCT TAC TAA AGT CAA CT
Mogat2	TAC AGC TTT GGC CTC ATG C	AGG GCT GTG GTG TCA TCT G
Dgat2	GGC TAC GTT GGC TGG TAA CTT	TTC AGG GTG ACT GCG TTC TT
Abhd5	ATC TTT GGA GCC CGA TCC T	CTT CTG GCT GAT CTG CAT ACA C
Cidec	GGG TCA CAG CTT GGA GGA	CTC CAC GAT TGT GCC ATC T
Mttp	ATA CAT GCA AAA TTG AGC GGT CT	CCT GGT CTC TTC TGC AAG CAC
Apoc3	AGG AGT CCG ATA TAG CTG TGG T	TGC TCC AGT AGC CTT TCA GG
Lpcat3	GGC CTC TCA ATT GCT TAT TTC A	AGC ACG ACA CAT AGC AAG GA
Lpcat4	GGC CTC CAG AGG GTT AAG TT	AAA AGC TAG AAG TAC TCG GAT TGG
Cd36	TGG CTA AAT GAG ACT GGG ACC	ACA TCA CCA CTC CAA TCC CAA G
Slc27a4	GAA GGG GGA CCA AGC CTA	AGT TCC TGG CAC CTC AAC AC
Fabp6	GGC AAA GAA TGT GAA ATG CAG	CCG AAG TCT GGT GAT AGT TGG
Slc10a2	AGC TGG TCA ACC CTG GTA CA	GGG GGA GAA GGA GAG CTG
Slc51a	GCT GCC CAC CTC TCA TAC TT	GAA GAA GGC GTA CTG GAA AGG
Slc51b	GAG CAT CCT GGC AAA CAG A	TGC AGG TCT TCT GGT GTT TCT
Nr0b2	CGA TCC TCT TCA ACC CAG AT	AGC CTC CTG TTG CAG GTG T
Cyp7a1	ACA CCA TTC CTG CAA CCT TC	TCT TGG CCA GCA CTC TGT AA
Cyp8b1	GCA GCA CTG AAT ACC CAT CC	TCT GAG AGC TGG GGA GAG G
Cyp27a1	CTT TCC TGA GCT GCT TTT GG	CAC CAG TCA CTT CCT TGT GC

3.2.4 Fatty acid absorption

Intestinal fatty acid absorption was quantified as described previously (Yen et al., 2009), with modifications. Briefly, male control and CTα^{IKO} mice were fasted for 4 h before receiving an oral gavage of 150µL of olive oil containing 5µCi [^{3}H]-labelled triolein. Blood was collected at 30-, 60-, 90- and 120-mins post-gavage. At 120 mins post-gavage, the small intestine was excised, flushed with 40ml PBS containing 0.5mM sodium taurocholate and cut into 2cm segments. Segments were dissolved in 1N NaOH for 3 days at 60℃. Plasma and tissue [^{3}H]-label was measured by liquid scintillation counting (Beckman Coulter Inc, CA). For poloxamer studies, mice were fasted for 10 h before receiving an intraperitoneal injection of Poloxamer-407 (1g/kg; Sigma, MO, USA) and an oral gavage of 150µL olive oil. Blood was collected before, and every hour for 4 h after the oil challenge. Plasma TG concentrations were assessed using a commercially available kit (Sekisui Diagnostics, MA). To visualize intestinal lipid droplets after a fat challenge, 4-h fasted female mice were gavaged (7.5µL/g mouse) with olive oil containing 20% Bodipy 500/510 C1, C12 (D3823, Invitrogen, CA, USA). Sections of the proximal small intestine were embedded in optimum cutting temperature (OCT) and sliced on cryostat at -20 degrees Celsius. Slides were mounted with Prolong Diamond Antifade Mountant containing DAPI (P36962, Invitrogen, CA, USA) before images were obtained with a fluorescence microscope (Olympus, Markham, ON) equipped with Suveyor and ImagePro Plus software.

3.2.5 Cholesterol absorption

Individually-housed male control and CTα^{IKO} mice received an oral dose of [^{3}H]-cholesterol (2 µCi) with 6mg unlabeled cholesterol in 150µL of olive oil, and an intravenous injection of [^{14}C]-cholesterol (1µCi) mixed in Intralipid®. Blood and feces were collected at 24, 48 and 72 h after cholesterol administration. Blood was centrifuged at 3000g for 10 minutes and plasma was

collected. Plasma [^{3}H]-label and [^{14}C]-label was measured by liquid scintillation counting (Beckman Coulter Inc, CA). The [^{3}H]-label and [^{14}C]-label in fecal neutral sterol and fecal bile salt (aqueous) fractions were measured by liquid scintillation counting (Beckman Coulter Inc, CA) after separation of the fractions by the method of Folch (FOLCH et al., 1957).

3.2.6 Gallbladder cannulations and bile measurements

For gallbladder cannulations, male mice were anesthetized by intraperitoneal injection of ketamine (100mg/kg)/ xylazine (10mg/kg). Gallbladders were cannulated with silastic tubing (Dow Corning, MI; internal diameter 0.5mm) after ligation of the common bile duct beside the duodenum. Mice were placed in a humidified incubator and bile was collected for 30 mins in pre-weighed vials. Bile acid species composition (including taurohyodeoxycholic acid (THDCA), tauro-β-muricholic acid (TB-MCA), tauro-α-muricholic acid (TA-MCA), β-muricholic acid (β-MCA), taurodeoxycholic acid (TDCA), taurochenodeoxycholic acid (TCDCA), taurocholic acid (TCA), tauroursodeoxycholic acid (TUDCA) and cholic acid (CA)) was determined by liquid chromatography tandem mass spectrometry (LC-MS/MS) as described previously (Alnouti et al., 2008). The hydrophobicity of bile acids was determined according to the method of Heuman (Heuman, 1989). Biliary lipids were extracted by the method of Folch (FOLCH et al., 1957). Biliary phospholipid concentrations were assessed by phosphorous assay, as described previously (Zhou and Arthur, 1992). Biliary cholesterol concentrations were measured with a commercially available kit (WAKO Chemicals, USA). Biliary secretion rates were calculated by multiplying biliary bile acid, phospholipid, or cholesterol concentrations by bile flow (μL/min). Bile acid concentrations in feces and plasma were determined by enzymatic fluorometric assay (Mashige et al., 1976).

3.2.7 Plasma, tissue, and fecal lipid analysis

Plasma TG (Sekisui Diagnostics, MA, USA), cholesterol (Wako Chemicals, USA) and non-esterified fatty acids (Wako Chemicals, USA) were measured with commercially available kits. Lipoprotein fractions were separated from pooled plasma samples (5 mice per group) by size exclusion fast protein liquid chromatography (FPLC) before measuring TG and cholesterol concentrations by commercially-available kits (Lipidomics Core Facility, University of Alberta). Total protein concentrations of tissue homogenates were determined by bicinchoninic acid assay before tissue lipids were extracted from homogenates (1mg/ml) by the method of Folch (FOLCH et al., 1957). PC and phosphatidylethanolamine (PE) were separated by thin layer chromatography using the solvent system chloroform: methanol: acetic acid: water (50:30:8:4). Bands were visualized after exposure to iodine and measured by phosphorous assay, as described previously (Zhou and Arthur, 1992). Intestinal TG concentrations were measured using a commercially available kit (Sekisui Diagnostics, MA, USA). The relative abundance of PC species in jejunal epithelial cells was measured by mass spectrometry at The Metabolomics Innovation Centre, University of Alberta. To analyze the fatty acid composition of intestinal PC, lipids were extracted from intestinal scrapings and separated by thin-layer chromatography as described above. PC bands were recovered from the plates and converted to fatty acid methyl esters by incubation with 2 ml of hexane (Sigma Aldrich, 293253) and 1.5ml boron trifluoride (Sigma-Aldrich, B1252) in methanol for 1 h at 110 °C. Fatty acid methyl esters were then analyzed by gas-liquid chromatography (results are shown as the percentage of total fatty acids measured). Intestinal non-esterified cholesterol and cholesterol esters were measured by gas-liquid chromatography with flame ionization detection (GC-FID) after derivatization with bis(trimethylsilyl)trifluoroacetamide and adjustment of peaks with the internal standard tridecanoin, as described previously (Myher

and Kuksis, 1984). Fecal samples (50mg) were dried and ground to a powder before extraction of lipids by the method of Folch (FOLCH et al., 1957). Non-esterified fatty acid and cholesterol concentrations in feces of female mice collected over 72 h were determined with commercially available kits (Wako Chemicals, USA).

3.2.8 Measurement of intestinal CT activity

Intestinal CTα activity was determined by measuring the conversion of [^{3}H]phosphocholine into [^{3}H]CDP-choline, as described previously (Jacobs et al., 2004).

3.2.9 Plasma biochemical measurements

Blood glucose was measured from the tail vein with a glucometer (Accu-chek, Roche, Switzerland) after a 10-h fast, after 2 h re-feeding or after 2 h re-feeding followed by an oral gavage of glucose (2mg/g body weight). Blood was collected by cardiac puncture in tubes containing EDTA, dipeptidyl peptidase 4 inhibitor (EMD Millipore, MA, USA) and Complete® general protease inhibitor (Sigma, MO, USA) before being centrifuged at 3000g for 10 minutes to obtain plasma. Plasma insulin (Meso Scale Diagnostics, MD, USA), leptin (Meso Scale Diagnostics, MD, USA), active GLP-1 (EMD Millipore, MA, USA), PYY (Crystal Chem IL, USA), and FGF21 (Abcam, Cambridge, UK) were measured by enzyme-linked immunosorbent assay, according to the manufacturer's instructions. Plasma (4μL) was resolved on a sodium dodecyl sulfate polyacrylamide gel (5% gel for ApoB and 10% for ApoA1). Proteins were transferred to a to a polyvinylidene difluoride membrane and probed with either anti-Apolipoprotein B (dilution 1:7500, AB742; Chemicon, MA, USA) or Anti-Apolipoprotein A1 (dilution 1:2000; K23001R, BioDesign/Meridian). ApoB and ApoA1 were run on separate gels. Immunoreactive proteins were

detected with Enhanced ChemiLuminescence (ECL; Amersham, GE Healthcare, UK), and blots were imaged with a ChemiDoc MP Imager, (Bio-rad Laboratories, CA, USA).

3.2.10 Statistics

Data are expressed as mean ± standard error of the mean using Graphpad Prism 7 (GraphPad Software, La Jolla, CA); $p < 0.05$ was considered statistically significant. Student's t-test was used to compare two independent groups. A one-way ANOVA was used when comparing 3 groups with one experimental outcome. A two-way ANOVA with Bonferroni post-test was used to determine the effect of genotype and time on an experimental outcome.

3.3 Results

3.3.1 Intestinal PC concentrations are reduced in CTα ^{IKO} mice fed a chow diet, but fat absorption capacity is unaltered.

Induction of Cre recombinase with tamoxifen in *Pcyt1a*$^{LoxP/LoxP}$;villin-CreER^{T2} mice (herein referred to as CTα^{IKO} mice) reduced *Pcyt1a* mRNA abundance and CTα enzyme activity in the jejunum and ileum by >90% compared to tamoxifen-treated *Pcyt1a*$^{LoxP/LoxP}$ control mice (Figure 3.1A-3.1C). *Pcyt1a* mRNA remained repressed in the intestines of CTα^{IKO} mice for at least 50 days following Cre induction (Figure 3.1D). Characterization of mice with a heterozygous deletion of intestinal CTα fed either a chow diet (Figure 3.2A-3.2E) or a HFD (Figure 3.2F-3.2J) revealed no differences in body weight, blood glucose, plasma lipids, or fat absorption after an oral lipid bolus despite a ~50% decrease in intestinal *Pcyt1a* mRNA abundance compared to control mice (Figure 3.1E).

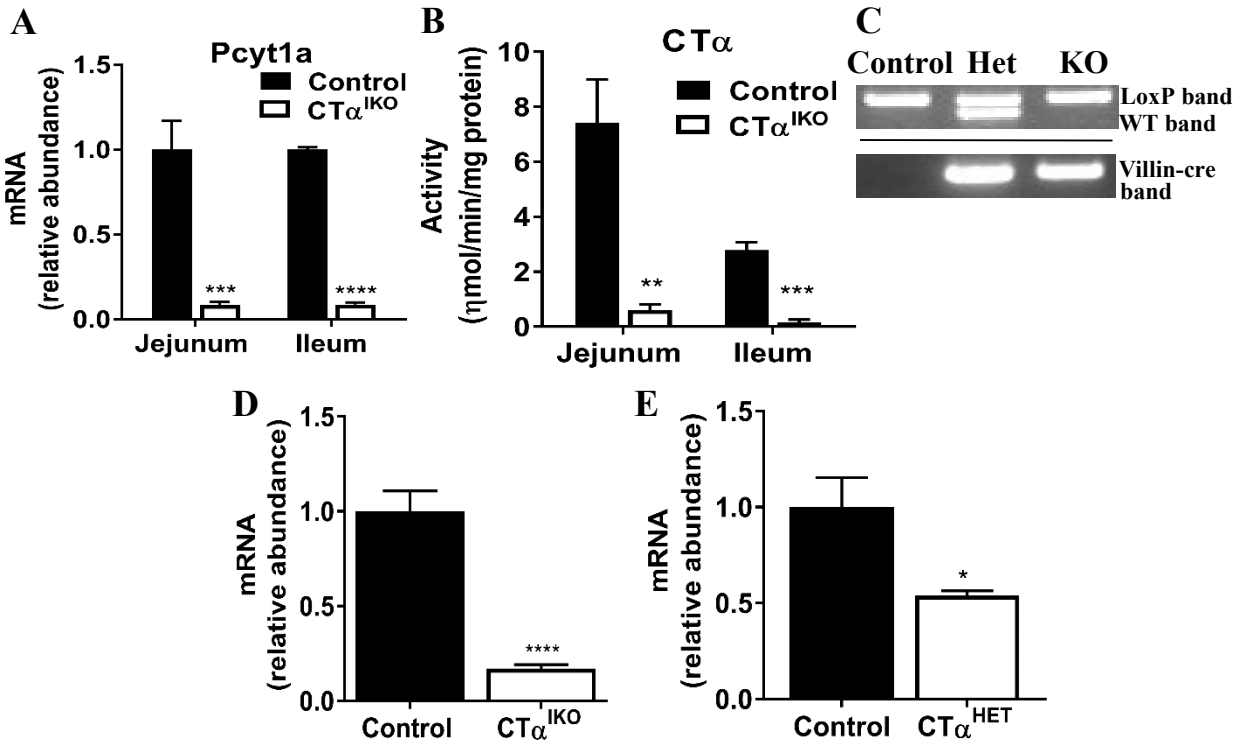

Figure 3.1. Generation of *Pcyt1a*$^{LoxP/LoxP}$;villin-CreER^{T2} (CTα^{IKO}) mice, *Pcyt1a*$^{LoxP/WT}$;villin-CreER^{T2} mice (CTα^{HET}) *and* *Pcyt1a*$^{LoxP/LoxP}$ (Floxed control) mice. (A) *Pcyt1a* mRNA abundance, and (B) CTα enzyme activity in the jejunum and ileum of control and CTα^{IKO} mice 5 days after Cre induction. (C) Representative genotyping of *Pcyt1a*$^{LoxP/LoxP}$ without Villin-Cre (Control), *Pcyt1a*$^{LoxP/WT}$ with Villin-Cre (CTα^{Het}) and *Pcyt1a*$^{LoxP/LoxP}$ with Villin-Cre (CTα^{IKO}). (D) *Pcyt1a* mRNA in the jejunum of control and CTα^{IKO} mice 50 days after Cre induction (n=5/group). (E) *Pcyt1a* mRNA in jejunums of vehicle-treated *Pcyt1a*$^{LoxP/WT}$;Villin-CreER^{T2} (control) mice and CTα^{Het} mice (n=5/group). Values are means ± SEM. *p<.05, **p<.01. Values are means ± SEM. *p<.05, ****p<.0001.

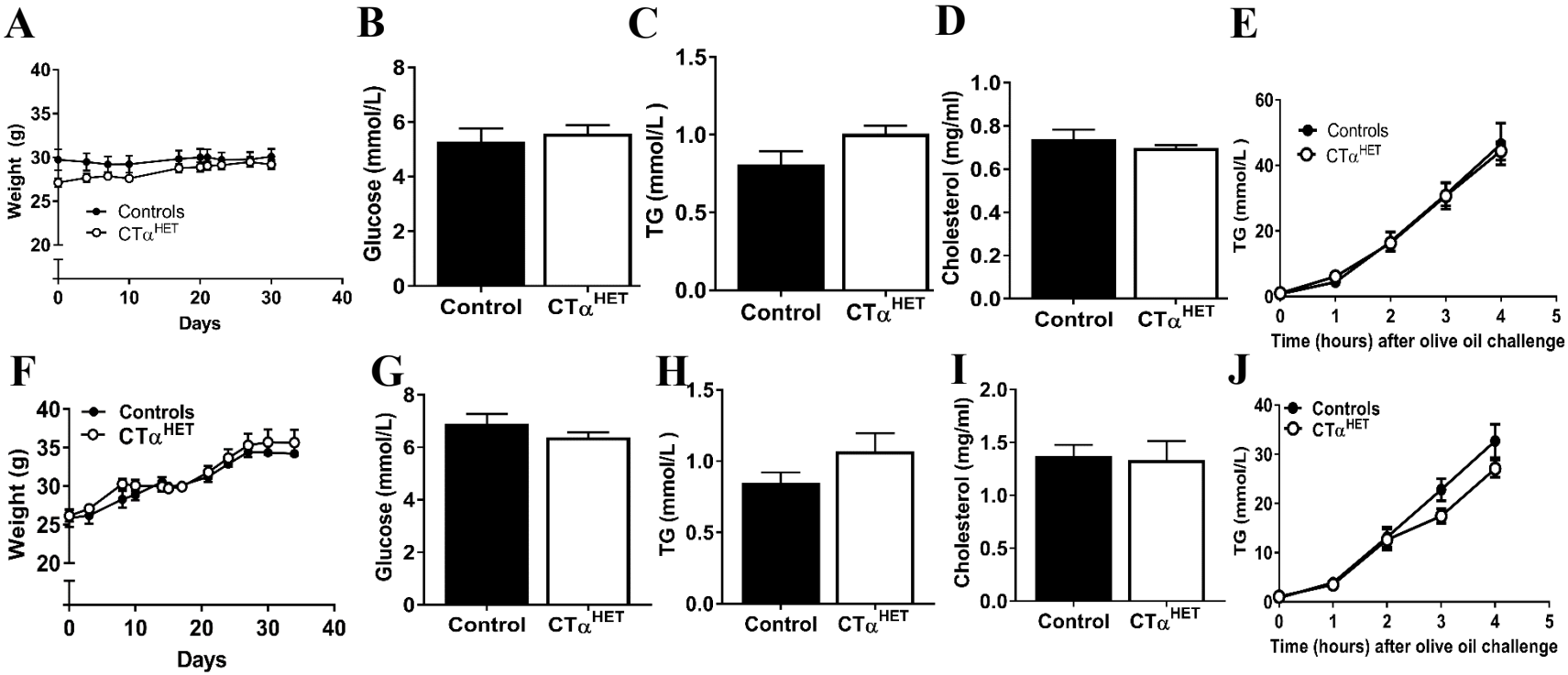

Figure 3.2 Metabolic characterization of mice with a heterozygous deletion of intestinal CTα fed either a chow diet or a high fat diet. (A) Growth curves, (B) fasting blood glucose, (C) fasting plasma TG concentrations, (D) fasting plasma cholesterol concentrations, and (E) plasma TG before and 1, 2, 3, 4 h after an oral bolus of olive oil and intraperitoneal injection of Poloxamer-407 in CTα^{Het} mice and vehicle-treated *Pcyt1a*$^{LoxP/WT}$;Villin-CreER^{T2} (control) mice fed the chow diet (n=5/group). (F) Growth curves, (G) fasting blood glucose (H) fasting plasma TG concentrations, (I) fasting plasma cholesterol concentrations, and (J) plasma TG before and 1, 2, 3, 4 h after an oral bolus of olive oil and intraperitoneal injection of Poloxamer-407 in CTα^{Het} mice and vehicle-treated *Pcyt1a*$^{LoxP/WT}$;Villin-CreER^{T2} (control) mice fed the HFD (n=5-6/group). Values are means ± SEM.

Loss of intestinal CTα did not impact survival of the mice fed the chow diet, and body mass of $CT\alpha^{IKO}$ mice was comparable to that of controls after 6 weeks (Figure 3.3A). Furthermore, histological examination of H&E-stained jejunum sections at 6 weeks after Cre induction revealed no overt morphological abnormalities in $CT\alpha^{IKO}$ intestines compared to control intestines (Figure 3.3B). This finding suggests that intestinal CTα activity is not required for maintenance of epithelial cell turnover in adult mice. PC concentrations were lower in the jejunum and ileum of $CT\alpha^{IKO}$ mice compared to controls after an oil challenge (Figure 3.3C). Furthermore, PE concentrations were higher in the jejunum of $CT\alpha^{IKO}$ mice (Figure 3.3D). This resulted in a relatively large decrease in the molar ratio of PC-to-PE in the intestines of $CT\alpha^{IKO}$ mice (Figure 3.3E). Fasting plasma cholesterol concentrations (Figure 3.3F) were significantly lower in $CT\alpha^{IKO}$ mice fed the chow diet, while fasting plasma TG (Figure 3.3G), insulin and blood glucose concentrations (not shown) were unchanged compared to control mice.

Unexpectedly, despite the absence of intestinal CTα activity and a dramatic decrease in the PC/PE ratio of intestinal membranes, TG appearance in plasma was comparable between $CT\alpha^{IKO}$ mice and control mice after an oral bolus of olive oil (Figure 3.3H). This finding suggests that, in contrast to the liver where 'new' PC synthesis is required for VLDL secretion (Jacobs et al., 2004), intestinal *de novo* PC synthesis is dispensable for intestinal TG secretion in the setting of a low-fat diet. These results also suggest that PC supplied to the intestine in bile is sufficient for chylomicron formation and secretion under these conditions.

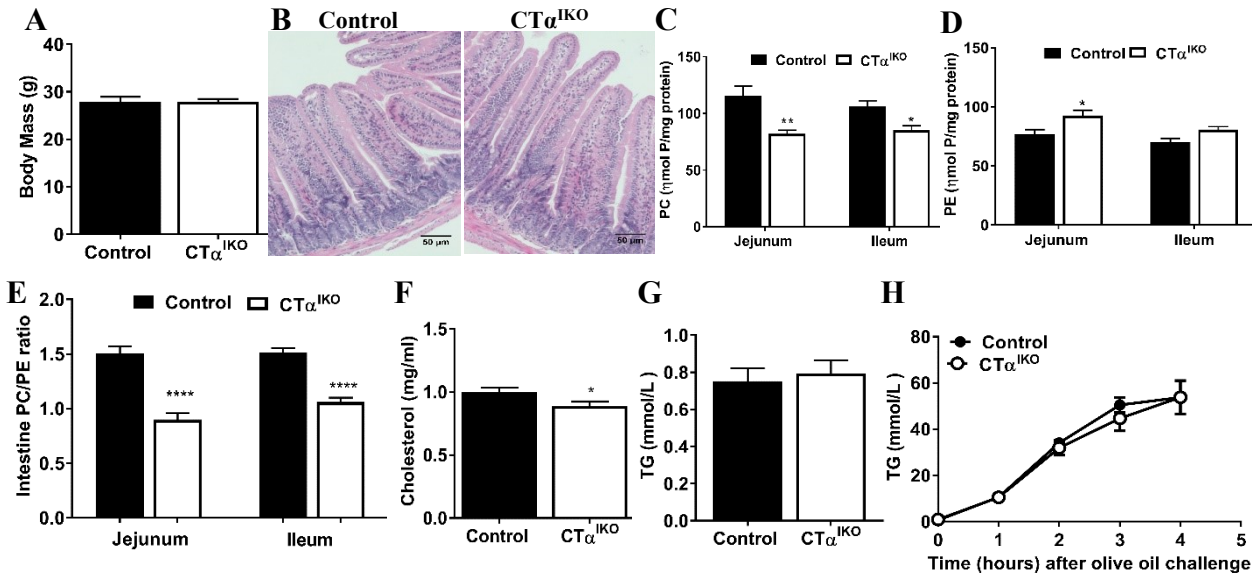

Figure 3.3. Intestinal PC concentrations are reduced in CTα^{IKO} mice fed a chow diet, but fat absorption capacity is unaltered. Male control and CTα^{IKO} mice were fed a chow diet for 6 weeks following Cre induction before being fasted for 10 h, intraperitoneal injected with Poloxamer-407, and gavaged with olive oil. (A) Fasting body mass of male control and CTα^{IKO} mice fed the chow diet for 6 weeks (n = 10 or 11 per group). (B) Representative H&E-stained jejunum sections from control and CTα^{IKO} mice fed the chow diet for 6 weeks. (C) PC concentrations in the jejunum and ileum (n = 5 per group). (D) PE concentrations in the jejunum and ileum (n = 5 per group). (E) Molar ratio of PC-to-PE in the jejunum and ileum (n = 5 per group). Fasting plasma cholesterol (F), fasting plasma TG (G), and plasma TG (H) before and 1, 2, 3, and 4 h following the oral bolus of olive oil in mice fed the chow diet for 6 weeks. All mice were male. Values are means ± SEM, n=5/group. *p<.05, **p<.01, ****p<.0001.

3.3.2 HFD feeding initially induces rapid weight loss, while chronic HFD feeding reduces weight gain, in CTα^{IKO} mice

We hypothesized that feeding mice a HFD would increase demand for lipid droplet and chylomicron PC and therefore induce more pronounced changes to the intestinal epithelium of CTα^{IKO} mice. Analysis of 3 independent experiments showed that 26% of CTα^{IKO} mice (5 of 19) lost ~25% body weight and displayed steatorrhea between days 4 and 5 following initiation of the HFD, forcing termination of these mice under protocol requirements (Figure 3.4A). Interestingly, the remaining 74% of CTα^{IKO} mice experienced only modest weight loss but had significantly less visceral fat mass and lower plasma leptin concentrations than did control mice on day 6 following initiation of the HFD (Figure 3.4B and 3.4C).Weight loss in CTα^{IKO} mice coincided with reduced food intake (2.76 g ± 0.25 by control mice compared to 1.36 g ± 0.21 by CTα^{IKO} mice between 48 and 72 h after initiation of the HFD, P=0.0009).

The CTα^{IKO} mice fed a HFD for 50 days gained 20% less body weight and had 40% less visceral fat mass compared to controls (Figure 3.4D and 3.4E). The lower food intake observed in CTα^{IKO} mice compared to control mice in the first week following initiation of the HFD appeared to be an acute response to the diet, as food intake was similar between CTα^{IKO} mice and control mice by week 6 following HFD initiation (2.257± 0.16 g/day for control mice compared to 2.034 ± 0.17 g/day for CTα^{IKO} mice, P=0.36). Nevertheless, fasting blood glucose was significantly lower in CTα^{IKO} mice (Figure 3.4F). In contrast to liver-specific CTα knockout mice (Jacobs et al., 2004), fasting plasma TG and cholesterol concentrations were not reduced in CTα^{IKO} mice after being fed a HFD for 6 days or 50 days (Figure 3.4G and 3.4H). Taken together, these data show that HFD feeding induces weight loss, while chronic feeding reduces weight gain in CTα^{IKO} mice.

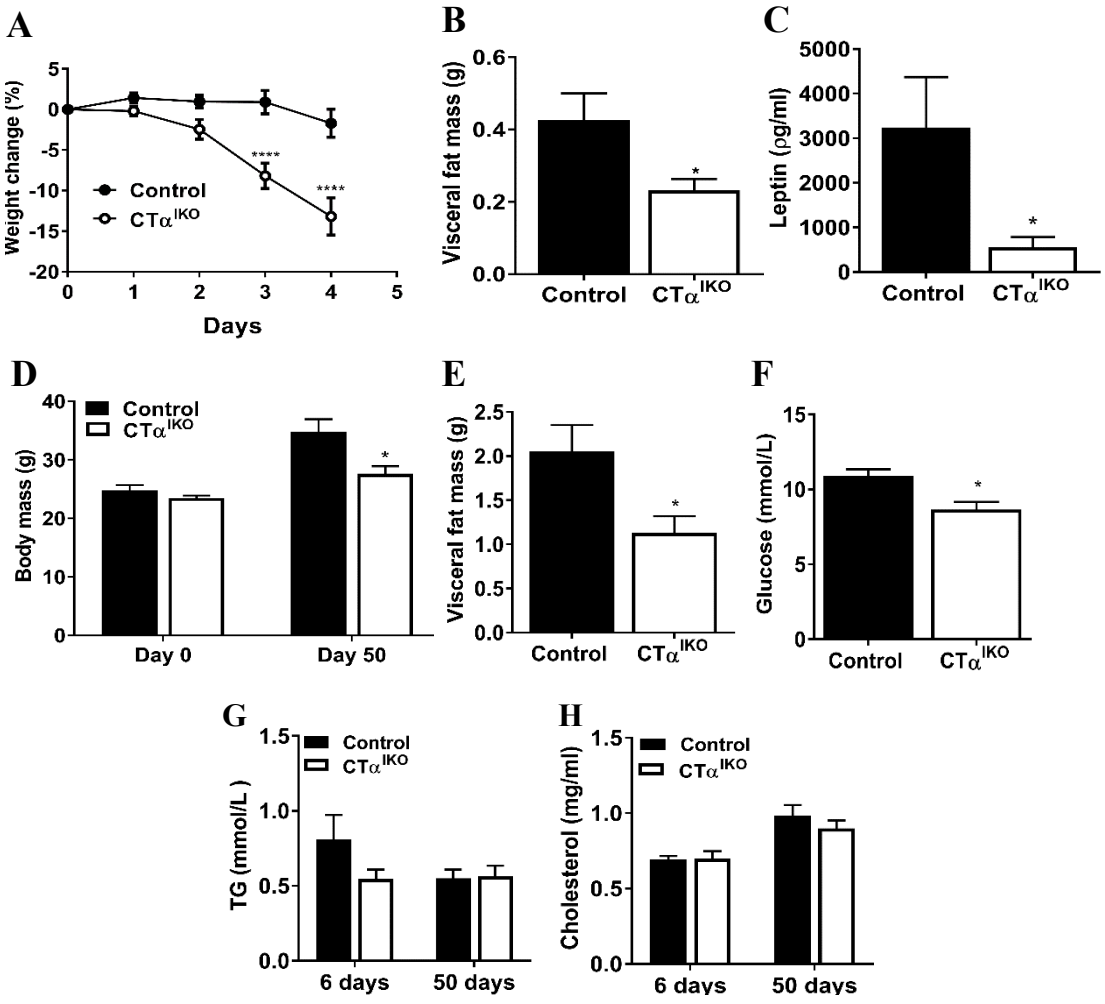

Figure 3.4. HFD feeding initially induces rapid weight loss, while chronic HFD feeding reduces weight gain, in CTα^{IKO} mice. (A) Body mass change relative to total body mass at time of initiation of the HFD; graph is representative of 2 independent experiments (n=12 control and 14 CTα^{IKO} mice). (B) Visceral fat mass, and (C) plasma leptin concentrations of control and CTα^{IKO} mice on day 6 following initiation of the HFD (n=6/group). (D) Body mass of control mice and CTα^{IKO} mice on day 0 and day 50 after initiation of the HFD (n=5/group). (E) Visceral fat mass, and (F) blood glucose concentrations of control mice and CTα^{IKO} mice on day 50 after initiation of the HFD (n=5/group). (G) Fasting plasma TG, and (H) fasting plasma cholesterol concentrations of control mice and CTα^{IKO} mice on day 6 and day 50 after initiation of the HFD (n≥5/group). All mice were female. Values are means ± SEM. *p<.05, ****p<.0001.

3.3. Loss of intestinal CTα reduces chylomicron lipidation during HFD feeding

To determine whether impaired dietary lipid absorption contributed to the acute weight loss in $CT\alpha^{IKO}$ mice fed the HFD, we assessed postprandial plasma lipid concentrations. Control and $CT\alpha^{IKO}$ mice were fasted overnight on day 3 following initiation of the HFD (prior to onset of severe weight loss) before re-feeding them for 2 h. Food intake was similar between $CT\alpha^{IKO}$ mice and control mice during HFD re-feeding (not shown). Plasma TG concentrations were 60% lower and plasma non-esterified fatty acid concentrations were 30% lower in $CT\alpha^{IKO}$ mice compared to controls after 2 h HFD re-feeding (Figure 3.5A). Furthermore, $CT\alpha^{IKO}$ mice had markedly less TG in the largest lipoprotein fraction compared to controls (Figure 3.5B). Interestingly, despite a dramatic difference in plasma TG concentrations between genotypes after re-feeding, plasma ApoB concentrations were not different (Figure 3.5C). This finding strongly suggests that lipidation of the intestine-derived lipoprotein particles was impaired in $CT\alpha^{IKO}$ mice, whereas the number of particles secreted was not reduced. Total plasma cholesterol concentrations were similar between $CT\alpha^{IKO}$ mice and control mice after HFD re-feeding (Figure 3.5A). However, $CT\alpha^{IKO}$ mice had less cholesterol in the largest lipoprotein fraction, while high-density lipoprotein (HDL) cholesterol (Figure 3.5D) and plasma ApoA1 concentrations were not different between genotypes (Figure 3.5C). To determine whether enhanced particle clearance contributed to the low postprandial plasma TG in $CT\alpha^{IKO}$ mice, we challenged the mice with an intra-gastric bolus of olive oil after intra-peritoneal injection of Poloxamer-407, an inhibitor of lipoprotein lipase. Intestinal TG secretion remained blunted in $CT\alpha^{IKO}$ mice over the 4 h study period (Figure 3.5E). Lower plasma TG after re-feeding was also observed in $CT\alpha^{IKO}$ mice compared to controls fed a HFD for 6 weeks (1.14 ± 0.06 mmol/L in control mice compared to 0.83± 0.09 mmol/L in $CT\alpha^{IKO}$ mice, P=0.03). Taken together, these data show that the intestine secretes TG-poor ApoB-containing particles in the absence of *de novo* PC synthesis.

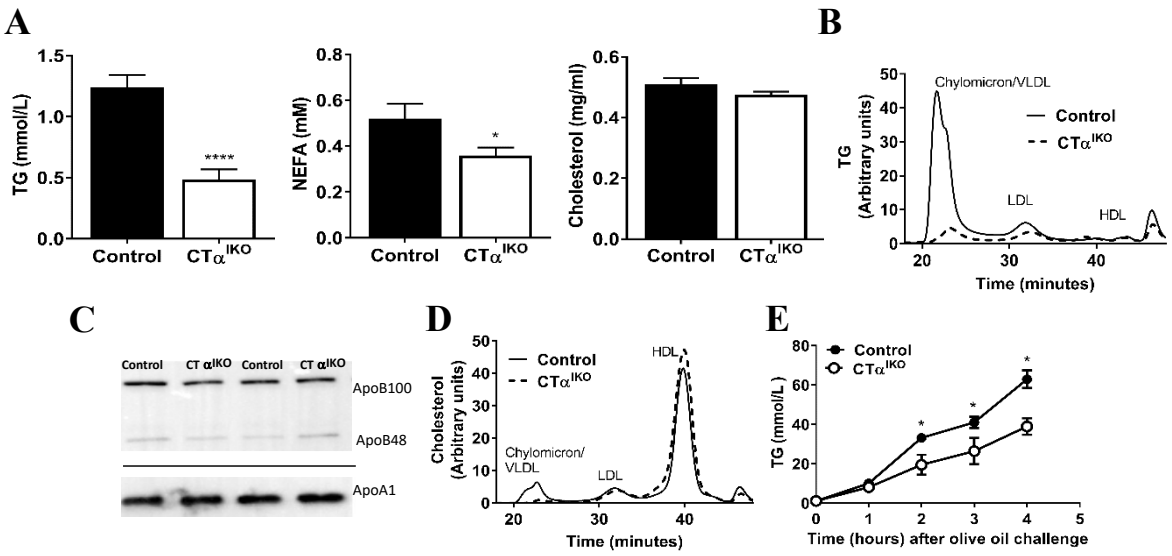

Figure 3.5. CTα^{IKO} mice fed a HFD have impaired intestinal TG secretion after a meal. Control and CTα^{IKO} mice were fasted overnight and re-fed the HFD for 2 h. (A) Plasma TG, non-esterified fatty acids, and cholesterol after re-feeding (n=10/group). (B) FPLC of plasma lipoprotein TG after re-feeding (pooled plasma samples from n=5/group). (C) Representative immunoblot of plasma apolipoproteins after re-feeding (ApoB and ApoA1 were run on separate gels). (D) FPLC of plasma lipoprotein cholesterol after re-feeding (pooled plasma samples from n=5/group). (E) Plasma TG before and 1, 2, 3, 4 h following an oral bolus of olive oil and intraperitoneal injection of Poloxamer-407. (n=4/group). All mice were female. Values are means ± SEM. *p<.05, ****p<.0001.

3.3.4 Loss of intestinal CTα impairs fatty acid uptake from the intestinal lumen into enterocytes

We hypothesized that impaired intestinal TG secretion in CTα^{IKO} mice was due to TG accumulation in enterocytes. However, CTα^{IKO} mice had significantly lower jejunal TG concentrations than control mice after fasting and re-feeding (Figure 3.6A and 3.6B). To track the spatial distribution of fatty acid uptake in the small intestine, we gavaged mice with [^{3}H]-labeled triolein. The radioactivity in the mid-intestine of CTα^{IKO} mice, where fatty acid absorption is typically highest, was strikingly lower than in control mice (Figure 3.6C). As expected, based on

previous results, appearance of [^{3}H]-label in plasma was significantly blunted in CTα^{IKO} mice (Figure 3.6D). Taken together, these data indicate that loss of intestinal CTα impairs fatty acid uptake from the intestinal lumen into enterocytes in the setting of a HFD.

Histological examination of jejunal sections 2 h after a bolus of olive oil revealed fewer lipid droplets in enterocytes of CTα^{IKO} mice compared to control mice fed the HFD (Figure 3.6E). We also detected markedly fewer fluorescent lipid droplets in enterocytes of CTα^{IKO} mice gavaged with Bodipy-labeled fatty acids (Figure 3.6F). Moreover, electron microscopy showed a general absence of cytosolic lipid droplets in HFD-fed CTα^{IKO} enterocytes 2 h after an oil challenge (Figure 3.6G). Consistent with lower fatty acid concentrations in enterocytes, the mRNA levels of *monoacylglycerol O-acyltransferase 2* (*Mogat2)*, *diacylglycerol O-acyltransferase 2* (*Dgat2)* and lipid droplet-associated *abhydrolase domain containing 5* (*Abhd5*) and *cell death-inducing DFFA-like effector c* (*Cidec*) were significantly lower in CTα^{IKO} intestines 2 h after a meal (Figure 3.6H). These data suggest that impairment of intestinal PC synthesis reduces fatty acid uptake from the intestinal lumen and thereby limits fatty acid supply for lipid droplet and chylomicron formation. In further support of impaired fat absorption, non-esterified fatty acids were elevated in feces of CTα^{IKO} mice compared to control mice (Figure 3.6I).

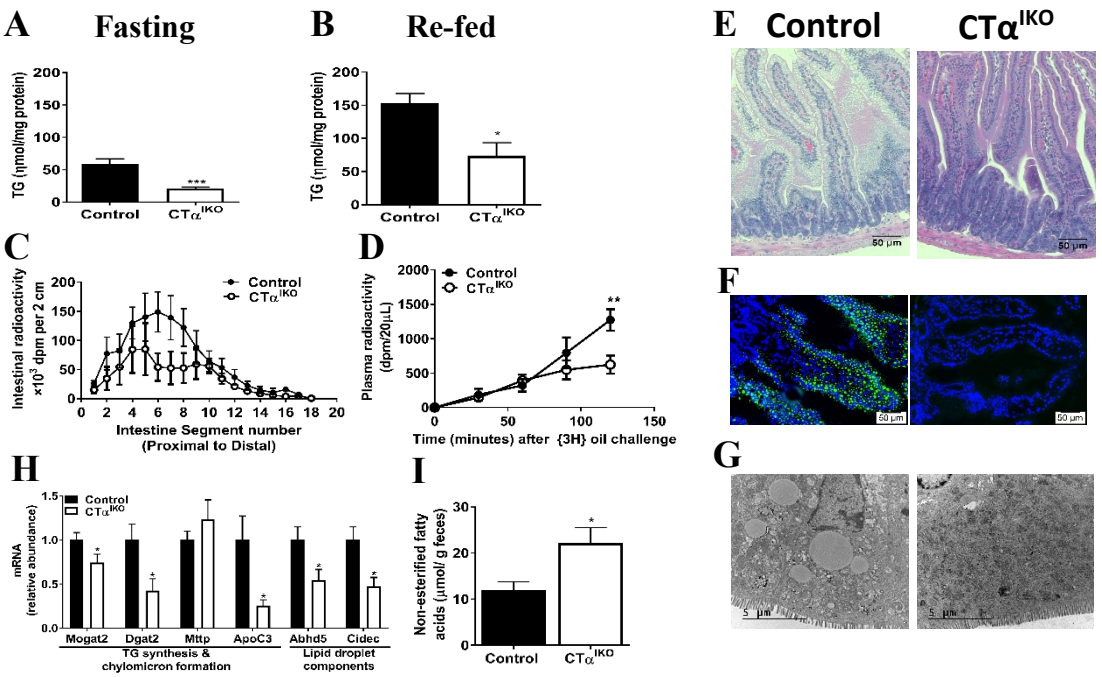

Figure 3.6. CTα^{IKO} mice fed a HFD have impaired passage of fatty acids from the intestinal lumen into enterocytes. Jejunal TG concentrations of female control and CTα^{IKO} mice after (A) fasting or (B) 2 h after re-feeding the HFD (n=5-6/group). Male control and CTα^{IKO-} mice were fasted for 4 h on day 3 following initiation of the HFD before receiving an oral bolus of 5μCi [^{3}H]-labeled triolein in 150μL olive oil for 2 h (n=5/group). (C) Radiolabel in intestine segments 2 h after [^{3}H]-labeled triolein gavage. (D) Plasma radiolabel before and 30, 60, 90 and 120 mins after a [^{3}H]-labeled triolein gavage. Female control and CTα^{IKO} mice were fasted for 4 h on day 3 following initiation of the HFD before receiving an oral bolus of Bodipy-labelled olive oil. (E) Representative H&E stained proximal intestine sections of control and CTα^{IKO} mice 2 h after oil gavage. (F) Fluorescence microscope images of jejunal sections 2 h after Bodipy-labelled oil gavage. (G) Representative Transmission Electron Micrographs of jejunal enterocytes 2 h after oil gavage. (H) Jejunal mRNA abundance of genes involved in chylomicron and lipid droplet formation (n=≥5/group (I) Fatty acids in fecal samples from female control and CTα^{IKO} mice (n=5/group). Values are means ± SEM. *p<.05, **p<.01, ***p<.001.

3.3.5 Loss of intestinal CTα impairs intestinal cholesterol absorption

Cholesterol concentrations were also significantly higher in feces of CTα^{IKO} mice compared to control mice (Figure 3.8A). To examine cholesterol turnover, control and CTα^{IKO} mice were administered with an oral gavage of [^{3}H]-cholesterol and an intravenous injection of [^{14}C]-cholesterol, and the labels were measured in plasma and feces over 72 h. After 24 h, the appearance of [^{3}H]-cholesterol in the circulation of CTα^{IKO} mice was significantly lower than in control mice (Figure 3.8B). Furthermore, cumulative fecal excretion of [^{3}H]-neutral sterols over 72 h was higher in CTα^{IKO} mice (Figure 3.8C). These data suggest that luminal cholesterol absorption is impaired with loss of intestinal CTα. The rate of disappearance of radiolabel from plasma after intravenous injection of [^{14}C]-cholesterol and cumulative [^{14}C] accumulation in fecal neutral sterols was not different between genotypes (Figure 3.8D and 3.8E) suggesting that hepatic cholesterol uptake was unaltered by loss of intestinal CTα. Furthermore, there was no difference in appearance of [^{3}H] or [^{14}C] in fecal bile salts between genotypes, suggesting that hepatic bile acid synthesis was not changed in response to reduced intestinal cholesterol absorption in CTα^{IKO} mice (Figure 3.8F and 3.8G). Despite impaired cholesterol absorption, jejunal cholesterol and cholesterol ester concentrations were unchanged in CTα^{IKO} mice relative to controls (Figure 3.8H and 3.8I). Accordingly, mRNA abundance of several genes linked to cholesterol biosynthesis, including *mevalonate kinase (Mvk)*, *farnesyl diphosphate synthase* (*Fdps)*, *cytochrome P450, family 51 (Cyp51)* and *NAD(P) dependent steroid dehydrogenase-like (Nsdhl),* were induced in the jejunum of CTα^{IKO} mice relative to controls (Figure 3.8J). Induction of intestinal cholesterol biosynthesis in CTα^{IKO} mice might be a compensatory response to impaired cholesterol absorption, or it might be due to altered intestinal phospholipid homeostasis in epithelial cells, as has recently been described (Wang et al., 2018) .

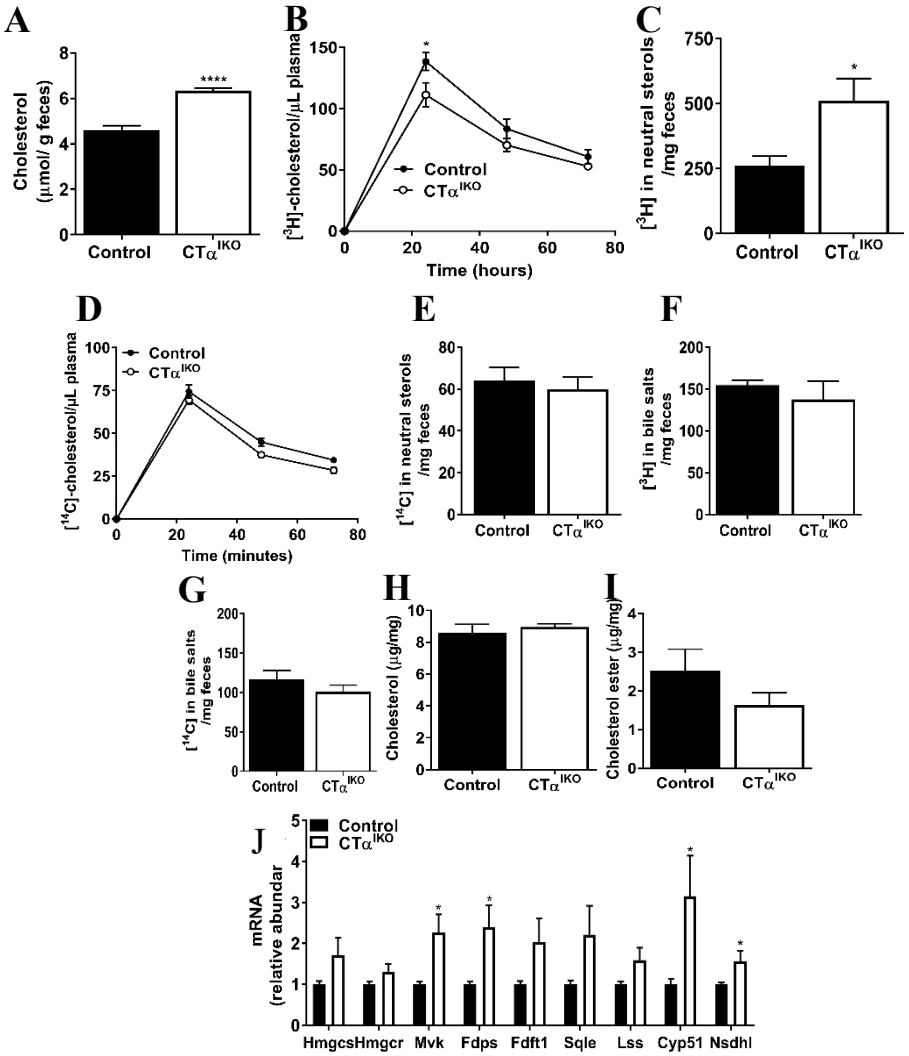

Figure 3.8. **Loss of intestinal CTα reduces dietary cholesterol absorption.** (A) Cholesterol in fecal samples from female control and CTα^{IKO} mice (n = 5 per group). Male control and CTα^{IKO} mice received an oral dose of [^{3}H]-cholesterol (2 µCi) with 6 mg of unlabeled cholesterol in 150 µl of olive oil and an intravenous injection of [^{14}C]-cholesterol (1 µCi) mixed in Intralipid®. Blood and feces were collected at 24, 48, and 72 h after cholesterol administration (n = 4 per group). (B) Plasma [^{3}H]-labeled at 24, 48, and 72 h. (C) Cumulative counts in fecal [3H]-labeled neutral sterols over 72 h. (D) Plasma [^{14}C]-labeled at 24, 48, and 72 h. (E) Cumulative counts in fecal [^{14}C]-labeled neutral sterols over 72 h. Cumulative [^{3}H] labeled in bile salts (F) and cumulative [^{14}C]-labeled in bile salts (G) over 72 h. Non-esterified cholesterol (H), cholesterol esters (I), and mRNA abundance of cholesterol synthetic genes (J) in the jejunum of control and CTα^{IKO} mice refed the HFD (n = 4 or 5 per group). Values are means ± SEM. * $P < 0.05$; **** $P < 0.0001$.

Cyp51, cytochrome P450, family 51; Fdft1, farnesyl-diphosphate farnesyltransferase 1; Fdps, farnesyl diphosphate synthase; Hmgcr, 3-hydroxy-3-methylglutaryl-CoA reductase; Hmgcs, 3-hydroxy-3-methylglutaryl-CoA synthase; Lss, lanosterol synthase; Mvk, mevalonate kinase; Nsdhl, NAD(P)-dependent steroid dehydrogenase-like; Sqle, squalene epoxidase.

3.3.6 Impaired lipid absorption in CTα^{IKO} mice is linked to reduced expression of plasma membrane lipid transporters

Microvillus length and structure were comparable between genotypes, suggesting that lipid malabsorption in CTα^{IKO} mice is not due to structural damage to the intestinal epithelium (Figure 3.8A). Intestinal *Lpcat3* deletion in mice reduces passive lipid diffusion into enterocytes by reducing the incorporation of polyunsaturated fatty acids into intestinal PC (Wang et al., 2016, Li et al., 2015). The mRNA levels of *Lpcat3* were not altered in CTα^{IKO} mice relative to controls (Figure 3.8B). As expected, jejunal PC concentrations were significantly lower in CTα^{IKO} mice re-fed the HFD, while PE concentrations were unchanged compared to controls (Figure 3.8C and 3.8D). However, the relative abundance of PC molecular species as measured by mass spectrometry was only minimally different between genotypes, with small increases in the relative abundance of PC (36:2) and PC (38:3) observed in CTα^{IKO} mice (Figure 3.8E). To validate our mass spectrometry results we performed gas-liquid chromatography analysis of the acyl chain constituents of PC and confirmed that the composition of fatty acids acylated to PC in the proximal intestine was comparable between genotypes (Figure 3.8F). Therefore, impaired lipid absorption in CTα^{IKO} mice is not due to changes in the relative abundance of PC molecular species as observed in mice deficient in intestinal *Lpcat3*. However, altered PC/PE ratio of intestinal membranes may impair passive lipid diffusion into enterocytes of CTα^{IKO} mice.

The membrane lipid transporters *Cluster-determinant 36* (*Cd36*), *Solute Carrier Family 27 Member 4* (*Slc27a4;* Fatty acid transport protein 4) and *NPC1 like intracellular cholesterol transporter 1* (*Npc1l1*) promote lipid absorption in intestinal epithelial cells (Drover et al., 2005, Stahl et al., 1999, Altmann et al., 2004). The mRNA levels of jejunal *Cd36* were dramatically lower in CTα^{IKO} mice relative to control mice (Figure 3.8G). Furthermore, *Slc27a4* and *Npc1l1* mRNA levels were modestly lower in CTα^{IKO} mice (Figure 3.8G). Therefore, reduced expression of membrane transporters involved in intestinal lipid uptake relative to controls may at least partially account for impaired lipid absorption in CTα^{IKO} mice.

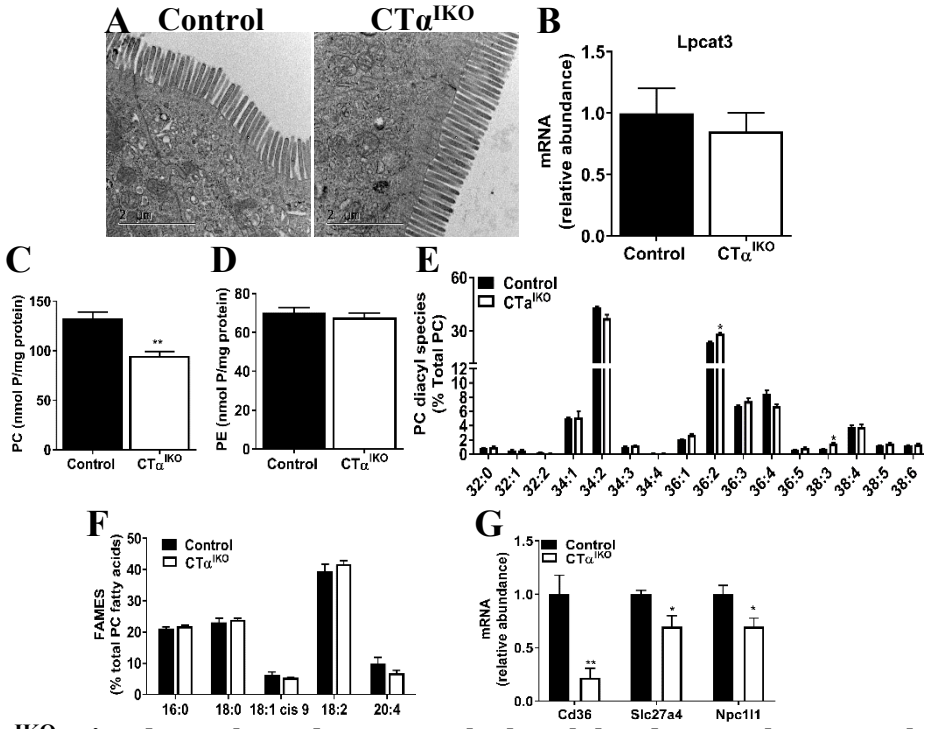

Figure 3.8. CTα^{IKO} mice do not have damage to the brush border membrane or changes to relative abundance of jejunal PC molecular species but do have reduced jejunal expression of genes encoding proteins involved in lipid uptake. (A) Representative transmission electron micrographs of intestinal microvilli of control and CTα^{IKO} mice fed the HFD. (B) *Lpcat3* mRNA abundance in the jejunum of control and CTα^{IKO} mice (n = 5 per group). PC (C) and PE (D) concentrations in the jejunum of control and CTα^{IKO} mice 2 h after HFD refeeding (n = 5 per

group). The relative abundance of jejunal PC molecular species (E) and FA methyl esters (FAMES) (F) in PC of control and CT IKO mice refed the HFD for 2 h (n = 4 or 5 per group). (G) mRNA abundance of *Cd36*, *Slc27a4*, and *Npc1l1* in the jejunum of control and CTα^{IKO} mice 2 h after HFD refeeding (n ≥ 4 per group). All mice were female. Values are means ± SEM. * P < 0.05; ** P < 0.01.

3.3.7 Loss of intestinal CTα enhances postprandial enteroendocrine hormone secretion

Dietary fatty acids in the lumen of the distal intestine stimulate secretion of enteroendocrine hormones including glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) from L cells to control food intake and satiety (Cummings and Overduin, 2007). Since CTα^{IKO} mice have impaired fatty acid absorption in the proximal small intestine resulting in increased fatty acid content of feces, we hypothesized that the higher abundance of fatty acids in the distal small intestine of CTα^{IKO} mice would trigger GLP-1 and PYY secretion. CTα^{IKO} mice had strikingly elevated active GLP-1 (~9-fold) and PYY (~9-fold) in plasma 2 h after re-feeding the HFD (Figure 3.9A and 3.9B). A major physiological function of GLP-1 is to promote insulin secretion from the pancreas (Cummings and Overduin, 2007). Accordingly, CTα^{IKO} mice had a strong trend towards higher postprandial plasma insulin concentrations compared to controls Figure 3.9C). Fibroblast-like growth factor 21 (FGF21) is a hepatokine with multiple systemic effects on carbohydrate and lipid metabolism and is highly responsive to changes in nutrient intake (Fisher and Maratos-Flier, 2016, Badman et al., 2007, Kharitonenkov et al., 2005). Circulating concentrations of FGF21 were 4.5-fold higher in CTα^{IKO} mice compared to controls after HFD re-feeding (Figure 3.9D). To test whether the observed changes to circulating hormone concentrations altered postprandial glucose metabolism, we re-fed mice a HFD for 2 h before administering them with an oral glucose

challenge. CTα^{IKO} mice had significantly lower peak blood glucose concentrations compared to controls after the glucose challenge (Figure 3.9E). Importantly, re-fed plasma TG and GLP-1 were not different between tamoxifen-treated floxed controls and mice with a heterozygous deletion of intestinal CTα (Figure 3.10A and 3.10B). These data link impaired lipid uptake in the absence of intestinal CTα to enhanced postprandial enteric and hepatic hormone secretion, likely contributing to reduced body weight in CTα^{IKO} mice relative to control mice.

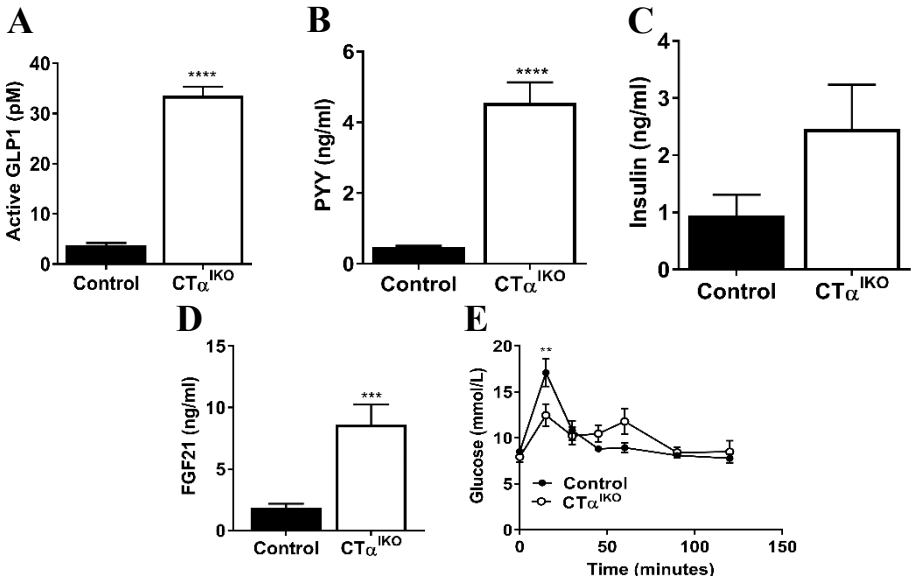

Figure 3.9. CTα^{IKO} mice have enhanced GLP-1, PYY and FGF21 secretion after a high-fat meal. Control and CTα^{IKO} mice were fasted overnight on day 3 following initiation of the HFD before being re-fed for 2 h. (A) Plasma active GLP-1 (n=5/group). (B) Plasma PYY (n=10/group). (C) Plasma insulin (n=10/group). (D) Plasma FGF21 (n=10/group). (E) Blood glucose concentrations in control and CTα^{IKO} mice re-fed the HFD for 2 h followed by an oral bolus of glucose. All mice were female. Values are means ± SEM. **p<.01, ***p< .001, ****p<.0001.

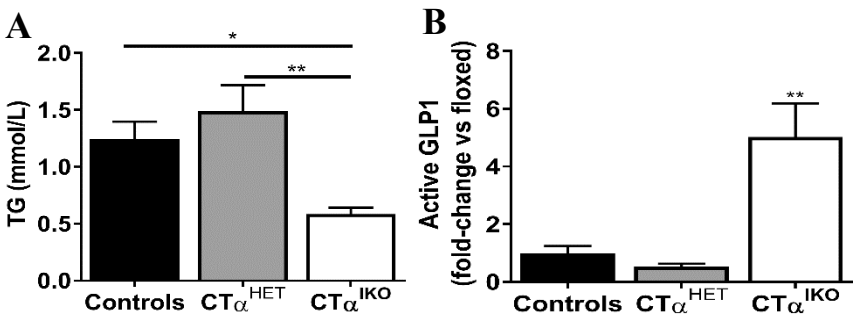

Figure 3.10. Lower plasma TG concentrations and higher plasma GLP-1 after a meal does not occur in CTα^{HET} mice, suggesting that the villin-Cre promoter does not drive this phenotype. (A) Plasma TG and (B) active GLP-1 2 h after HFD re-feeding in tamoxifen-treated floxed controls, CTα^{HET} mice and CTα^{IKO} mice fed the HFD (n=5/group). All mice were female. Values are means ± SEM. *p<.05, **p<.01.

3.3.8 Loss of intestinal CTα alters enterohepatic circulation of bile acids and increases biliary PC secretion into the intestinal lumen

We were initially surprised that CTα^{IKO} mice can maintain epithelial cell turnover and intestinal viability in the absence of *de novo* PC synthesis. Thus, we hypothesized that increased biliary PC secretion may contribute to the maintenance of intestinal PC concentrations and intestinal epithelial cell viability in CTα^{IKO} mice. To examine biliary lipid and bile acid secretion, we cannulated the gallbladders of mice fed the HFD. The rate of bile flow in CTα^{IKO} mice was strikingly higher (~25%) than in control mice (Figure 3.11A), coinciding with a doubling of biliary bile acid secretion, a 1.5-fold increase in phospholipid secretion and a 1.3-fold increase in cholesterol secretion (Figure 3.11B-3.11D). Consistent with increased biliary lyso-PC delivery to the intestinal epithelium, the mRNA levels of jejunal *Lpcat4*, which catalyzes the addition of oleic acid to lyso-PC (Hishikawa et al., 2008), was significantly higher in CTα^{IKO} mice 2 h after HFD re-feeding (Figure 3.11E). Therefore, a higher rate of delivery of biliary PC to the intestines of

CTα^{IKO} mice may contribute to their capacity to maintain intestinal epithelial cell viability in the absence of *de novo* intestinal PC synthesis.

While total biliary bile acid concentrations were higher in CTα^{IKO} mice, the bile acid species composition was largely unaltered, except for taurodeoxycholic acid, which was reduced (Figure 3.11F). Consequently, the bile acid hydrophobicity index was similar between control and CTα^{IKO} mice (Figure 3.11G). Additionally, a non-significant trend towards higher plasma bile acid concentrations in CTα^{IKO} mice fed the HFD was observed (Figure 3.11H). There was no change in the hepatic expression of genes involved in bile acid synthesis, and total fecal bile acid concentrations were not different between CTα^{IKO} mice and control mice fed the HFD (Figure 3.11I and 3.11J). These data strongly indicate that enhanced biliary bile acid and lipid secretion in CTα^{IKO} mice is not driven by an increase in hepatic bile acid synthesis.

There was a robust increase in mRNA levels of the apical sodium-bile acid transporter, *solute carrier family 10 member 2* (*Slc10a2/*Asbt*;* mainly restricted to the ileum under normal conditions), in the jejunum of CTα^{IKO} mice (Figure 3.11K). An increase in jejunal Asbt is predicted to increase bile acid absorption in the proximal small intestine and to accelerate enterohepatic cycling of bile acids (Out et al., 2015). In line with increased Asbt-mediated bile acid uptake in the proximal small intestine, mRNA levels of the bile-acid transporter *Fabp6* had a strong tendency towards an in increase the jejunum of CTα^{IKO} mice compared to controls 2 h after re-feeding. Furthermore, ileal *Slc10a2*, *Fabp6* and *Slc51b* mRNA levels were not different, while ileal *Slc51a* were lower in CTα^{IKO} mice than in ileums from control mice. Thus, loss of intestinal CTα stimulates the secretion of bile acids and lipids from the liver by a mechanism that involves a shift in reabsorption of bile acids to the proximal small intestine to promote enterohepatic cycling.

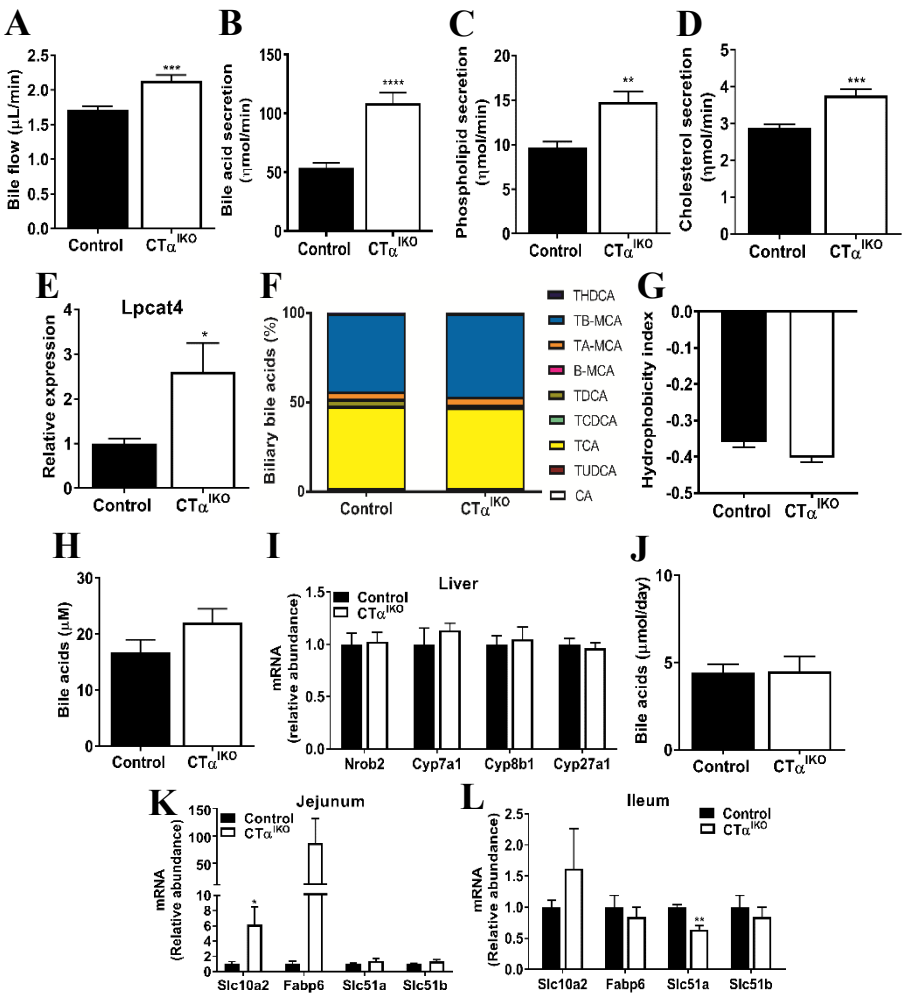

Figure 3.11. Loss of intestinal CTα enhances biliary bile acid secretion. Gallbladders of male control and CTα^{IKO} mice were cannulated, and bile was collected in pre-weighed containers (n = 6–8 per group). (A) Bile flow. (B) Bile salt secretion. (C) Phospholipid secretion. (D) Cholesterol secretion. (E) Jejunal *Lpcat4* expression. (F) Relative biliary bile acid composition. (G) Bile acid hydrophobicity index. (H) Plasma bile acids. (I) mRNA abundance of hepatic genes involved in bile acid synthesis. (J) Fecal bile acids. mRNA abundance of jejunal bile-acid responsive genes (K) (n ≥ 4 per group) and mRNA abundance of ileal bile acid-responsive genes in control and CTα^{IKO} mice 2 h after refeeding the HFD (L) (n = 3 per group). Values are means ± SEM. * P < 0.05; ** P < 0.01; *** P < 0.001; **** P < 0.0001. B-MCA, β-muricholic acid; CA, cholic acid; Cyp7a1, cytochrome P450, family 7 subfamily a member 1; Cyp8b1, cytochrome P450, family 8 subfamily b member 1; Cyp27a1, cytochrome P450, family 27 subfamily a member 1; Fabp6, FA binding protein 6; Nrob2, nuclear receptor subfamily 0 group b member 2; TA-MCA, tauro-α-muricholic acid; TB-MCA, tauro-β-muricholic acid; TCA, taurocholic acid; TCDCA, taurochenodeoxycholic acid; THDCA, taurohyodeoxycholic acid; TUDCA, tauroursodeoxycholic acid..

3.4 Discussion

In this study we show that intestinal PC synthesis *via* CTα plays a crucial role in coordinating the metabolic response of mice to HFD feeding. Our data suggest that the metabolic response to a HFD in $CT\alpha^{IKO}$ mice is driven by a combination of impaired dietary lipid absorption, increased secretion of the fat-induced satiety hormones GLP-1 and PYY, and reduced food intake. Furthermore, a shift in expression of bile-acid responsive genes to the proximal small intestine of $CT\alpha^{IKO}$ mice is linked to accelerated enterohepatic bile acid cycling. Loss of intestinal CTα is not well tolerated by some mice fed a HFD, indicating that *de novo* PC synthesis is specifically required for normal intestinal metabolic function. Furthermore, our data show that re-acylation of lyso-PC from the intestinal lumen cannot compensate for loss of CTα-derived PC in enterocytes.

Mice with intestinal deletion of the gene encoding the PC remodeling enzyme Lpcat3 have reduced chylomicron secretion when fed a chow diet due to failure to incorporate polyunsaturated fatty acids into intestinal PC, which alters membrane dynamics and fatty acid uptake (Li et al., 2015, Wang et al., 2016). In contrast, we found that *de novo* PC synthesis is not required for chylomicron secretion in the setting of a low-fat diet. This observation suggests that the re-acylation of lyso-PC derived from bile can fully accommodate chylomicron assembly under these conditions. However, an increase in cellular fatty acid concentrations increases cellular demand for PC to maintain membrane lipid homeostasis and produce the surface monolayer for lipid droplets and lipoproteins (Cornell and Ridgway, 2015). Accordingly, HFD-fed $CT\alpha^{IKO}$ mice have disrupted intestinal metabolic function (likely due to constant influx of fatty acids into intestinal epithelial cells which increases cellular PC demands) including impaired dietary lipid uptake (despite minimal changes to the relative abundance of polyunsaturated PC species or *Lpcat3* expression in the intestine). Therefore, both pathways for intestinal PC formation (i.e. *de novo* PC synthesis and PC remodeling) are independently required for dietary lipid absorption when

consuming a HFD. Furthermore, Lpcat3-deficient mice do not experience changes in bile acid concentrations (Wang et al., 2016), suggesting that the pathways of *de novo* PC synthesis and PC remodeling also control distinct aspects of enterohepatic physiology.

We used a radiolabeled tracer to demonstrate that CTα^{IKO} mice fed a HFD have impaired jejunal fatty acid uptake, resulting in the production of chylomicrons with lower TG content. Impaired lipid uptake in mice lacking intestinal *de novo* PC synthesis is not due to gross damage to the intestinal epithelium, as electron microscopy revealed an intact brush border membrane. Instead, loss of intestinal CTα is linked to intestinal metabolic dysfunction in the setting of a HFD including reduced expression of genes involved in dietary lipid absorption. Targeted deletion of *Mogat2 (Yen et al., 2009)*, *Cd36* (Drover et al., 2005), *Abhd5* (Xie et al., 2014) and *Npc1l1* (Altmann et al., 2004), all of which are lower in the intestines of CTα^{IKO} mice relative to controls, impairs dietary lipid absorption. Therefore, intestinal *de novo* PC synthesis apparently maintains a network of genes involved in dietary lipid uptake.

The intestinal epithelium is highly active in cell division, growth, differentiation, and secretion i.e. processes that require adequate PC supply (Cornell and Ridgway, 2015). Therefore, we were surprised that CTα^{IKO} mice can maintain epithelial cell turnover and intestinal viability in the absence of *de novo* intestinal PC synthesis. Nevertheless, increased biliary PC secretion may contribute to the maintenance of intestinal PC concentrations in CTα^{IKO} mice. We unexpectedly found that deletion of intestinal CTα doubles the rate of biliary bile acid secretion in CTα^{IKO} mice compared to control mice. Because bile acids drive bile flow as well as biliary cholesterol and phospholipid secretion, these parameters were also significantly higher in CTα^{IKO} mice (Verkade et al., 1995). The higher rate of delivery of bile acids (with similar composition to bile acids in controls) into the intestinal lumen of CTα^{IKO} mice implies that impaired fatty acid absorption with

loss of intestinal CTα is not due to a deficiency of mixed micelles required for efficient fat absorption. Furthermore, since the cholesterol labeling study, the analysis of fecal bile output, and measurement of expression of bile acid-synthetic genes revealed that hepatic bile acid synthesis is not induced by loss of intestinal CTα, enterohepatic cycling of bile acids is likely accelerated. This hypothesis is supported by a shift in expression of *Slc10a2* and *Fabp6* towards more proximal parts of the small intestine. A shift in the expression of bile-acid responsive genes to the proximal intestine has been reported previously in mice lacking intestinal GATA4, a transcription factor that controls jejunal-ileal identity (Bosse et al., 2006, Battle et al., 2008). The change in spatial expression of bile-acid responsive genes in intestinal GATA4 deficient mice is also linked to impaired lipid absorption (Battle et al., 2008) and increased bile flow (Out et al., 2015). Clearly, the molecular mechanisms that regulate crosstalk between the intestine and liver in controlling bile formation in $CT\alpha^{IKO}$ mice require further research. Changes to enterohepatic circulation of bile acids can influence lipid, carbohydrate, and energy metabolism (Cariou et al., 2006). Furthermore, bile acids increase the circulating level of FGF21 (Cyphert et al., 2012), a hepatokine that is increased in $CT\alpha^{IKO}$ mice and that reduces adiposity when administered pharmacologically (Fisher and Maratos-Flier, 2016). Therefore, changes in bile acid metabolism may contribute to metabolic adaptations in the $CT\alpha^{IKO}$ mice fed a HFD.

GLP-1 and PYY are hormones that can influence energy balance through both the enteric nervous system and peripheral targets after nutrient-induced secretion from L cells (Cummings and Overduin, 2007). Under normal conditions, most dietary fatty acids are absorbed in the proximal small intestine (Fig. 4C). However, if high concentrations of fatty acids reach L cells of the distal intestine they can enhance GLP-1 and PYY secretion (Cummings and Overduin, 2007). Accordingly, mice with intestinal deletion of genes involved in dietary fat absorption, including

Mogat2 (Yen et al., 2009), *Dgat1* (Ables et al., 2012) and *Lpcat3* (Wang et al., 2016), have relatively high postprandial plasma GLP-1 concentrations. Similarly, impaired HFD absorption in the proximal small intestine of CTα^{IKO} mice allows fatty acids to reach the distal intestine to potentiate enteroendocrine hormone secretion. The stimulation of GLP-1 and PYY secretion by fatty acids in CTα^{IKO} mice likely contributes to their relatively more severe response to a HFD compared to a chow diet, as was observed in intestine-specific Lpcat3-deficient mice (Wang et al., 2016). High postprandial plasma GLP-1 is associated with increased insulin secretion and lower blood glucose concentrations in CTα^{IKO} mice compared to control mice fed a HFD. Furthermore, enhanced GLP-1-mediated insulin secretion might account for the lower plasma free fatty acid concentrations in CTα^{IKO} mice compared to controls by suppressing lipolysis in adipocytes. Additionally, enhanced PYY secretion in CTα^{IKO} mice could contribute to their reduced body weight by interacting with brain regions that control energy balance (Stadlbauer et al., 2013). Taken together, our findings demonstrate that modulation of intestinal phospholipid metabolism can influence whole-body physiology by enhancing the secretion of metabolically active hormones.

3.5 Conclusions

In conclusion, this study demonstrates that intestinal *de novo* PC synthesis plays a central role in dietary lipid absorption during HFD feeding. Loss of intestinal CTα amplifies postprandial GLP-1 and PYY secretion and alters circulating glucose and free fatty acid concentrations, linking gut phospholipid synthesis to whole-body nutrient disposal and metabolism. Our studies also reveal an unexpected role for intestinal *de novo* PC synthesis in the maintenance of normal enterohepatic circulation of bile acids, potentially by governing the spatial expression of the bile-acid transporter *Slc10a2* in the intestine. Therefore, a physiologically normal response to dietary

fat intake depends on *de novo* PC synthesis in the intestinal epithelium, and the re-acylation of PC derived from extra-intestinal sources cannot fulfill this requirement.

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CHAPTER 4: Spontaneous colitis in mice with intestinal epithelial cell-specific disruption of the phospholipid synthetic enzyme CTP: phosphocholine cytidylyltransferase-α

4.1 Introduction

Inflammatory bowel diseases (IBD), comprising Chron's disease and ulcerative colitis (UC), are increasing in incidence and prevalence worldwide (Molodecky et al., 2012). Evidence from human genetics and animal models suggests that IBD arise from defects in mucosal barrier function or failure to appropriately regulate immune tolerance, epithelial restitution, or inflammation (Khor et al., 2011). The gastrointestinal mucus layer, a critical component of the mucosal barrier, restricts exposure of the intestinal epithelium and underlying immune system to luminal antigens (Johansson and Hansson, 2016). Mucus is largely produced by goblet cells, which secrete heavily O-glycosylated Mucin 2 (MUC2) polymers into the intestinal lumen to form a gel-like scaffold containing various molecules including antibacterial peptides and phospholipids (Johansson and Hansson, 2016, Ehehalt et al., 2010). Loss of goblet cell mucus granules is a typical feature of UC, suggesting that defective mucus production might be involved in the development of UC (Johansson et al., 2014) or that loss of granules is a pathological response to the disease. Furthermore, loss of specific components of mucus, including MUC2, trefoil factor 3 (TFF3), or zymogen granulae protein 16 (ZG16), increases susceptibility to intestinal injury in mice (Van der Sluis et al., 2006, Mashimo et al., 1996, Bergström et al., 2016). However, the role that endogenous production of the major lipid component of the mucus layer, phosphatidylcholine (PC) (Ehehalt et al., 2004, Braun et al., 2009), plays in intestinal barrier function, and the factors that control mucus layer PC concentrations, are poorly understood.

Patients with UC have lower PC concentrations in their mucus layer of the distal intestine relative to controls without UC (Braun et al., 2009, Ehehalt et al., 2004). Furthermore, clinical trials have shown that PC delivery to the distal intestine reduces disease severity in patients with

UC (Stremmel et al., 2005, Stremmel et al., 2007, Karner et al., 2014). It has been proposed that exogenous PC is incorporated into the colonic mucus layer and acts to increase mucus hydrophobicity and reduce microbial interaction with the intestinal epithelium (Treede et al., 2007). On the other hand, the addition of exogenous PC to intestinal epithelial cells (IECs) inhibits tumor necrosis factor-α (TNF-α) induced inflammation, suggesting that PC might be incorporated into cellular membranes where it dampens inflammatory processes (Treede et al., 2007). The role that *de novo* PC synthesis plays in maintaining immune homeostasis *in vivo* has not been investigated.

To determine the role of endogenous PC in intestinal barrier function and immune homeostasis, we examined mice with IEC-specific deletion of the rate-limiting enzyme in the major pathway for *de novo* PC synthesis, CTP:phosphocholine cytidylyltransferase-α ($CT\alpha^{IKO}$ mice). We hypothesized that $CT\alpha^{IKO}$ mice would have lower mucus PC concentrations leading to impaired mucus barrier integrity. We found that loss of CTα in the intestinal epithelium results in rapid and spontaneous colitis in mice. Colitis development is linked to induction of endoplasmic reticulum (ER) stress in IECs, increased IEC apoptosis, impaired goblet cell differentiation and maturation, and loss of the mucus barrier integrity. These data show that *de novo* PC synthesis and IEC lipid homeostasis is important for maintaining goblet cell abundance and mucosal barrier integrity.

4.2 Materials and methods

4.2.1 Mice. Generation of *Pcyt1a*$^{LoxP/WT}$;villin-CreER^{T2} mice (with the capacity to induce a heterozygous deletion of IEC CTα after tamoxifen treatment) and *Pcyt1a*$^{LoxP/LoxP}$;villin-CreER^{T2} mice (with the capacity to induce a homozygous deletion of IEC CTα after tamoxifen treatment) has been described (Kennelly et al., 2018). Genomic DNA was extracted from tail biopsies with a

DNeasy Blood and Tissue Kit (Qiagen, UK). PCR products were identified on a 3% agarose gel (floxed *Pcyt1a* band) or a 1.5% agarose gel (Villin Cre-inducible band). Cre was induced in 8-12-week-old female mice fed a chow diet (5001, Lab Diet, St. Louis, MO) by intraperitoneal injection of tamoxifen (1mg/day in sunflower oil for 5 days; Sigma, St. Louis, MO), while tamoxifen-treated *Pcyt1a*$^{LoxP/LoxP}$ mice were used as controls. Twenty-four hours after the end of tamoxifen treatment control and CTα^{IKO} mice were placed on a semi-purified diet (40% fat, 20%, protein, 40% carbohydrate; (Kennelly et al., 2018)) until termination. Mice were housed in a temperature-controlled room with 12-h light/dark cycle and free access to food and water. Small intestines were excised, flushed with phosphate buffered saline (PBS) containing protease inhibitor cocktail (Sigma, St. Louis, MO), and kept on ice. The small intestine was divided into 3 portions of length ratio 1:3:2 (corresponding to duodenum: jejunum: ileum) before jejunum and ileum sections were fixed in 10% neutral-buffered formalin for histology. IECs were collected in liquid nitrogen after segments were opened longitudinally. A cross section from the distal colon was fixed in 10% neutral-buffered formalin for histology. Colonic tissue and caecal contents were snap frozen in liquid nitrogen. For antibiotic experiments, CTα^{IKO} mice and control 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 has been used previously (Out et al., 2015). For 4-phenyl butyric acid experiments, CTα^{IKO} mice and control mice were administered with PBA (500 mg per kg body weight) dissolved in phosphate buffered saline or vehicle twice daily from the time of first tamoxifen injection until termination (10 days). 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.

4.2.2 Microscopy. Formalin-fixed, paraffin-embedded tissue slices (5µm thick) were stained with hematoxylin and eosin (H&E) or Alcian blue/Period acid-Schiff and visualized with a light microscope. Crypt depth was measured as the distance from the bottom of the crypt unit to the villus junction in at least 20 crypts per intestine segment using ImageJ software (US National Institute of Health, MD, USA). Standard immunofluorescent staining methods were used to visualize crypt PCNA (Ab18197; Abcam) and images were obtained on a fluorescence microscope (Olympus, Markham, ON) equipped with Suveyor and ImagePro Plus software. For electron microscopy, 2cm jejunal rings were fixed with 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1M phosphate buffer (pH 7.2). Sections were cryo-sectioned with an ultramicrotome (Ultracut E, Reichert-Jung) equipped with an FC4D attachment, and images were obtained using a Philips 410 transmission electron microscope.

4.2.3 mRNA isolation and quantification by PCR. Total RNA was isolated from frozen tissue using Trizol (Invitrogen, CA, USA). RNA was reversed-transcribed using Superscript II (Invitrogen, CA, USA). Quantitative PCR was run on an Applied Biosystems StepOne Plus for 40 cycles using a Power SYBR Green PCR Master Mix (Applied Biosystems, MA, USA), in triplicate. Relative mRNA expression was normalized to cyclophilin (for small intestine) or Rplp0 (for colon). Quantitation was performed using the standard curves method. Primer sequences and gene names are listed in Table 4.1.

Table 4.1. qPCR primer sets used In Chapter 4

Gene Symbol	*Gene name*	*Forward sequence*	*Reverse sequence*
Pcyt1a	Phosphate cytidylyltransferase 1, choline, alpha	GCT AAA GTC AAT TCG AGG AA	CAT AGG GCT TAC TAA AGT CAA CT
Reg3b	Regenerating islet-derived 3 beta	GACAAGATGCTGCCTCCAA	CGTGCGGAGGGTATATTCTT
Reg3g	Regenerating family member 3 gamma	GCTTCCCCGTATAACCATCA	GCATCTTTCTTGGCAACTTCA
Duox2	Dual oxidase 2	GGACAGCATGCTTCCAACAAGT	GCCTGATAAACACCGTCAGCA
Duoxa2	Dual oxidase maturation factor 2	CCTGTTCATCTTGCCTGGA	CACGAACCAGTCTCCACTGA
Nos2	Nitric oxide synthase 2	GAAGGTCGCCAGTCGTGT	GGAGCCATTTTGGTGACTCTT
Fut2	Fucosyltransferase 2	GCGGTTCGTCCATTCCTA	AAAGGTACCTGGGCACTCG
Oas1g	Oas1g 2'-5' oligoadenylate synthetase 1G	ATCCGCCTGGTCAAACACT	TACATCCATTCCCCTGTTCC
Muc2	Mucin 2	CCATTGAGTTTGGGAACATGC	TTCGGCTCGGTGTTCAGAG
Tff3	Trefoil factor 3	CTGGGATAGCTGCAGATTACG	CATTTGCCGGCACCATAC
Agr2	anterior gradient 2, protein disulphide isomerase family member	CCTCAACCTGGTCTATGAAACA	ACCGTCAGGGATGGGTCT
Atoh1	Atonal bHLH transcription factor 1	TGCGATCTCCGAGTGAGAG	TCTCTTCTGCAAGGTCTGATTTT
Hes1	Hes family bHLH transcription factor 1	ACACCGGACAAACCAAAGAC	CGCCTCTTCTCCATGATAGG
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
Atf6	Activating transcription factor 6	GGACGAGGTGGTGTCAGAG	GACAGCTCTTCGCTTTGGAC
Ddit3	DNA damage inducible transcript 3	GCGACAGAGCCAGAATAACA	GATGCACTTCCTTCTGGAACA
Eif2ak3	Eukaryotic translation initiation factor 2 alpha kinase 3	CCTTGGTTTCATCTAGCCTCA	TTGGTTTCGGATCAACATTCT
Hspa5	Heat shock protein family A (Hsp70) member 5	CTGAGGCGTATTTGGGAAAG	TCATGACATTCAGTCCAGCAA
Ern1	Endoplasmic reticulum to nucleus signaling 1	CAGAGCCTCCAACCACTCC	TCACCATTCTGGTTTCCTCA
Ern2	Endoplasmic reticulum to nucleus signaling 2	CTGCCTCCAGCTACCAAGA	TCCCCACATACAGTGTCATCA
Bcl2	B-cell lymphoma 2	AGTACCTGAACCGGCATCTG	GGGGCCATATAGTTCCACAAA
Rplp0	ribosomal protein lateral stalk subunit P0	ACTGGTCTAGGACCCGAGAAG	CTCCCACCTTGTCTCCAGTC

4.2.4 Cytokines analysis. 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 after centrifugation at 10 000 rpm for 10 min to remove debris. Cytokines concentrations were determined using Multiplex LASER Bead Technology (Eve Technology, Calgary, Canada).

4.2.5 Lipid measurements. Total protein concentrations of tissue homogenates were determined by bicinchoninic acid assay before tissue lipids were extracted from homogenates by the method of Folch (FOLCH et al., 1957). PC and phosphatidylethanolamine (PE) were separated by thin layer chromatography using the solvent system chloroform: methanol: acetic acid: water (50:30:8:4) as described previously (Kennelly et al., 2018). Plates were exposed to iodine for visualization before being measured by phosphorous assay (Zhou and Arthur, 1992).

4.2.6 Plasma metabolite measurements. Blood glucose was measured from the tail vein with a glucometer (Accu-chek, Roche, Switzerland). Blood was collected by cardiac puncture in tubes containing EDTA, dipeptidyl peptidase 4 inhibitor (EMD Millipore, MA, USA) and Complete® general protease inhibitor (Sigma, MO, USA) before being centrifuged at 3000g for 10 minutes to obtain plasma.

4.2.7 Intestinal permeability assay. Mice were fasted for 12 h before, weighed, and orally gavaged with 4 kDA fluorescein isothiocyanate (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. The appearance of the non-digestible FITC-dextran in plasma after oral administration is a measure of paracellular permeability (Woting and Blaut, 2018).

4.2.8 IEC Microarray data acquisition and analysis. Small intestines 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 from the geometric middle of the intestine (jejunum) was collected and immediately frozen in liquid nitrogen. IECs 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). RNA was sent to TCAG microarray facility (Sick Kids Hospital, Toronto, Canada). Whole genome expression profiles were obtained using the Affymetrix mouse Gene 2.0 ST chips (Affymetrix, Thermo Fischer Scientific, CA, USA). Differentially expressed genes were identified with Affymetrix Transcriptome Analysis Console (TAC) 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 (Huang et al., 2009). Significantly changed genes (1.5-fold change, ANOVA P value <0.05) are listed in Appendix 1.

4.2.9 Statistics. Data are expressed as mean ± standard error of the mean using Graphpad Prism (Version 7). Student's t-test or 2-way ANOVA with Tukey's post-test was used, where appropriate.

4.3 Results

4.3.1 Hyperproliferation of the crypt compartment and activation of the immune system in the small intestines of mice lacking *de novo* PC synthesis in IECs

To delete CTα specifically in murine IECs, mice carrying floxed *Pcyt1a* alleles (encoding the CTα protein) were crossed with mice carrying a tamoxifen-inducible Cre recombinase transgene driven by an villin promotor (el Marjou et al., 2004), as described previously (Kennelly et al., 2018). Induction of Cre recombinase with tamoxifen resulted in the generation of adult CTα^{IKO} mice with lower *Pcyt1a* mRNA abundance in both the small intestine and colon compared with tamoxifen-treated floxed *Pcyt1a* control mice (Figure 1A). CTα^{IKO} mice experienced modest body weight loss (~5%) while *Pcyt1a* floxed controls did not experience body weight loss after tamoxifen treatment (Figure 4.1A). However, CTα^{IKO} mice began to regain body weight at day 5 and were a similar body weight to controls by day 7 (Figure 4.1B).

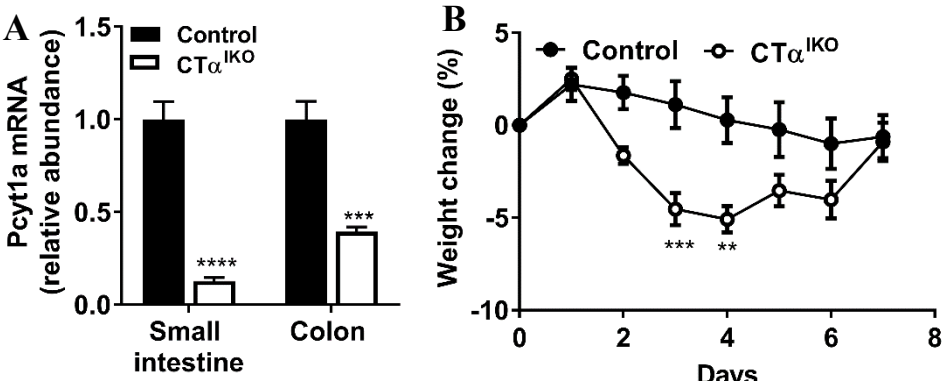

Figure 4.1. Lower expression of Pcyt1a in the small intestine and colon of CTα^{IKO} mice relative to controls is associated with transient body weight loss. (A) *Pcyt1a* mRNA abundance in the small intestine (n=10/group) and colon (n=5/group) of control and CTα^{IKO} mice 5 days after the end of tamoxifen treatment. (B) Body mass change relative to total body mass at the end of tamoxifen treatment (n=7/group). Values are means ± SEM. ***p<.001, ****p<.0001.

To gain insight into the role of *de novo* PC synthesis in intestinal function, we performed unbiased whole genome expression analysis of IECs isolated from the small intestine of control and CTα^{IKO} mice. Pathway analysis indicated a significant increase in mRNAs linked to cell cycle progression and cancer (Figure 4.2A and Appendix 1). Accordingly, histological examination of H&E-stained jejunal segments revealed prominent elongation of small intestinal crypts and regions of massive crypt hyperproliferation in CTα^{IKO} mice (Figure 4.2B and 4.2C). Furthermore, immunofluorescence staining for PCNA, a marker of proliferation, showed increased staining intensity in ileal crypts of CTα^{IKO} mice compared to control crypts (Figure 4.2D). These data implicate *de novo* PC synthesis in control of intestinal proliferation.

Hyperplasia of the intestinal epithelium is a characteristic response to mucosal injury following microbial invasion (Khor et al., 2011, Elinav et al., 2013). The mRNA levels of genes involved in the intestinal defense response, including regenerating islet-derived protein 3-β (*Reg3b*), regenerating islet-derived protein 3-γ (*Reg3g*), dual oxidase 2 (*Duox2*), nitric oxide synthase 2 (*Nos2*) were robustly higher in the small intestine of CTα^{IKO} mice compared with controls (Figure 4.2E). Furthermore, the mRNA abundance of interferon-inducible 2'-5' oligoadenylate synthetase 1G (*Oas1g*) was higher in CTα^{IKO} intestines compared to control intestines (Figure 4.2E). Taken together, these data suggest that loss of IEC CTα increases microbial interaction with the gut epithelium leading to induction of a proliferative and pro-inflammatory gene expression profile.

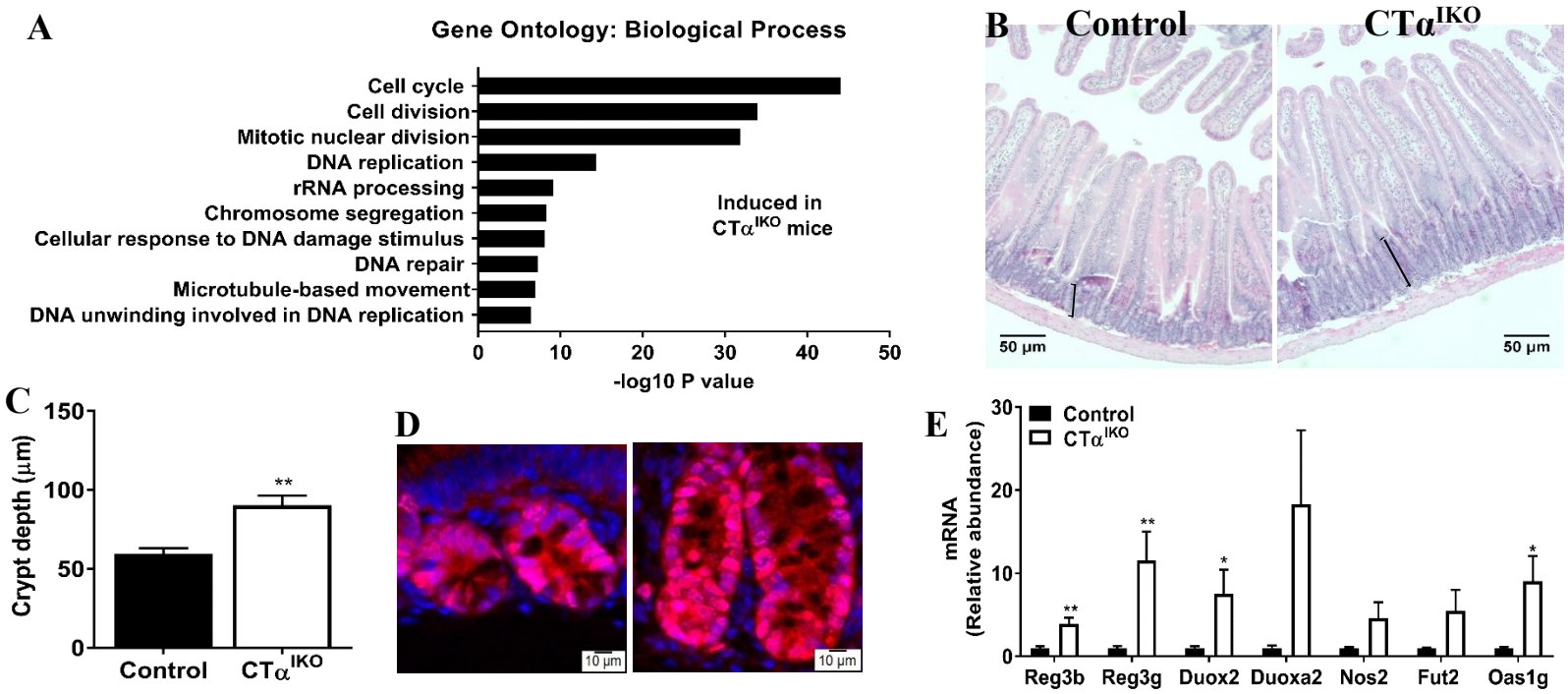

Figure 4.2. Hyperproliferation of the crypt compartment and activation of the immune system in the small intestines of mice lacking *de novo* PC synthesis in IECs. (A) Gene Ontology enrichment analysis (Biological process) of significantly more abundant mRNAs in the jejunum of control mice compared to CTα^{IKO} mice according to DAVID (n=5/group). (B) Representative H&E stained jejunal sections (black lines demark crypts), and (C) crypt depth of control mice compared to CTα^{IKO} mice (n≥4/group). (D) Representative PCNA staining of ileal crypts from control mice and CTα^{IKO} mice. (E) mRNA abundance of genes linked to epithelial immune system activation in control mice and CTα^{IKO} mice as determined by qPCR (n≥9/group). Values are means ± SEM.*p<.05, **p<.01.

4.3.2 Spontaneous colitis in mice lacking CTα in IECs

The uncontrolled proliferation and induction of transcripts linked to the intestinal defense response in the small intestines of $CT\alpha^{IKO}$ mice suggested that *de novo* PC synthesis might be involved in intestinal barrier function. Accordingly, the colons of $CT\alpha^{IKO}$ mice were opaque and weighed ~30% more than those of control mice 7 days after Cre induction (0.24± 0.01g in control mice compared to 0.30g ± 0.02g in $CT\alpha^{IKO}$ mice P=0.041). Furthermore, $CT\alpha^{IKO}$ mice had lighter caeca than controls (0.26±0.01g in control mice compared to 0.19± 0.02g in $CT\alpha^{IKO}$ mice P=0.022). Red blood cells, hemoglobin and hematocrit levels were significantly lower in $CT\alpha^{IKO}$ mice compared to control mice, while blood reticulocyte concentrations were robustly higher, which is indicative of anemia; this suggested that $CT\alpha^{IKO}$ mice may have lost blood through the injured bowel (Table 4.1). A panel of other blood components remained unchanged between $CT\alpha^{IKO}$ mice and control mice (Table 4.1).

The most striking observation upon assessment of H&E-stained distal colon sections was a profound loss of typical goblet cells in all $CT\alpha^{IKO}$ mice, such that $CT\alpha^{IKO}$ mice could be easily identified by this feature (Figure 4.3A). Furthermore, there was extensive damage to the colonic epithelium in $CT\alpha^{IKO}$ mice including loss of epithelial morphology, mononuclear cell infiltration, crypt dysplasia and crypt abscesses, which are typical features of murine colitis (Figure 4.3B). These data show that *de novo* PC synthesis plays an important role in protecting the colon against injury.

Table 4.2. Complete blood cell count data

Complete Blood Cell Count	***Control***	***CTα^{IKO}***
Red blood cells (x10E12 cells/L)	10.38 ± 0.26	9.72 ± 0.11*
Hemoglobin (g/L)	156.7 ± 3.06	147.8 ± 1.8*
Hematocrit (%)	54 ± 1.31	50.32 ± 0.48*
Reticulocytes (x10E09 cells/L)	149.4 ± 20.05	295.8 ± 29.5**
Reticulocytes (%)	1.46 ± 0.22	3.05 ± 0.31**
White blood cell count peroxidase method (x10E09 cells/L)	2.85 ± 0.88	2.33 ± 1.1
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 ± 0.1	15.23 ± 0.8
Mean corpuscular hemoglobin concentration (g/L)	290.3 ± 1.86	293.8 ± 1.99
Platelets (x10E09 cells/L)	701 ± 11.85	796.2 ± 44.19
Neutrophils (%)	9.9 ± 1.83	11.38 ± 2.51
Lymphocytes (%)	80.32 ± 2.20	79.13 ± 1.86
Monocytes (%)	2.9 ± 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 ± 0.5	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

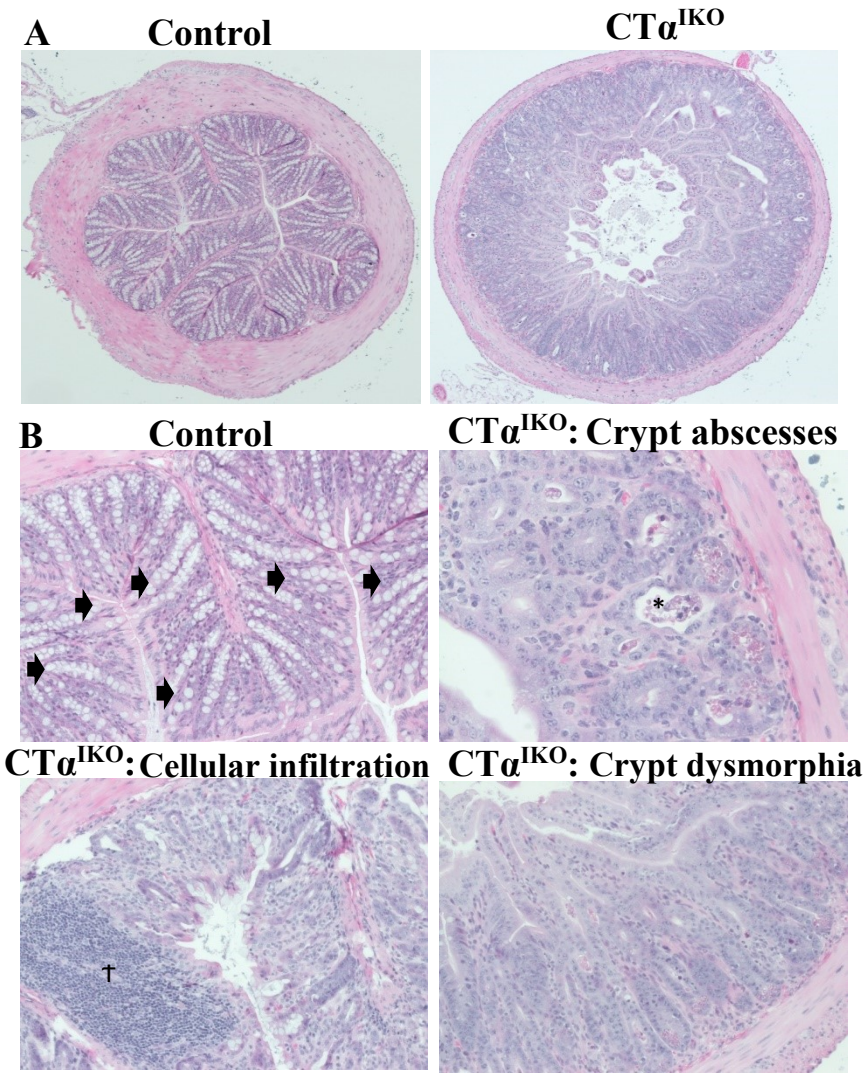

Figure 4.3. Spontaneous colitis in mice lacking CTα in IECs. (A) Representative H&E stained colon sections of control mice and CTα^{IKO} mice 4 days after the end of tamoxifen treatment. (B) Representative H&E stained colon sections from control mice and examples of crypt abscesses, cellular infiltration and crypt dysmorphia in CTα^{IKO} mice 4 days after the end of tamoxifen treatment. Arrow indicates goblet cells in crypts, * indicates crypt abscesses and † indicates immune cells. (n=6 control mice and 9 CTα^{IKO} mice).

4.3.3 Loss of mucus granules and ultrastructural damage to theca in goblet cells of mice lacking *de novo* PC synthesis

Goblets cells comprise ~15% of cells in the colon and secrete various proteins including mucins and trefoil factors to protect the intestinal epithelium from microbial invasion and tissue damage (Karam, 1999). To further probe the apparent loss of normal goblet cell morphology in $CT\alpha^{IKO}$ mice, we stained small intestine and colon sections with Alcian Blue, which identifies acidic glycans on mucin glycoproteins in goblet cells and pre-goblet cells. Alcian Blue/Period Acid Schiff staining showed a marked decrease in abundance of staining in small intestinal sections from $CT\alpha^{IKO}$ mice compared to controls (Figure 4.4A). Moreover, the mucus staining that was present in the small intestines of $CT\alpha^{IKO}$ mice was largely restricted to the crypt region and reflected small, immature goblet cells (Figure 4A). Control mice had abundant Alcian Blue positive cells in the colon (Figure 4.4B). However, very few Alcian Blue positive cells were observed in the colons of $CT\alpha^{IKO}$ mice (Figure 4.4B). These data suggest that *de novo* PC synthesis might be involved in maintaining goblet cell abundance in the intestinal epithelium.

To characterize the subcellular effects of loss of CTα on goblet cells, we conducted electron microscopy. Mucin proteins are synthesized at the rough ER and travel to the Golgi where they are glycosylated and packaged into secretory vacuoles called theca (the major site of Alcian Blue staining) which are bound to the apical membrane (Karam, 1999). As expected, these thecae were abundant in goblet cells of control mice; however, $CT\alpha^{IKO}$ mice lacked typical secretory vacuoles (Figure 4.4C). Instead, the ultra-structural integrity of mucus-containing theca in $CT\alpha^{IKO}$ mice appeared damaged, with loss of mucus granules and infiltration of cellular debris and vacuoles (Figure 4.4C). This atypical appearance of the mucus granules, with unidentified material of variable electron density, has been observed previously in patients with UC and might be due to impaired mucin packaging in the Golgi (Heazlewood et al., 2008). These data provide evidence

that *de novo* PC synthesis is important for maintaining the structure of mucus granules in goblet cells.

The mRNA abundance of *Muc2* was significantly lower in the colons of CTα^{IKO} mice compared to control mice (Figure 4.4D). Furthermore, the mRNA abundance of trefoil factor 3 (*Tff3)*, encoding a protective trefoil factor, which is secreted from goblet cells into mucus, was lower in CTα^{IKO} mice (Figure 4.4D). Interestingly, the mRNA abundance of anterior gradient protein 2 homolog (*Agr2)*, a component of secreted mucus that is expressed in Paneth cells and enteroendocrine cells in addition to goblet cells, was not different between CTα^{IKO} mice and control mice (Figure 4.4D). The lower mRNA abundance of the protective mucus components *Muc2* and *Tff3*, but not *Agr2*, suggests that there might be a specific defect in goblet cells after loss of intestinal *de novo* PC synthesis.

A hallmark of colon cancer is uncontrolled stem cell proliferation and loss of ability to differentiate into the different intestinal epithelial cell subtypes (Reya and Clevers, 2005). The mRNA levels of *Hes1* and *Atoh1*, which are required for intestinal secretory cell lineage commitment, were lower in the colons of CTα^{IKO} mice compared to control mice. (Figure 4.4E). Accordingly, and consistent with the dramatically lower abundance of goblet cells in the intestinal epithelium, the mRNA levels of growth factor independent 1 transcriptional repressor (*Gfi1)*, SAM pointed domain containing ETS transcription factor (*Spdef)*, and Kruppel like factor 4 (*Klf4)*, genes involved in goblet cell maturation, were also lower in the colons of CTα^{IKO} mice compared with control mice (Figure 4.4E). These data suggest that loss of *de novo* PC synthesis in IEC impairs epithelial cell differentiation from progenitor cells *via* transcriptional repression of the intestinal secretory cell differentiation program, as seen during colon cancer progression (Reya and Clevers, 2005).

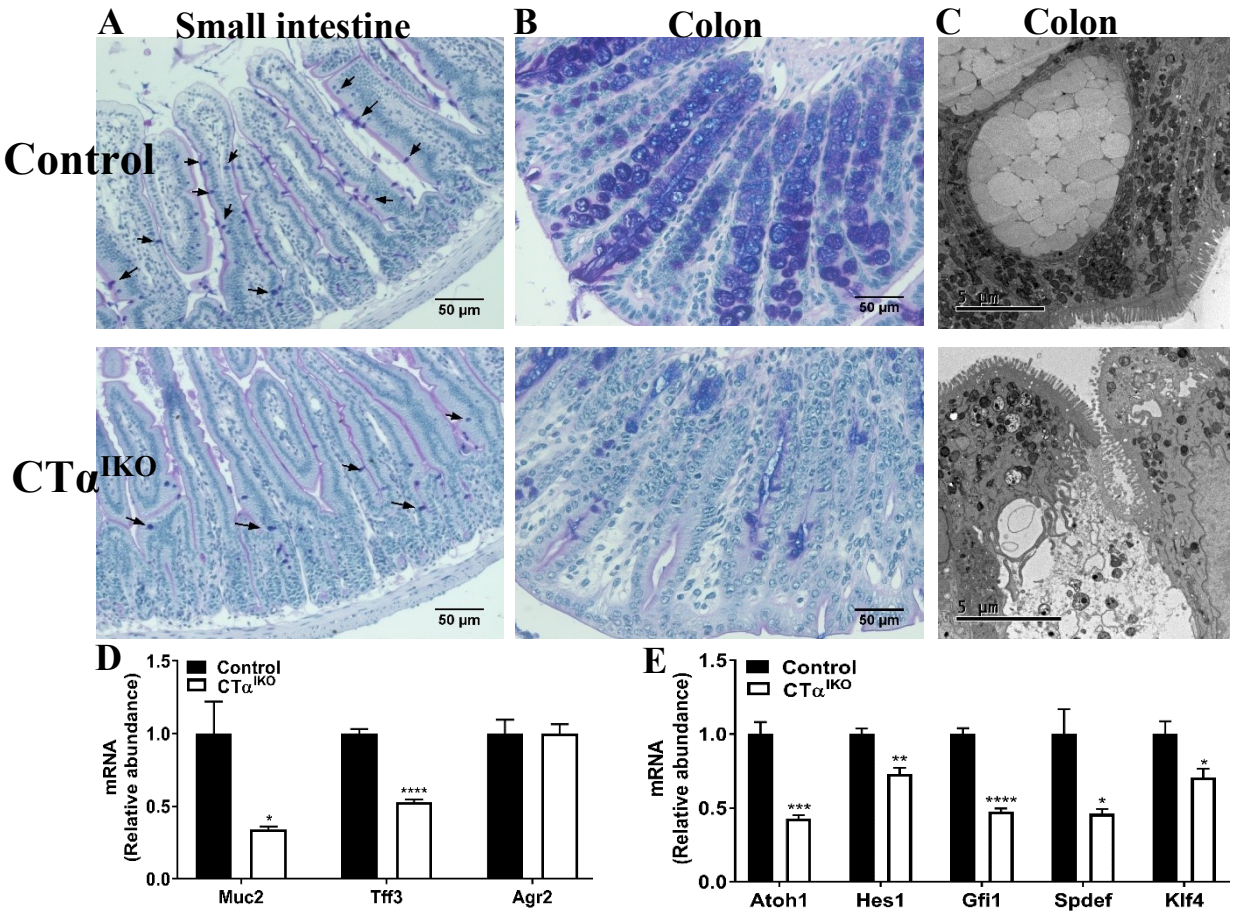

Figure 4.4. Loss of mucus granules and ultrastructural damage to theca in goblet cells of mice lacking de novo PC synthesis. (A) Alcian Blue/Period acid-Schiff staining of small intestinal sections from control mice and CTα^{IKO} mice (black arrows indicate goblet cells). (B) Alcian Blue/Period acid-Schiff staining of colon sections from control mice and CTα^{IKO} mice. (C) Transmission electron micrographs of goblet cell theca in the colons of control mice and CTα^{IKO} mice. (D) mRNA abundance of *Muc2*, *Tff3* and *Agr2* in the colons of control mice and CTα^{IKO} mice (n=5/group). (E) mRNA abundance of genes linked to intestinal epithelial cell differentiation and goblet cell maturation in the colons of control mice and CTαIKO mice (n=5/group). Values are means ± SEM.*p<.05, ***p<.001, ****p<.0001.

4.3.4 Increased intestinal permeability and microbial invasion of the colonic epithelium in response to loss of *de novo* PC synthesis in IECs

Carnoy's fixation (preserves the mucus layer) followed by Alcian Blue staining revealed that the mucus layer of CTα^{IKO} mice tended to be thinner than that of control mice (Figure 4.5A). Consistent with a compromised mucus barrier, electron microscopy showed extensive damage to the apical brush border of colonic epithelial cells in CTα^{IKO} mice (Figure 4.5B). Accordingly, CTα^{IKO} mice had increased intestinal permeability, as assessed by appearance of FITC-labeled dextran in circulation following oral administration (Figure 4.5C).

The mRNA abundance of the bacterial DNA sensor Z DNA binding protein (*Zbp1),* which is activated in response to bacteria in the cytosol of epithelial cells and functions to induce interferon-regulatory factors (Takaoka et al., 2007), was 3-fold higher in colonic tissue of CTα^{IKO} mice relative to controls (Figure 4.5D). Consistent with increased *Zbp1*, bacterial cells of varying morphology were observed by electron microscopy in individual colonic epithelial cells of CTα^{IKO} mice (Figure 4.5E), as has been reported in humans with UC (Swidsinski et al., 2002). Cells adjacent to bacteria-colonized cells did not appear to contain bacteria. Taken together, these data suggest that a depleted mucus barrier leads to microbial invasion of the colonic epithelium and activation of the innate immune system in response to impaired *de novo* PC synthesis.

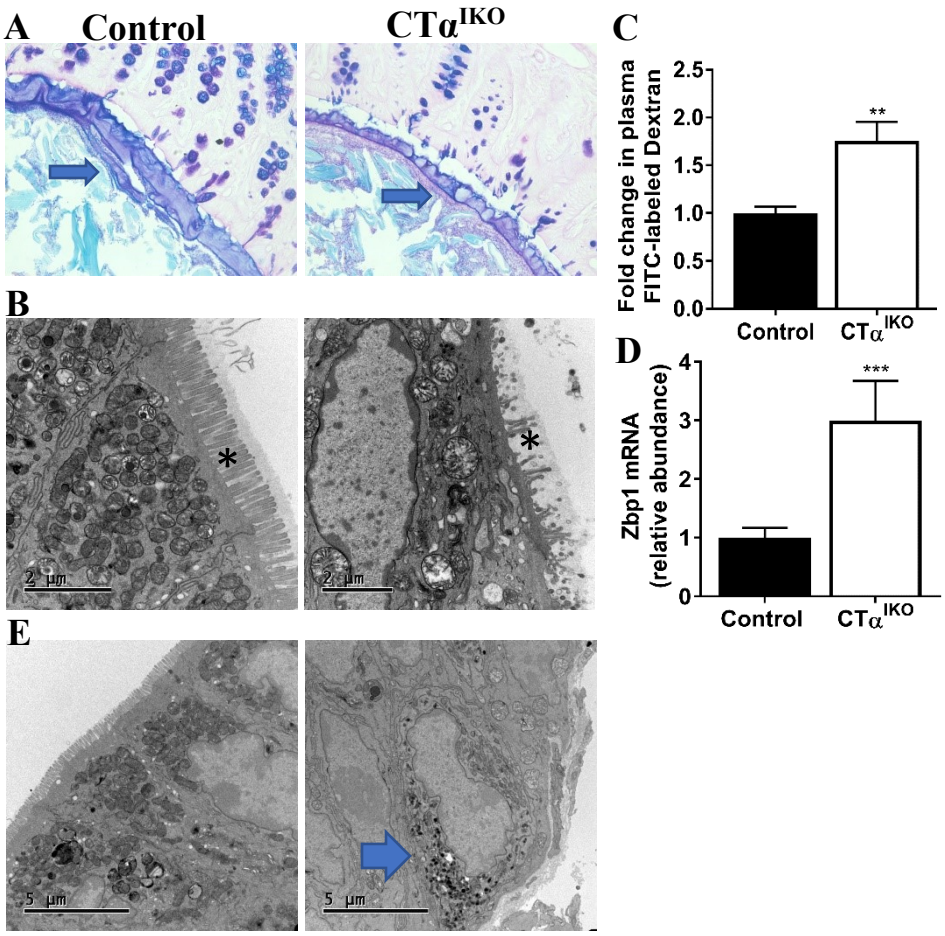

Figure 4.5. Increased intestinal permeability and microbial invasion of the colonic epithelium in response to loss of *de novo* PC synthesis in IECs. (A) Light microscope images of the colonic mucus layer in control mice and CTα^{IKO} mice after Carnoy's fixation and Alcian Blue/Period acid-Schiff staining. Arrow indicates mucus layer (B) Electron micrographs of the colonic microvilli in control mice and CTα^{IKO} mice. * indicates microvilli. (C) Relative fluorescence in plasma of control mice and CTα^{IKO} mice 2 hr after an oral gavage of FITC-labelled dextran (n=5/group). (D) mRNA abundance of *Zbp1* in the colons of control mice and CTα^{IKO} mice (n=5/group). (E) Electron micrographs of bacteria-free IECs from control mice and bacteria-laden IECs from CTα^{IKO} mice. Arrow indicates bacteria. Values are means ± SEM. **p<.01, ***p<.001.

4.3.5 Antibiotics partially ameliorate colonic inflammatory cytokine secretion but do not reverse loss of goblet cells or colitis in CTα^{IKO} mice

To further characterize the inflammatory response observed in the colons of CTα^{IKO} mice, and to determine the extent to which bacteria contributed to this inflammation, we profiled inflammatory cytokine concentrations under both normal conditions and broad-spectrum antibiotic-treated conditions Antibiotic treatment induced weight loss in both control mice and CTα^{IKO} mice during tamoxifen treatment; however, both groups re-gained weight at the same rate after tamoxifen treatment (Figure 4.6A). Increased intestinal permeability and exposure of the epithelium to luminal antigens is a major driver of tissue injury (Khor et al., 2011). Granulocyte-macrophage colony-stimulating factor (GM-CSF), primarily produced in the intestinal mucosa by dendritic cells and macrophages in response to tissue injury (Hirata et al., 2010), was ~8-fold higher in the distal colon of CTα^{IKO} mice compared with control mice (Figure 4.6B). Furthermore, monocyte chemoattractant protein-1 (MCP-1), a chemokine that regulates the migration and infiltration of macrophages to inflamed colonic tissue, tended to be higher in the colons of CTα^{IKO} mice relative to controls (Figure 4.6B). In line with the enhanced proliferation observed in the crypts of CTα^{IKO} mice, leukemia inhibitory factor (LIF), a member of the interleukin (IL)-6 family of pro-tumorigenic cytokines, was increased 18-fold in the colons of CTα^{IKO} mice compared with control mice (Figure 4.6B). Treatment with antibiotics ameliorated LIF concentrations in the colons of CTα^{IKO} mice, suggesting that bacteria might play a partial role in inflammatory cytokine secretion (Figure 4.6B). However, antibiotics did not reduce colonic GM-CSF or MCP-1 concentrations, suggesting that exposure of the colonic epithelium to non-bacterial luminal antigens or tissue-intrinsic factors was also causing mucosal damage.

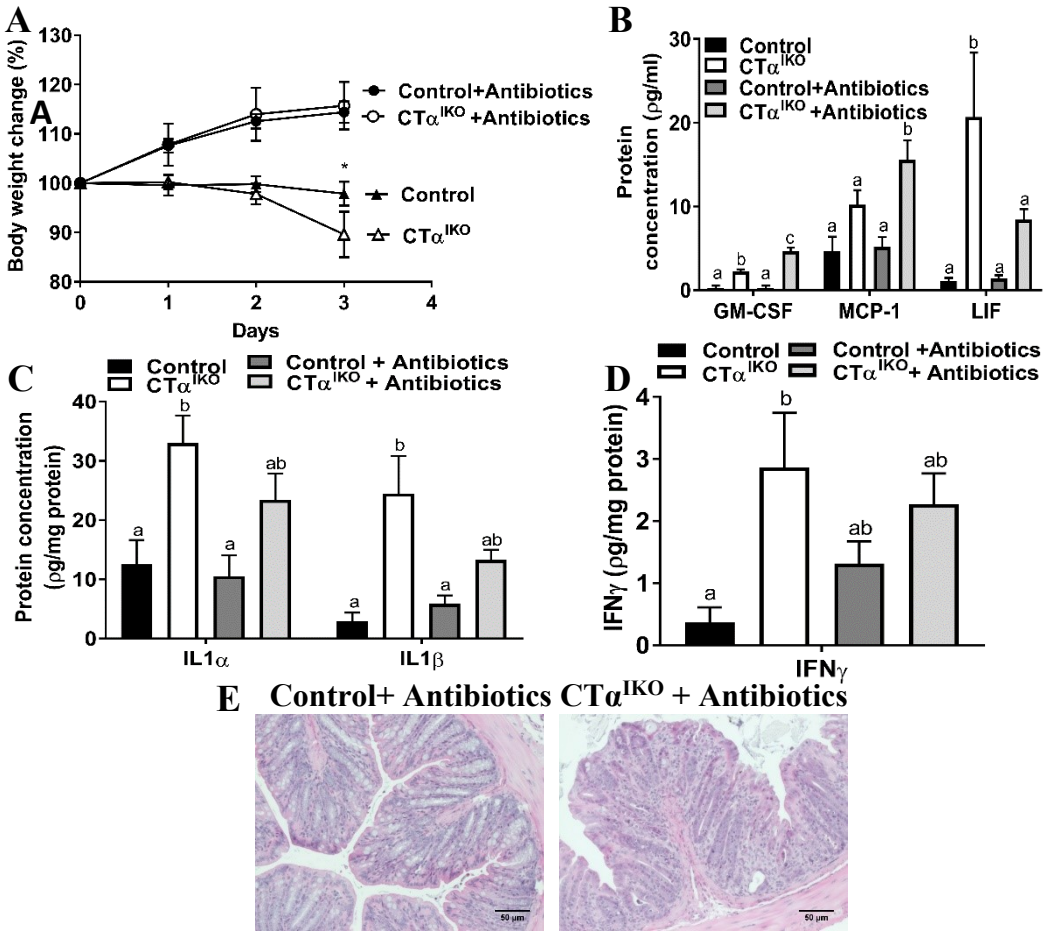

Figure 4.6 Antibiotics ameliorate colonic inflammatory cytokine secretion but do not reverse loss of goblet cells or colitis in CTα^{IKO} mice. (A) Body weight relative to body weight at the end of tamoxifen treatment in control mice and CTα^{IKO} mice with and without antibiotic treatment (n≥4/group). (B) GM-CSF, MCP-1 and LIF protein concentrations in the colons of control mice and CTα^{IKO} mice either with or without antibiotic treatment (n≥4/group). (C) IL-1α and IL-1β protein concentrations in the colons of control mice and CTα^{IKO} mice with and without antibiotic treatment (n≥4/group). (D) IFN-γ protein concentrations in the colons of control mice and CTα^{IKO} mice with and without antibiotic treatment (n≥4/group). (E) Representative H&E stained colon sections from antibiotic-treated control mice and CTα^{IKO} mice. Arrows indicate goblet cells. Values are means ± SEM.*p<.05 In Figure 4.6B-4.6D, columns that do not share a letter (a or b) are significantly different. α=0.05.

Protein concentrations of the inflammatory cytokines IL-1α, IL-1β and interferon-γ (IFNγ) were robustly higher in the colons of CTα^{IKO} mice compared with control mice (Figure 4.6C and 4.6D). These master inflammatory cytokines are not secreted by colonic epithelial cells and so their increased abundance provides further evidence of immune cell infiltration to a site of tissue injury. Antibiotic treatment partially ameliorated the increase in IL1α, IL1β and IFNγ, suggesting that luminal bacteria might exacerbate the inflammatory response observed in the colons of CTα^{IKO} mice (Figure 4.6C and 4.6DC). However, histological assessment of the colonic epithelium showed that there was still extensive loss of goblet cells following antibiotic treatment (Figure 4.6E), and antibiotics did not ameliorate the production of several other cytokines and chemokines in the colons of CTα^{IKO} mice (Table 4.2). Taken together, these data suggest that bacteria exacerbate inflammation in CTα^{IKO} mice by enhancing IFNγ, IL1α, IL1β secretion, but that loss of goblet cells and colonic damage occurs independent of bacterial invasion of the epithelium.

Table 4.3. Colonic cytokines and chemokines in control mice and CTα^{IKO} with and without antibiotics

Protein name	***Control***	***CTα^{IKO}***	***Control + Antibiotics***	***CTα^{IKO} + Antibiotics***
Eotaxin	4.47 ± 2.62	3.94 ± 3.47	1.25 ± 0.79	2.10 ± 0.82
G-CSF	0.85 ± 0.09	1.29 ± 0.22	0.96 ± 0.07	1.97 ± 0.52
IL-6	1.37 ± 0.54	1.88 ± 0.54	1.46 ± 0.42	2.29 ± 0.29
IL-9	16.03 ± 8.58	3.23 ± 2.88	0.48 ± 0.15	0.258 ± 0.12
IL-10	3.82 ± 1.42	6.04 ± 1.07	5.27 ± 1.40	7.15 ± 0.94
IL-12 (p40)	6.40 ± 1.18	6.81 ± 2.23	7.12 ± 1.16	12.00 ± 2.94
IL-12 (p70)	2.41 ± 1.29	5.19 ± 1.42	4.08 ± 0.79	6.99 ± 2.15
M-CSF	1.63 ± 0.72	5.78 ± 3.02	1.08 ± 0.26	3.03 ± 1.11
RANTES	1.60 ± 0.77	1.24 ± 0.43	0.93 ± 0.27	0.65 ± 0.10
MIP-2	32.40 ± 4.48	50.31 ± 5.72	38.48 ± 3.14	63.55 ± 16.13
MIP-1α	6.75 ± 2.95	12.29 ± 2.04	8.07 ± 1.44	12.95 ± 1.85

[1]Data are mean ± SEM. A 2-way ANOVA was used.

4.3.6 CHOP is induced in the colon in response to loss of IEC *de novo* PC synthesis

Electron microscopy showed that the ER in colonic IECs of CTα^{IKO} mice appeared dilated and distended (Figure 4.7A). Furthermore, the ER network contained numerous vacuoles and aggregates of variable size and density (Figure 4.7A), a hallmark of the unfolded protein response and ER stress (Heazlewood et al., 2008). Accordingly, the ER-stress-induced transcription factor C/EBP homologous protein (CHOP; DNA damage inducible transcript 3 *Ddit3* gene) was 2-fold higher in the colons of CTα^{IKO} mice relative to control mice (Figure 4.7B). Interestingly, the mRNA abundance of other mediators of the ER stress response, including activating transcription factor 6 (*Atf6*), eukaryotic translation initiation factor 2 alpha kinase 3 (*Eif2ak3)* and heat shock protein family A (Hsp70) member 5 (*Hspa5)*, were not increased in CTα^{IKO} mice, while the mRNA levels of endoplasmic reticulum to nucleus signaling 1 (*Ern1,* encoding IRE1α) and endoplasmic reticulum to nucleus signaling 2 (*Ern2,* encoding IRE1β*)* were significantly lower in the colons of CTα^{IKO} mice compared to controls (Figure 4.7B). IRE1β has been implicated in normal MUC2 biosynthesis, and loss of IRE1α in IECs results in spontaneous colitis in mice due to loss of goblet cells, and so the lower abundance of these transcripts in the colons of CTα^{IKO} mice could contribute towards disease progression (Tsuru et al., 2013, Zhang et al., 2015). Importantly, it has been reported previously that disruption of CTα in cells specifically induces CHOP, but not other mRNAs linked to ER stress, prior to apoptosis (van der Sanden et al., 2003). Interestingly, the pathological changes that occur the colons of CTα^{IKO} mice were not associated with changes in the total mass of PC or phosphatidylethanolamine (PE) in the colonic epithelium of CTα^{IKO} mice relative to controls (Figure 4.7C and 4.7D). Biliary PC is primarily taken up in the proximal small intestine and so re-acylation of lyso-PC in colonic epithelial cells is unlikely to make a substantial contribution towards total colon PC mass. However, enhanced uptake of lipoproteins from venous circulation could contribute towards maintenance of colon PC concentrations after impairment of

intestinal epithelial cell CTα. Furthermore, the high abundance of muscle and immune cells (that still carry functional CTα) in total colon homogenates could explain the lack of change to total colon PC concentrations in $CT\alpha^{IKO}$ mice relative to control mice.

If the unfolded protein response fails to sufficiently reduce the burden of ER stress, apoptosis will proceed (Schröder and Kaufman, 2005). CHOP has been shown to induce apoptosis by downregulating B-cell lymphoma 2 (*Bcl2)* (McCullough et al., 2001). The mRNA levels of *Bcl2* were dramatically lower in the colons of $CT\alpha^{IKO}$ mice relative to controls (Figure 4.7E). Furthermore, the proinflammatory cytokine TNFα, which activates death receptor signaling pathways to promote apoptosis in IECs, was significantly higher in colons of $CT\alpha^{IKO}$ mice compared with control mice, and this rise in colonic TNFα concentrations was unaffected by antibiotic treatment (Figure 4.7F). Electron microscopy showed that the abundance of mitochondria in intestinal crypts from $CT\alpha^{IKO}$ mice was greatly reduced (Figure 4.7A), and that the mitochondria that were present were swollen and distended with damaged cristae, suggesting that necrosis of IECs was occurring in addition to apoptosis (Elmore, 2007). Mitochondrial damage in IECs is a feature of both human and murine UC (Heazlewood et al., 2008). Taken together, these data show that loss of CTα promotes ER stress and apoptosis in the intestinal epithelium.

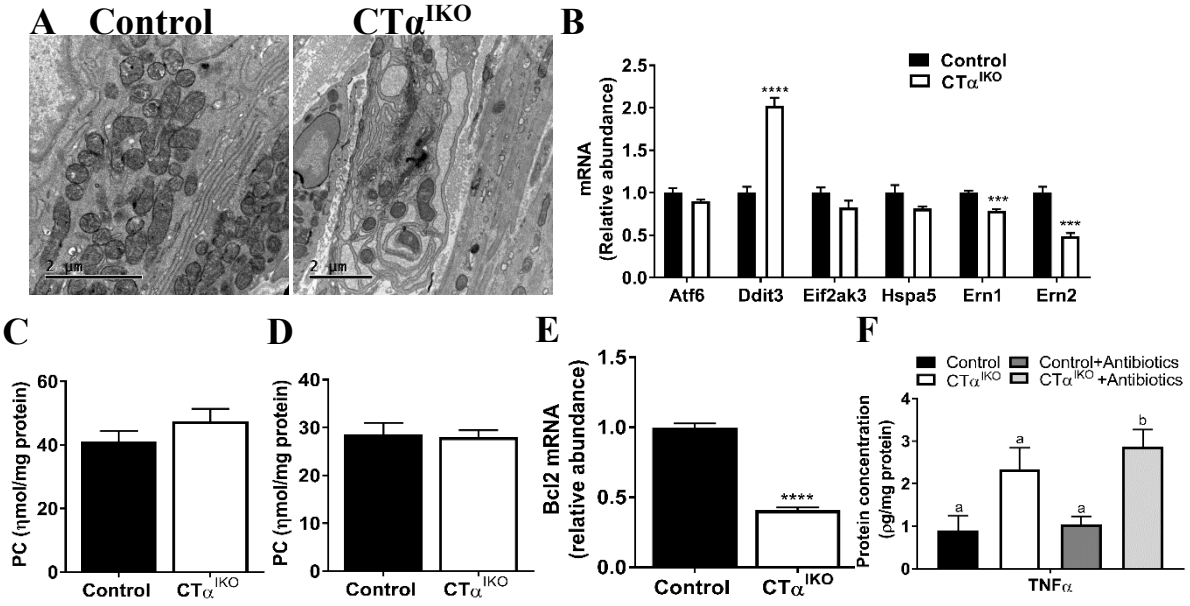

Figure 4.7 CHOP is induced in the colon in response to disruption of IEC CTα. (A) Electron micrographs of the ER in colonic epithelial cells of control mice and CTα^{IKO} mice. (B) mRNA abundance of genes linked to ER stress and the unfolded protein response in the colons of control mice and CTα^{IKO} mice (n=5/group). (C) PC and (D) PE concentrations in the colons of control mice and CTα^{IKO} mice (n=9/group). (E) mRNA abundance of *Bcl2* in the colons of control mice and CTα^{IKO} mice (n=5/group). (F) TNF-α protein concentrations in the colons of control mice and CTα^{IKO} mice with and without antibiotic treatment (n≥4/group). Values are means ± SEM.***p<.001, ****p<.0001. In Figure 4.7F, columns that do not share a letter (a or b) are significantly different. α=0.05.

4.3.7 Administration of the chemical chaperone 4-phenylbutyrate normalizes colon mass and reduces inflammatory tone in the colons of CTα^{IKO} mice

ER stress typically arises due to accumulation of misfolded proteins. However, the preponderance of evidence suggests that alterations to ER membrane lipid composition can indirectly cause misfolded protein accumulation by disrupting the protein folding environment

(Han and Kaufman, 2016). The chemical chaperone 4-phenylbutyrate (PBA) has been used previously to reduce ER stress and improve disease activity in the setting of colitis (Cao et al., 2013). We hypothesized that an altered ER membrane lipid composition was causing misfolded protein accumulation in CTα^{IKO} mice (leading to ER stress and inflammation) and that providing the PBA would alleviate ER stress and disease severity. Like antibiotic-treated mice, control mice and CTα^{IKO} mice initially lost weight during PBA and tamoxifen treatment; however, both groups re-gained weight at the same rate after the end of tamoxifen treatment (Figure 4.8A).. Control and CTα^{IKO} mice receiving the vehicle control lost weight at the same rate, likely due to the mild discomfort of twice daily oral gavages (Figure 8A). Treatment with PBA completely reversed the increase in colon weight observed in untreated CTα^{IKO} mice (Figure 4.8B). Consistent with this normalization of colon mass, PBA ameliorated the increase in colonic MCP-1 observed in CTα^{IKO} mice (Figure 4.8C). Importantly, colonic IL-1β concentrations, which were 10-fold higher in untreated CTα^{IKO} mice compared to controls, were normalized with PBA treatment (Figure 4.8D). However, PBA treatment failed to maintain goblet cell abundance (Figure 4.8E) or to normalize GM-CSF, TNFα, LIF and several other cytokines and chemokines in the colons of CTα^{IKO} mice (Table 4.3). These data suggest that impaired protein folding contributes to the inflammatory changes observed in response to loss of *de novo* PC synthesis, but that additional pathogenic factors drive colonic inflammation.

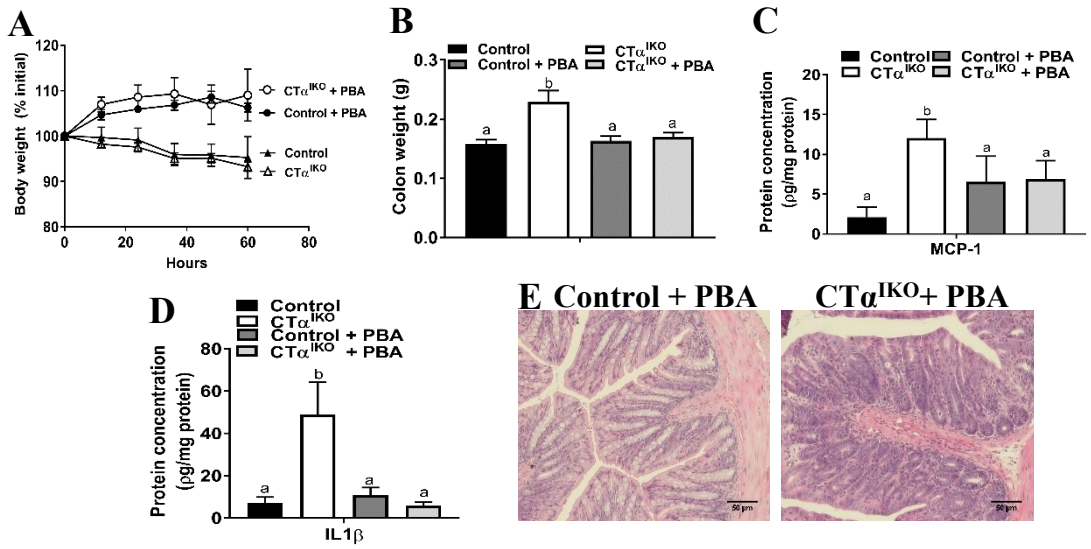

Figure 4.8. Administration of the chemical chaperone 4-phenylbutyrate normalizes colon mass and reduces inflammatory tone in the colons of CTα^{IKO} mice. (A) Body mass relative to body mass at the end of tamoxifen treatment in control mice and CTα^{IKO} mice with and without PBA treatment (5 control mice, 5 CTαIKO mice, 3 control mice + PBA and 4 CTα^{IKO} mice + PBA). (B) Colon weights of control mice and CTα^{IKO} mice with and without PBA treatment (n≥3/group). (C) MCP-1, (D) IL-1β protein concentrations in the colons of control mice and CTα^{IKO} mice with and without PBA treatment (n≥3/group). (E) Representative H&E stained colon sections from PBA-treated control mice and CTα^{IKO} mice. (n≥3/group). Values are means ± SEM. In Figure 4.8B, 1.8C, 4.8D. columns that do not share a letter (a or b) are significantly different. α=0.05.

Table 4.4 Colonic cytokines and chemokines in control mice and CTα^{IKO} with and without PBA[1]

Protein name	***Control***	***CTα^{IKO}***	***Control + PBA***	***CTα^{IKO} + PBA***
Eotaxin	5.07 ± 2.51	22.15 ± 10.87	0.61 ± 0.26	15.44 ± 4.82
G-CSF	1.23 ± 0.33	1.91 ± 0.43	1.07 ± 0.25	1.50 ± 0.23
IFNγ	0.41 ± 0.08	0.96 ± 0.19	0.77 ± 0.37	0.62 ± 0.10
IL-1α	20.14 ± 1.90	26.57 ± 2.89	22.04 ± 7.77	25.99 ± 5.38
IL-2	2.88 ± 0.66	2.11 ± 0.66	1.52 ± 0.68	4.21 ± 0.64
IL-6	0.85 ± 0.41	2.76 ± 1.12	2.18 ± 0.70	1.18 ± 0.33
IL-9	16.91 ± 4.77	8.00 ± 2.83	11.62 ± 9.76	18.17 ± 6.27
IL-10	2.86 ± 0.97	5.46 ± 1.33	6.74 ± 0.44	2.21 ± 0.41
IL-12 (p40)	5.33 ± 2.28	8.31 ± 2.80	12.50 ± 0.78	1.38 ± 0.45
IL-12 (p70)	0.60 ± 0.50	4.17 ± 2.19	6.37 ± 3.21	0.01 ± 0.01
M-CSF	0.41 ± 0.23	1.36 ± 0.33	0.94 ± 0.38	0.52 ± 0.02
RANTES	1.57 ± 0.87	2.60 ± 0.72	1.22 ± 0.21	2.26 ± 0.53
MIP-1α	2.92 ± 0.81	4.07 ± 0.92	2.44 ± 1.01	5.36 ± 0.42
MIP-2	28.70 ± 8.55	55.26 ± 9.93	42.53 ± 3.31	41.02 ± 1.86

[1]Data are mean ± SEM. A 2-way ANOVA was used.

4.4 Discussion

In this study we show that genetic disruption of CTα in IECs induces spontaneous colitis in mice. Colonic inflammation in $CT\alpha^{IKO}$ mice is linked to ER stress, damage to goblet cell mucus granules, and infiltration of the intestinal epithelium by microbes. ER stress, microbial invasion of the colonic epithelium and inflammatory cytokine secretion can independently and collectively contribute to colonic damage in human UC (Heazlewood et al., 2008). Indeed, the phenotype of $CT\alpha^{IKO}$ mice closely mimics that seen in human UC (reduced goblet cell abundance, reduced mucus integrity, immune cell infiltration) and so $CT\alpha^{IKO}$ mice will provide a useful model for studying aspects IBD pathogenesis. Our initial hypothesis was that $CT\alpha^{IKO}$ mice would have lower PC in their colonic mucus layer leading to a compromised mucus barrier. We found that there is loss of the entire mucus barrier after loss of intestinal CTα, suggesting that *de novo* PC synthesis plays a crucial role in colonic protection.

The ER is the primary site of cellular lipid and protein synthesis. Patients with IBD have elevated markers of ER stress in their distal intestinal epithelium (Shkoda et al., 2007, Hu et al., 2007, Heazlewood et al., 2008). Furthermore, genome-wide association studies have linked abnormalities in *Agr2*, X-box binding protein 1 (*Xbp1)* and ORMDL sphingolipid biosynthesis regulator 3 (*Ormdl3)*, genes that encode proteins involved in ER stress, to IBD (Kaser et al., 2008, McGovern et al., 2010). Misfolding of MUC2 induces spontaneous colitis in mice, demonstrating that ER stress induced by the accumulation of misfolded proteins can promote intestinal inflammation (Heazlewood et al., 2008). In addition to protein misfolding, changes to ER membrane lipid composition have been shown to induce ER stress in pancreatic beta cells loaded with saturated fatty acids (Cunha et al., 2008), macrophages exposed to cholesterol (Feng et al., 2003), and the livers of mice fed a high fat diet (Fu et al., 2011b). However, whether disturbed ER

membrane lipid homeostasis can contribute to proinflammatory changes in the gastrointestinal tract had not been investigated. Our data show that impairment of *de novo* PC synthesis in IECs induces the ER stress-associated transcription factor CHOP and promotes intestinal inflammation. Furthermore, we show that reducing ER stress with the chemical chaperone PBA normalizes clinically relevant markers of disease severity (including colon hyperproliferation and inflammatory cytokine secretion). Therefore, our data provide evidence of an important role for *de novo* PC synthesis in maintaining an appropriate ER microenvironment to prevent inflammation in IECs.

Mucus of the colon consists of two distinct layers (an inner microbe-free layer and a less dense outer layer that is colonized by bacteria) that restricts microbial access to host tissues (Johansson et al., 2008). We found that impaired *de novo* PC synthesis results in ultrastructural damage to theca in goblet cells, loss of mucus granules, and a compromised mucus barrier. Goblet cells are at increased risk of ER stress-induced apoptosis relative to other IEC types due to their high protein folding and secretory demands (Heazlewood et al., 2008, Kaser et al., 2008). The lower abundance of goblet cells mucus granules in $CT\alpha^{IKO}$ mice is associated with increased rates of apoptosis in the intestinal epithelium. Furthermore, $CT\alpha^{IKO}$ mice appear to be unable to replace mature goblet cells due to transcriptional repression of the goblet cell maturation factors *Gfi1*, *Klf4* and *Spdef*. Bacterial infiltration of the intestinal epithelium due to a compromised mucus barrier in $CT\alpha^{IKO}$ mice appears to contribute towards disease severity, since antibiotic treatment reduced colonic IL-1α, IL-1β and IFNγ concentrations relative to untreated $CT\alpha^{IKO}$ mice. However, extensive loss of goblet cells and high levels of a range of pro-inflammatory cytokines were still observed in $CT\alpha^{IKO}$ mice following antibiotic treatment and so tissue-intrinsic factors must also play a role in disease pathogenesis.

The mucus layer in the small intestine is discontinuous and thin relative to the large intestine and so secreted antimicrobial peptides such as REGIIIβ and REGIIIγ play an important role in restricting microbial access to the small intestinal epithelium (Vaishnava et al., 2011). Disruption of CTα in the small intestine induces *Reg3b* and *Reg3g*, as well as *Duox2*, *duox2a*, *Fut2,* indicative of impaired mucosal barrier function. Indeed, a FITC-labeled dextran permeability assay revealed a ~75% increase in intestinal permeability in $CT\alpha^{IKO}$ mice relative to control mice. Enhanced microbial interaction with the intestinal epithelium is linked to uncontrolled proliferation and cancer (Reinhardt et al., 2012, Stappenbeck et al., 2002). Furthermore, an intestinal environment with high inflammatory cytokine concentrations is linked to colon cancer initiation and progression (Elinav et al., 2013). The pro-tumorigenic cytokines LIF and MCP-1 (McClellan et al., 2012, Yu et al., 2014) were consistently found to be elevated in the colons of $CT\alpha^{IKO}$ mice. Accordingly, $CT\alpha^{IKO}$ mice have increased expression of genes linked to cell cycle progression, increased abundance of the PCNA protein, and hyperproliferation of intestinal crypts. These results are comparable to those observed in MUC2-deficient mice, as these mice also experience crypt hyperproliferation in both the small intestine and colon (Velcich et al., 2002).

4.5 Conclusions

In conclusion, our results show that endogenous PC synthesis is important for protecting against ER stress in the intestinal epithelium and that disruption of CTα in IECs results in goblet cell depletion and spontaneous colitis in mice. The factors underlying induction of intestinal inflammation in $CT\alpha^{IKO}$ mice include mucus barrier dysfunction, enhanced intestinal permeability, exposure of the intestinal epithelium to luminal antigens, ER stress and inflammatory cytokine secretion. Therefore, our data provide *in vivo* evidence that PC is an important anti-inflammatory component of the intestinal epithelium and provide molecular insight into the clinical benefits observed upon restoration of colonic PC homeostasis in UC patients (Stremmel et al., 2005, Stremmel et al., 2007, Karner et al., 2014).

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CHAPTER 5. Insufficient dietary choline aggravates disease severity in a mouse model of Citrobacter rodentium-induced colitis

T Ju*, JP Kennelly*, RL Jacobs and BP Willing

5.1 Introduction

The intestinal epithelium and gut-associated lymphoid tissue is spatially separated from luminal microbes by immunoglobulins, antimicrobial peptides, and a protective mucus barrier (Hooper and Macpherson, 2010). Mucus provides a physical barrier to prevent invasion of the epithelium by pathogens and microbial leakage into the lamina propria (Johansson and Hansson, 2016). The colonic mucus barrier is composed of two distinct layers: a sterile inner layer that adheres to the intestinal epithelium and a loose outer layer that contains bacteria (Johansson et al., 2008). Goblet cells establish and maintain the mucus layer by producing the major mucus glycoprotein Mucin 2 (MUC2) (Johansson and Hansson, 2016). Individuals with ulcerative colitis, a form of inflammatory bowel disease, have fewer typical goblet cells and a compromised mucus barrier compared to individuals without ulcerative colitis (McCormick et al., 1990, Pullan et al., 1994, Strugala et al., 2008). Furthermore, loss MUC2 induces spontaneous intestinal inflammation in mice, suggesting that goblet cells and the mucus barrier play an important role in protecting against intestinal injury (Van der Sluis et al., 2006).

Choline is a dietary factor required for the synthesis of membrane phospholipids, formation of the neurotransmitter acetylcholine, and methyl group metabolism (Li and Vance, 2008). After absorption in the intestine *via* active transport, most dietary choline is converted to PC, which accounts for 95% of the total choline pool in mammals (Li and Vance, 2008, van der Veen et al., 2017). PC is abundant in the gastrointestinal mucus layer where its hydrophobic properties are thought to play a role in maintaining mucus integrity (Treede et al., 2007, Stremmel et al., 2005).

Importantly, patients with ulcerative colitis have lower PC concentrations in the mucus layer of their distal intestine compared to non-inflamed controls, suggesting that choline supply or metabolism might be involved in maintaining mucus barrier integrity and intestinal immune homeostasis (Ehehalt et al., 2004). Furthermore, clinical trials have shown beneficial effects of supplying exogenous PC in a modified-release capsule (that prevents absorption in the proximal intestine) to the distal mucosa of ulcerative colitis patients (Stremmel et al., 2005, Stremmel et al., 2007, Karner et al., 2014). While these data suggest that exogenous PC might have anti-inflammatory effects in the setting of colitis, the role that choline, the diet-derived substrate for *de novo* PC synthesis, plays in protecting against colonic infection and inflammation remains poorly understood. It has been demonstrated that insufficient dietary choline leads to impaired liver and muscle function in humans and animals (da Costa et al., 2004, Zeisel et al., 1991). In the current study, we aimed to characterize the role that dietary choline plays in maintaining colonic immune homeostasis in the context of infectious colitis.

In addition to diet, choline availability for physiological processes is impacted by the gut microbiota (Romano et al., 2015). Certain gut microbes, including commensal *E. coli* strains, can convert dietary choline to trimethylamine (TMA) and decrease choline bioavailability to the host (Romano et al., 2017). Conversely, dietary choline levels could modulate gut microbial composition. In a human study in which dietary choline levels were manipulated, the abundance of *Gammaproteobacteria* and *Erysipelotrichia* were changed (Spencer et al., 2011). The microbiota is a diverse and dynamic ecosystem that makes important contributions to host metabolism and innate immunity (Round and Mazmanian, 2009, Nicholson et al., 2012). Commensal microbes prevent the proliferation of gut pathogens (Kamada et al., 2013) and can directly influence intestinal epithelial cell immune function (Cash et al., 2006). Individuals with

inflammatory bowel diseases have broadly different gut microbial compositions compared to healthy individuals, suggesting that microbial factors are involved in the origin or progression of intestinal inflammation (Frank et al., 2007, Willing et al., 2010). Diet is a major determinant of gut microbial composition (Turnbaugh et al., 2009), and defining the changes that occur to the microbiota in response to specific dietary nutrients in the context of intestinal inflammation can provide insight into the microbial factors that initiate or perpetuate disease. Indeed, the abundance of the commensal gut microbes *Gammaproteobacteria* and *Erysipelotrichia* were correlated with dietary choline intake in humans (Spencer et al., 2011). We aimed to clearly define changes to the gut microbial community in response to different levels of dietary choline intake in the setting of intestinal inflammation.

The aim of this study was to determine the effects of dietary choline deficiency on mucosal barrier function and immune responses during infection. We hypothesized mice fed a choline-deficient (CD) diet (i.e. lacking the key dietary substrate for *de novo* PC synthesis), would have increased susceptibility to colitis induced by *C. rodentium*, a murine attaching-effacing pathogen that induces pathological features comparable to those seen in ulcerative colitis (Mundy et al., 2005). We found that mice fed insufficient dietary choline developed more severe colitis following *C. rodentium* infection compared with mice fed diets containing sufficient (CS) or excess (CE) levels of choline. A CD diet was linked to greater loss of goblet cells and increased production of proinflammatory cytokines. Meanwhile, the enrichment of bacterial genera *Allobaculum* and *Turicibacter* have been identified as the indicators of dietary choline depletion. Our data shows that adequate levels of dietary choline are important for maintaining goblet cell abundance and colonic immune function during colitis development.

5.2 Materials and methods

5.2.1 Mice. Eight-week-old female C57BL/6J mice (Jackson Laboratory, Bar Harbor, ME) were housed in a Specific Pathogen Free (SPF) facility at the University of Alberta with a 12-h light/dark cycle and free access to food and water. Mice were randomly grouped into six cages with 4 to 5 mice per cage by a blinded lab animal technician and balanced for average body weight. Cages were allocated to one of three isocaloric diets differing only in their choline content (Table 1): choline deficient (CD; 0 g choline/kg diet), choline sufficient (CS; 1 g choline/kg diet), or excess choline (CE; 4 g choline/kg diet). In the first experimental arm of the study, mice were fed the CD, CS or CE diets for four weeks and were not inoculated with *C. rodentium*. Body weights were recorded weekly and mice were euthanized by before collection of tissues and gut contents. In the second experimental arm of the study, mice were fed the CD, CS, and CE diets for three weeks and before being inoculated with *C. rodentium* by oral gavage. Infected mice were euthanized 7 days post infection (DPI) for collection of tissues and gut contents, and for fecal *C. rodentium* enumeration. The infectious study was repeated twice with the total number of 18 mice in each treatment. The terminal 5 mm piece of the distal colon was collected in 10% neutral buffered formalin for histological analysis. Colon, small intestine and liver tissue was snap frozen in liquid nitrogen. Ileal, caecal, and colonic contents were snap frozen in liquid nitrogen for microbial composition analysis. 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.

Table 5.1. Composition of the experimental diets

Component	Choline deficient (CD)	Choline Sufficient (CS)	Choline Excess (CE)
Casein[1] (g)	270	270	270
Corn Starch[1] (g)	170	170	170
Sucrose[2](g)	195	195	195
Cellulose[1](g)	80	80	80
AIN-93-VX Vitamin Mix[1] (g)	19	19	19
Bernhart-Tomarelli Mineral Mix[1] (g)	50	50	50
Calcium Phosphate Dibasic[2] (g)	3.4	3.4	3.4
Myo-Inositiol[2] (g)	6.3	6.3	6.3
L-cysteine[2] (g)	1.8	1.8	1.8
Choline Bitartrate[2] (g)	0	5	19.5
Vegetable Oil[3] (g)	32	32	32
Corn Oil[4] (g)	10	10	10
Lard[5] (g)	155	155	155
DHAsco[6] (g)	1.5	1.5	1.5
Arasco[6] (g)	1.5	1.5	1.5
% energy from protein	25	25	25
% energy from carbohydrate	34	34	34
% energy from fat	41	41	41
Choline content of diet (g)	0	1	4

[1]Harlan Teklad (Indianapolis, IN, USA)

[2]Safeway (Edmonton, AB, Canada)

[3]Crisco J.M. Smucker Company (Orrville, OH, USA)

[4]Mazola ACH Food Companies Inc. (Oakbrook Terraces, IL, USA)

[5]TenderFlake (Chicago, IL, USA)

[6]DSM Nutritional Products Inc. (Heerlen, The Netherlands)

5.2.2 Bacterial strains. *C. rodentium* strain (DBS100) was cultivated in 5 ml of Luria-Bertani (LB) medium (Fisher Scientific, Nepean, ON) at 37℃ for 16 h. The culture medium contained approximately 1.0×10^9 colony forming units (CFU)/ml of *C. rodentium* and each mouse received 0.1 ml of culture. Enumeration of *C. rodentium* was conducted by serial dilutions of fecal samples plated on MacConkey agar (BD, Sparks, MD) and total CFUs per gram of feces were then calculated.

5.2.3 Microbial composition analysis. Total DNA was extracted from ileal, caecal, and colonic contents using the QIA stool extraction kit (Qiagen Inc., Valencia, CA) with an additional bead-beating step as described previously (Willing et al., 2011). Amplicon libraries were constructed that amplified the V3-V4 region of the 16S rRNA gene following Illumina 16S metagenomic Sequencing Library Preparation protocol. The paired-end sequencing and data analysis were performed using the protocols and pipelines published previously (Ju et al., 2017).

5.2.4 mRNA isolation and quantification by PCR. Total RNA was isolated from colon sections using Trizol (Invitrogen, CA, USA). RNA was reversed-transcribed using Superscript II (Invitrogen, CA, USA). Quantitative PCR was run on an Applied Biosystems StepOne Plus for 40 cycles using a Power SYBR Green PCR Master Mix (Applied Biosystems, MA, USA), in triplicate. Relative mRNA expression was normalized to Rplp0. Quantitation was performed using the standard curves method.

5.2.5 Microscopy. Formalin-fixed, paraffin-embedded tissue slices (5µm thick) were stained with hematoxylin and eosin (H&E). Images were obtained using an EVOS FL Auto Imaging System (Thermo Scientific, Nepean, ON). Well-oriented cross sections were assessed for pathology as previous described (Ju et al., 2017).

5.2.6 Measurement of colonic cytokines and chemokines. Colon tissue obtained from the infected mice were subjected to an assay targeting a panel of cytokines and chemokines. Distal colon tissue was 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 after centrifugation to remove debris. Cytokine concentrations were determined using Multiplex LASER Bead Technology (Eve Technology, Calgary, Canada).

5.2.7 Lipid measurements. Total protein concentrations of tissue homogenates were determined by bicinchoninic acid assay before tissue lipids were extracted from homogenates (1mg/ml) by the method of Folch (FOLCH et al., 1957). PC and phosphatidylethanolamine (PE) were separated by thin layer chromatography using the solvent system chloroform: methanol: acetic acid: water (50:30:8:4). Bands were visualized after exposure to iodine and measured by phosphorous assay, as described previously (Zhou and Arthur, 1992)

5.2.8 Statistical analysis and visualization. Body weight and cytokine data were assessed for normality by the Shapiro-Wilk test. To compare differences between treatments, a one-way analysis of variance (ANOVA) was used for parametric data and a Kruskal-Wallis test was used for nonparametric data, followed by a Bonferroni's post-hoc test. For microbial composition analysis, the comparison of individual taxa/OTUs between treatments were performed using the Kruskal-Wallis test. Nonparametric multivariate analysis of variance (NP-MANOVA) was used to identify the difference between groups using the Adonis function in the Vegan package (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 and Holmes, 2013). Correlation of colonic *C. rodentium* load with cytokine levels was analyzed by Spearman Rank Correlation using

SAS. *P* values indicate statistical significance as follows: **, $P < 0.01$; *, $P < 0.05$. R (v3.4.4) and GraphPad Prism were used for visualizing the results.

5.3 Results

5.3.1 Insufficient dietary choline exacerbates the severity of *C. rodentium*-induced colitis

To examine the effects of dietary choline on susceptibility to colitis, we fed mice isocaloric diets that differed only in choline content (Table 5.1 and Figure 5.1A): choline deficient (CD; 0 g choline/kg diet), choline sufficient (CS; 1 g choline/kg diet), or excess choline (CE; 4 g choline/kg diet). Three weeks after initiation of the dietary treatment, mice were exposed to *C. rodentium* by oral gavage before continuing the dietary treatment for one week. Three weeks after dietary treatment, there was no significant differences in body weight between treatments (data not shown). Following *C. rodentium* infection, mice fed the CD diet experienced moderate body weight loss (relative to pre-infection weight), while mice fed the CS or CE diets maintained their pre-infection body weight (Figure 5.1B). Seven days post infection, the CD group exhibited significantly higher *C. rodentium* loads in the feces than that in the CE group ($P < 0.05$, Figure 5.1C).

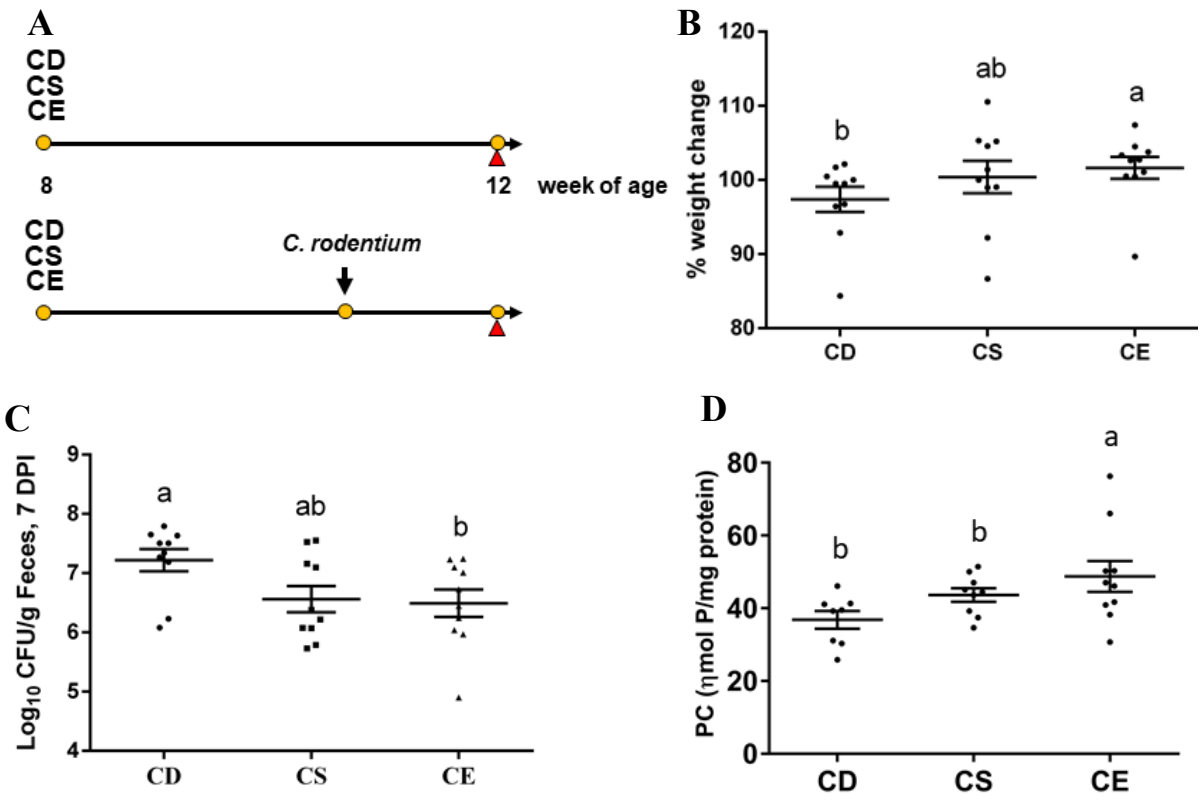
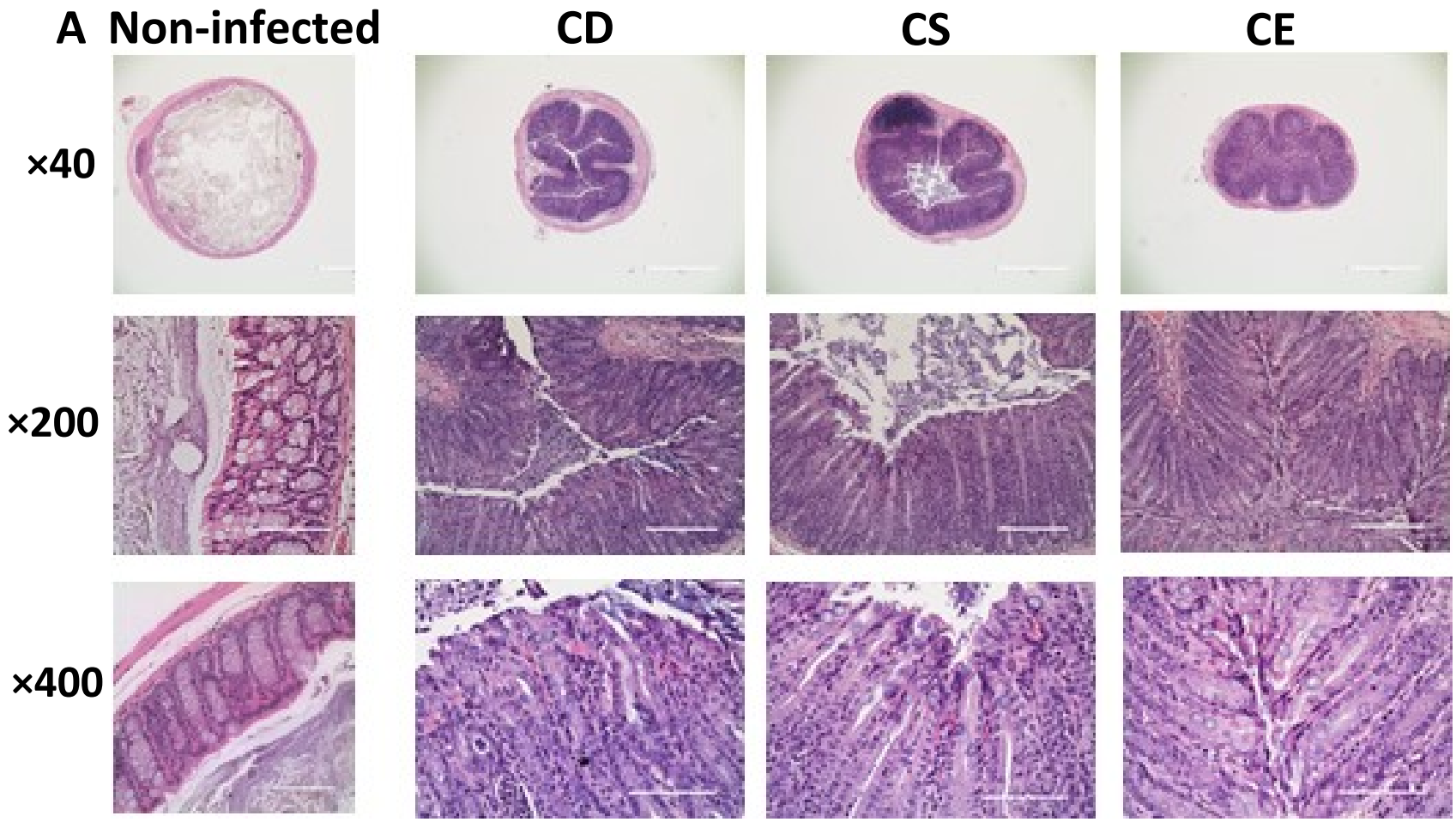


Figure 5.1. Insufficient dietary choline impairs the ability of mice to defend against *Citrobacter-rodentium* induced colitis. (A) Experimental timeline. Mouse feeding trail (top), mouse *C. rodentium* infection model (bottom) (B) Percent body weight change 7 days after *C. rodentium* infection relative to the time of infection (n = 8/group). (C) Enumeration of *C. rodentium* in the feces 7 days post infection (n = 8/group). (D) PC concentrations in the colon 7 days post infection (n = 8/group). Data are presented as mean ± SEM. Means that do not share a letter (a or b) are significantly different. $\alpha = 0.05$.

Analysis of H&E-stained colon sections after *C. rodentium* infection revealed that mice in all three diet groups displayed a thick colonic mucosa, a typical feature of *C. rodentium* infection (Figure 5.2A)(Mundy et al., 2005). However, there was more extensive damage to the colonic epithelium of mice fed the CD when compared to mice fed the CS or CE diets, as reflected by a significantly higher total pathology score (Figure 5.2B). Specifically, mice fed the CD diet had more damage to the surface epithelium than mice fed the CD or CE diets, as characterized by increased regenerative change, desquamation, and epithelial ulceration (Figure 5.2D). Furthermore, mice fed the CD diet had increased mucosal hyperplasia, as indicated by increased crypt length relative to mice fed the CS diet (Figure 5.2A and 2E). Interestingly, there was a dose response relationship between the level of dietary choline and the loss of goblet cells, with the most dramatic loss of goblet cells observed in the colons of mice fed the CD diet ($P < 0.05$, Figure 5.2A and 5.2G). Total pathology score was not different between mice fed the CS and CE diets at 7 days post infection (Figure 5.2B).



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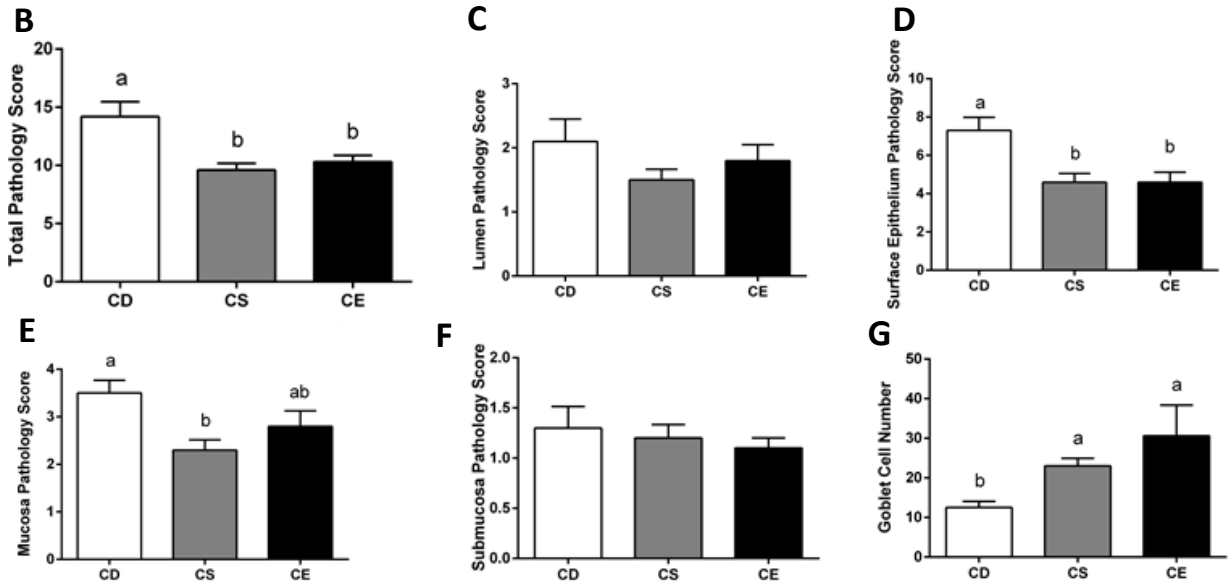

Figure 5.2. Insufficient dietary choline increases colonic damage relative to mice fed sufficient or excess dietary choline in response to *Citrobacter-rodentium* infection. (A) Distal colon sections from CD, CS, and CE mice 7 days after *C. rodentium* colonization were stained with Hematoxylin and Eosin. Original magnification and bars: Top: x 40, 1000 μm; Middle: x 200, 500 μm; Bottom: x 400, 100 μm. (B) Total pathology score (C) Lumen pathology score (D) Surface epithelium pathology score (E) Mucosa pathology score (F) Submucosa pathology score (G) Goblet cell pathology score 7 days post infection in the colons of mice fed the CD, CS or CE diets . For all the treatment, n = 8/group. Data are presented as mean ± SEM. Means that do not share a letter (a or b) are significantly different. $\alpha = 0.05$.

5.3.2 Mice fed a CD diet have enhanced production of proinflammatory cytokines and chemokines in response to *C. rodentium* infection

To determine whether dietary choline intake influences colonic inflammation and the innate immune response after infection, we profiled inflammatory cytokine and chemokine concentrations in the colon seven days after *C. rodentium* colonization. In general, mice fed a CD diet showed an enhanced production of proinflammatory cytokines and chemokines in response to *C. rodentium* infection compared to mice fed the CS or CE diets (Table 5.2). In line with greater abundance of fecal *C. rodentium* at 7 days post infection and more severe damage to the surface epithelium and mucosa, mice fed the CD diet had higher monocyte chemoattractant protein-1 (MCP-1) concentrations in colonic tissue compared to mice fed the CE diets ($P < 0.05$, Table 5.2). Furthermore, Eotaxin and RANTES (also known as CCL5), as well as the inflammatory cytokine interleukin-9 (IL-9), were elevated in the colons of mice fed the CD relative to mice fed the CE diet ($P < 0.05$, Table 5.2), suggesting an increased colonic inflammation in response to *C. rodentium* colonization in the CD group. Spearman's rank correlation analysis indicated significant correlations between *C. rodentium* load and levels of MCP-1 ($r = 0.480$, $P < 0.05$) and Macrophage inflammatory protein-2 (MIP-2; $r = 0.413$, $P < 0.05$; Figure 5.3). Collectively, there was a clear pattern of increased pro-inflammatory response in the CD mice after *C. rodentium* colonization. However, we did not observe significant differences in the cytokine and chemokine levels between the CS and CE group, which were consistent with the results from the pathological assessment (Table 5.2).

Table 5.2. Concentrations of cytokines/chemokines in the colon tissue 7 DPI *C. rodentium* infection.

	Cytokines/chemokines	CD	CS	CE	*P* value
MCP-1	Monocyte chemoattractant protein-1	25.60 ± 8.67[a]	12.41 ± 3.02[ab]	7.51 ± 1.44[b]	0.048
Eotaxin-1	Eotaxin-1	43.53 ± 11.37[a]	33.98 ± 9.68[ab]	9.78 ± 2.89[b]	0.035
IL-2	Interleukin 2	4.38 ± 0.52[a]	1.64 ± 0.44[b]	2.80 ± 0.75[ab]	0.017
IL-9	Interleukin 9	12.01 ± 2.39[a]	7.90 ± 1.59[ab]	3.78 ± 0.55[b]	0.013
RANTES	C-C Motif Chemokine Ligand 5	3.33 ± 0.82[a]	1.25 ± 0.16[ab]	1.11 ± 0.17[b]	0.039
MIP-2	Macrophage inflammatory protein 2-alpha	53.14 ± 15.24	19.57 ± 4.48	28.13 ± 5.67	0.068
MIP-1β	Macrophage inflammatory protein-1 beta	7.14 ± 1.39	4.26 ± 0.67	4.63 ± 0.58	0.082
IL-17	Interleukin 17	1.39 ± 0.83	0.44 ± 0.17	0.10 ± 0.04	0.043
IL-4	Interleukin 4	0.10 ± 0.02	0.079±0.01	0.08±0.01	0.164
IL-6	Interleukin 6	4.29 ± 2.16	1.31±0.48	1.29±0.45	0.495
IL-10	Interleukin 10	3.72 ± 1.04	3.67±1.03	3.54±0.76	0.990
IL-12 (P40)	The p40 Submit of Interleukin 12	1.37 ± 0.17	2.18±0.63	3.46±1.07	0.381
IL-12 (P70)	The p70 Submit of Interleukin 13	1.61 ± 0.41	1.31±0.42	1.78±0.61	0.796
IL-15	Interleukin 15	6.55 ± 1.12	5.41±0.41	5.28±0.71	0.462
KC	Keratinocyte chemoattractant (CXCL1)	15.54 ± 5.94	5.57±1.24	3.97±1.66	0.297
LIF	Leukemia inhibitory factor	5.16 ± 1.97	2.71±0.51	3.44±1.02	0.858
M-CSF	Macrophage colony-stimulating factor (CSF1)	0.95 ± 0.17	0.93±0.17	0.98±0.17	0.977
MIP-1α	Macrophage inflammatory protein 1-alpha (CCL3)	5.85 ± 2.49	4.61±1.75	3.06±1.38	0.588
TNFALPHA	Tumor necrosis factor-alpha	0.69 ± 0.14	0.54±0.10	0.65±0.20	0.797
VEGF	Vascular endothelial growth factor	0.60 ± 0.10	0.40±0.10	0.39±0.09	0.272

Data are presented as mean ± SEM. N=8/group. Means that do not share a letter (a or b) are significantly different. α = 0.05.

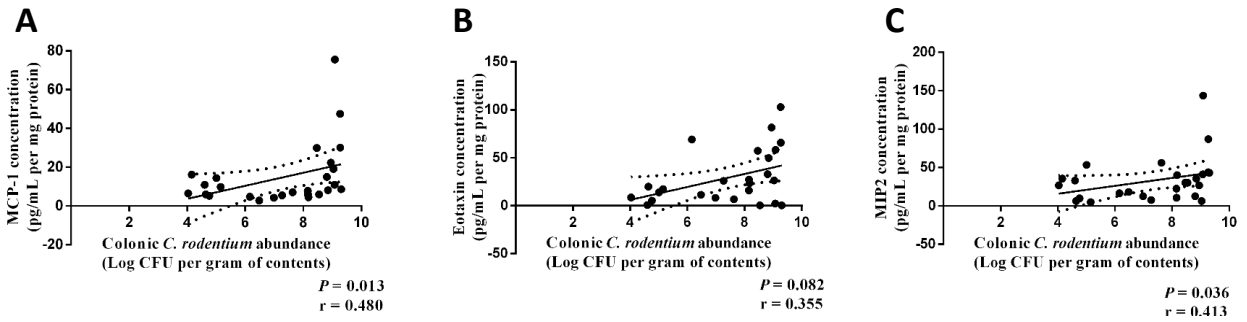

Figure 5.3. Correlation between colonic *C. rodentium* load with (A) MCP-1, (B) Eotaxin-1, and (C) MIP-2 expression levels. Spearman's correlation coefficient (r values) and significance *P* values are shown.

5.3.3 Insufficient dietary choline reduces PC concentrations in the colon following *C. rodentium* infection

In the dietary treatment trial (i.e. without infection), mice fed the CD deficient diet had lower hepatic PC concentrations as compared to mice fed the CS or CE diets, while hepatic PE concentrations were unchanged between groups (Table 5.3). Similarly, PC concentrations were lower in the proximal small intestines of mice fed the CD as compared to mice fed the CS diet (Table 5.3). These data show that feeding mice a CD diet for 4 weeks depletes hepatic and small intestinal PC pools. Interestingly, there was no difference in colonic PC concentrations between CD, CS, or CE groups without *C. rodentium* infection (Figure 5.4). However, after *C. rodentium* colonization, there was a dose-dependent decrease in colonic PC concentrations, with the CD group experiencing the largest decrease in PC concentrations (Figure 5.1D. These data suggest that homeostatic systems exist to maintain colonic PC concentrations in the setting of dietary choline deficiency (possibly by increasing uptake from circulation); however, under inflammatory conditions these homeostatic systems fail, and colonic PC concentrations drop. The colonic concentrations of PE were not different between groups either before or after *C. rodentium* infection (Figures 5.4).

Table 5.3. Phospholipid concentrations in liver and small intestine

4 weeks after dietary treatment (non-infection)

	CD	CS	CE
Liver phospholipids	*nmol/mg protein*	*nmol/mg protein*	*nmol/mg protein*
PC	91.8 ± 9.49^{b}	116.4 ± 7.97^{ab}	120.6 ± 3.54^{a}
PE	49.7 ± 3.37	54.5 ± 3.7	55.8 ± 2.21
Jejunal phospholipids			
PC	77.8 ± 2.2^{b}	87.5 ± 3.0^{a}	84.4 ± 2.6^{ab}
PE	55.7 ± 2.9	58.4 ± 2.6	58.6 ± 2.8

Data are presented as mean ± SEM (n=8/group) Means that do not share a letter (a or b) are significantly different. $\alpha = 0.05$.

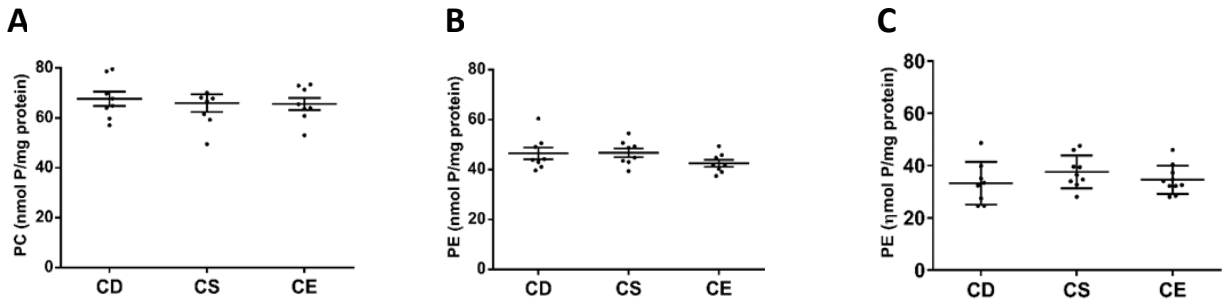

Figure 5.4. Colonic PC (A) and PE (B) concentration without *C. rodentium* infection. Colonic PE (C) concentrations after *C. rodentium* infection 7 DPI. For all treatment groups, n = 8/group. Each point represents an individual mouse. Data are shown as mean ± SEM.

5.3.4 Changes to dietary choline levels did not alter overall gut microbial structure

The gut microbial composition of the CD, CS, and CE group after 4 weeks of dietary treatment (without infection) was characterized by sequencing 16S rRNA gene amplicons (V3-V4 region) from ileal, caecal, and colonic contents. The sequencing obtained an average of 18,685 ± 6,019 (mean ± standard deviation) quality-controlled and chimera-checked reads per sample. At different intestinal segments, dietary choline levels did not cause a dramatic shift in gut microbial structure as determined by the PERMANOVA (Figure 5.5A-5.5C). Specifically, there was no difference in microbial structure between CD, CS or CE groups at the caecum ($P = 0.226$), colon ($P = 0.107$) or ileum ($P = 0.096$), as assessed using Bray-Curtis distance matrices with 999 random permutations (Adonis)(Figure 5.5A-5.5C). To evaluate phylogenetic richness and evenness of the microbial community, Chao1 diversity index and Shannon index were calculated in each sample at different intestinal segments. Chao1 and Shannon index did not vary among the treatment groups at each intestinal site, which suggested that dietary choline levels showed minimal effects on the diversity of the microbial community (Figure 5.5D-5.5F). Therefore, enhanced damage to the colonic epithelium of mice fed the CD diet compared to mice fed the CS or CE diets is not linked to broad changes to the structure of the gut microbiota and is instead likely linked to host-intrinsic factors

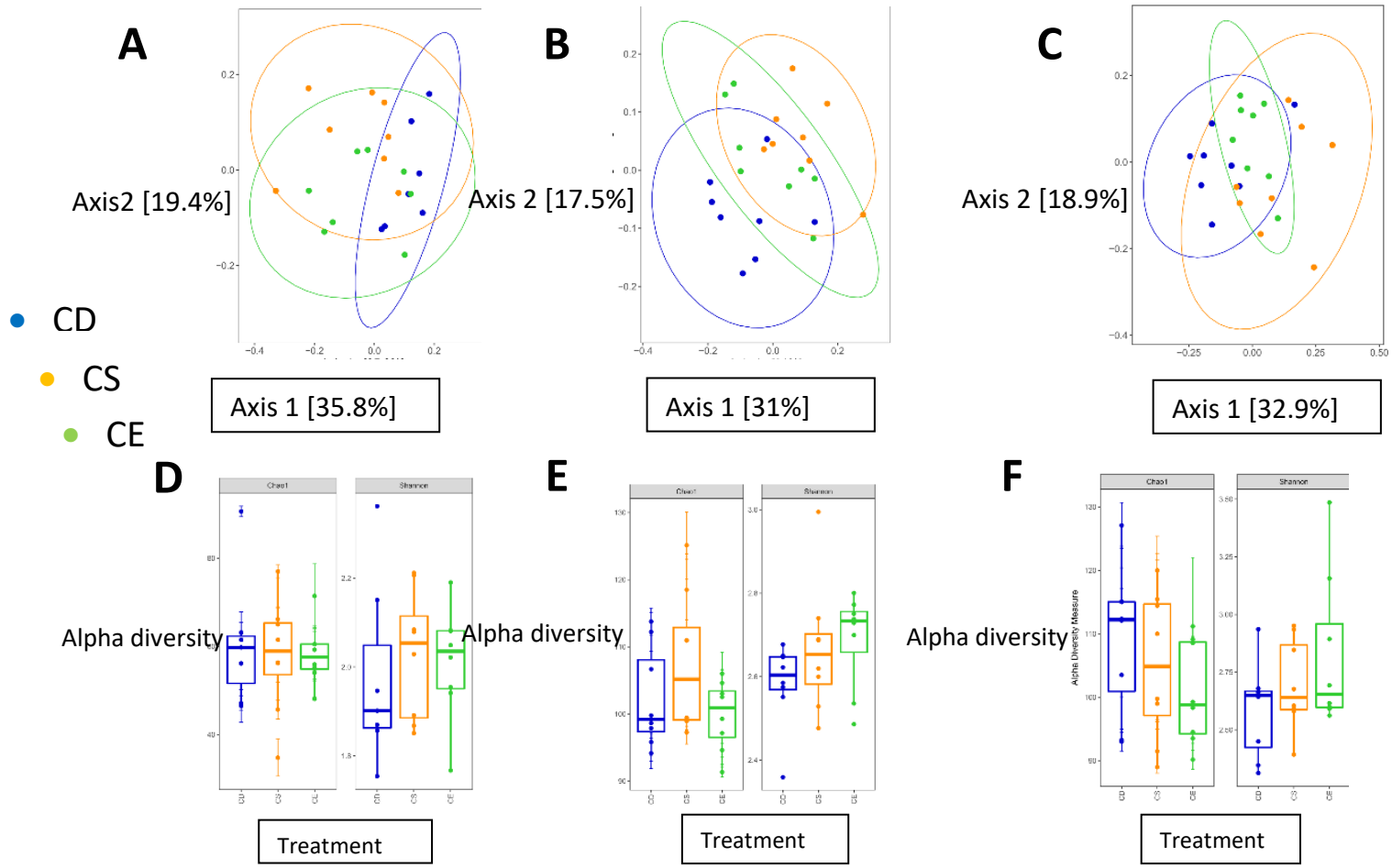

Figure 5.5 Dietary choline deficiency does not alter overall gut microbial structure compared to sufficient or excess dietary choline Principle component analysis (PCA) plots of the bacterial communities in the (A) ileum (B) caecum and (C) colon of mice fed the CD, CS or CE diets based on the Bray-Curtis distance matrix. Each point represents an individual mouse. Box-plots show the Chao1 and Shannon indexes of gut microbiota in the (A) ileum (B) caecum and (C) colon of mice fed the CD, CS or CE diets. For all treatment groups, n = 8/group. Data are shown as mean SEM.

Figure 5.5 Dietary choline deficiency does not alter overall gut microbial structure compared to sufficient or excess dietary choline Principle component analysis (PCA) plots of the bacterial communities in the (A) ileum (B) cecum and (C) colon of mice fed the CD, CS or CE diets based on the Bray-Curtis distance matrix. Each point represents an individual mouse. Box-plots show the Chao1 and Shannon indexes of gut microbiota in the (A) ileum (B) cecum and (C) colon of mice fed the CD, CS or CE diets. For all treatment groups, n = 8/group. Data are shown as mean ± SEM.

5.5.5 Dietary choline levels altered the relative abundance of certain bacterial taxonomies

To determine how the abundance of specific gut microbes change in response to changes in the level of dietary choline, the sequences were assigned to taxonomy using RDP Classifier. There were notable changes in the abundance of bacterial genera, *Allobaculum*, *Turicubacter*, *Planococcaeceae*, and *Oscillospira* in mice fed the CD diet compared to mice fed the CS or CE diets (Table 5.4). We observed a consistent increase in the relative abundance of *Allobaculum* and *Turicibacter* in the CD group at all three intestinal segments (Table 5.4). Specifically, the genus *Allobaculum* represented an average of 0.1%, 0.01%, and 0.06% of the total microbial community in the ileum, caecum, and colon, respectively, in mice fed the CE diets (Table 5.4). However, the average abundance of *Allobaculum* reached 1.73%, 0.19%, and 0.91% in the ileum, caecum, and colon, respectively, in mice fed the CD diet (Table 5.4). We were unable to detect the genus of *Turicibacter* in mice fed the CE diet at all three intestinal sites (Table 5.4). However, *Turicibacter* was abundant in mice fed the CD diet, representing 0.83% of total microbiota in the colon. In addition to the significant changes of *Allobaculum* and *Turicibacter* in response to a CD diet, we observed changes to certain bacterial taxonomies at specific intestinal sites. There was a decrease of unclassified *Planococcaceae* in mice fed the CD diet compared to mice fed the CS and CE diets.

Table 5.4. Relative abundance of bacterial taxonomies altered by dietary choline levels (Summarized down to the genus level)

Genus	CD (%)	CS (%)	CE (%)	*P* value	*P* value (FDR adjusted)
Ileum					
Allobaculum	1.73 ± 0.44^{a}	1.73 ± 0.44^{a}	0.10 ± 0.03^{b}	< 0.01	0.027
Plannococcaceae	0.03 ± 0.02^{b}	0.63 ± 0.42^{ab}	1.83 ± 0.42^{a}	< 0.01	0.133
Adlercreutzia	0.06 ± 0.03^{b}	0.08 ± 0.01^{ab}	0.14 ± 0.01^{a}	0.013	0.192
Turicibacter	0.68 ± 0.40^{a}	0.46 ± 0.24^{ab}	0.00 ± 0.00^{b}	0.019	0.192
Erysipelotrichaceae_unclassified	0.10 ± 0.03^{b}	0.15 ± 0.04^{ab}	0.46 ± 0.15^{a}	0.023	0.192
Peptostreptococcaceae_unclassified	6.81 ± 1.40	13.07 ± 3.63	13.96 ± 1.72	0.035	0.194
Ruminococcus	0.05 ± 0.00^{ab}	0.03 ± 0.00^{b}	0.09 ± 0.02^{a}	0.036	0.194
[Ruminococcus]	0.60 ± 0.14	0.58 ± 0.15	1.38 ± 0.32	0.036	0.194
Caecum					
Allobaculum	0.19 ± 0.06^{a}	0.09 ± 0.01^{a}	0.01 ± 0.00^{b}	< 0.01	0.015
Lactococcus	0.14 ± 0.01^{a}	0.11 ± 0.02^{ab}	0.07 ± 0.00^{b}	< 0.01	0.081
Planococcaceae_unclassified	0.03 ± 0.02^{b}	0.23 ± 0.09^{ab}	0.47 ± 0.14^{a}	< 0.01	0.081
Ruminococcus	2.49 ± 0.30^{ab}	1.55 ± 0.27^{b}	3.71 ± 0.55^{a}	0.013	0.126
Turcibacter	0.08 ± 0.03^{a}	0.01 ± 0.00^{ab}	0.00 ± 0.00^{b}	0.028	0.210
Erysipelotrichacese_unclassified	0.09 ± 0.03	0.19 ± 0.04	0.21 ± 0.03	0.037	0.210
Clostridiaceae_unclassified	0.07 ± 0.02^{ab}	0.14 ± 0.05^{a}	0.03 ± 0.00^{b}	0.046	0.221
Colon					
Allobaculum	0.91 ± 0.29^{a}	0.32 ± 0.08^{a}	0.01 ± 0.00^{b}	< 0.01	0.043
Turicibacter	0.21 ± 0.08^{a}	0.02 ± 0.02^{ab}	0.00 ± 0.00^{b}	< 0.01	0.043
Oscillospira	5.77 ± 0.59^{b}	8.33 ± 0.62^{ab}	10.05 ± 1.02^{a}	< 0.01	0.069
Ruminococcus	1.44 ± 0.18^{ab}	1.18 ± 0.19^{b}	2.99 ± 0.62^{a}	0.011	0.069

Data are presented as mean ± SEM (n = 8). Means that do not share a letter (a or b) are significantly different. $\alpha = 0.05$.

Furthermore, the colonic microbiota of mice fed the CD diet contained substantially less of the genus of *Oscillospira* compared to the colonic microbiota of the other two diet groups (Table 4). Therefore, while dietary choline levels did not significantly shift the overall microbial structure, our results identify specific gut bacteria that respond to changes in dietary choline supply.

5.4 Discussion

In this study we show that mice fed a CD diet have exacerbated severity of *C. rodentium*-induced colitis compared to mice fed a CS or CE diet. The enhanced inflammatory response in response to a CD diet was characterized by greater loss of goblet cells, increased production of proinflammatory cytokines and chemokines, and greater *C. rodentium* colonization compared to mice fed the CS or CE diets. Our results identify choline as a dietary factor that influences gut mucosal homeostasis and immune response to infection. However, no additional protection against *C. rodentium*-induced colitis was conferred by a CE diet relative to a CS diet. While we identified specific microbial taxonomies that respond to a CD diet, there were minimal changes to the overall structre of the gut microbiota with varying levels of dietary choline.

It was surprising that, in the absence of *C. rodentium* infection, mice fed the CD diet had normal colonic PC concentrations relative to mice fed the CS or CE diets after 4 weeks dietary treatment. It has been shown previously that mice fed a CD diet for 3 weeks maintain normal choline-containing metabolite concentrations in the brain by reducing choline oxidation to betaine and by sustaining the delivery of choline to the brain in blood (Li et al., 2007). Similarly, in the present study we found that mice fed the CD diet had normal colonic PC concentrations relative to mice fed the CS or CE diets after 4 weeks in the absence of *C. rodentium* infection. However, colonic PC concentrations were lower in mice fed the CD diet after *C. rodentium* infection,

indicating a failure of the homeostaic systems required to maintain colon PC concentrations. There is evidence that humans with ulcerative colitis have lower colonic PC concentrations relative to healthy controls (Ehehalt et al., 2004), and that delivering exogenous PC to the distal intestinal epithelium of ulcerative colitis patients reduces some metrics of disease severity (Stremmel et al., 2005, Stremmel et al., 2007, Karner et al., 2014). Our data suggest that low colonic PC concentrations in the setting of intestinal inflammtion was associated with adverse effects on the inflammatory disease progression.

Goblet cells produce and secrete critical components of gastrointestinal mucus and therefore function to protect the host from invasion by luminal microbes (Johansson and Hansson, 2016). Disruption of MUC2, a critical component of mucus, leads to microbial infiltration of the colonic epithelium and inflammation due to impaired mucus integrity (Van der Sluis et al., 2006, Heazlewood et al., 2008). In the current study, mice fed the CD diet had more severe loss of mucus granules in goblet cells and enhanced inflammatory cytokine production in response to *C. rodentium* infection relative to mice fed a CS or CE diet. A compromised mucus barrier has been shown to increase translocation of *C. rodentium* and lipopolysaccharide to colonic crypts and lamina propria (Khan et al., 2006). Consistent with greater infiltration of the intestinal epithelium by *C. rodentium* due to a loss of goblet cells and a compromised mucus barrier, mice fed the CD diet had greater colonic damage and enhanced production of pro-inflammatory cytokines in response to *C. rodentium* infection relative to mice fed adequte levels of dietary choline. It is also conceivable that an inability to produce and maintain PC concentrations of colon mucus due to insufficient dietary choline further compromises the integrity of the mucus layer. Taken together, our data suggest that dietary choline is important for maintaining goblet cell mucus granule abundance in the setting of *C. rodentium* infection.

Exacerbated colits severity in mice fed a CD diet was linked to impaired ability of the host to control *C. rodentium* proliferation, as indicated by higher fecal *C. rodentium* abundance at seven days post infection compared to mice fed the CS or CE diets. An inability to control *C. rodentium* proliferation suggests that immune function is impaired in mice fed a CD diet. Previous studies have shown that feeding pregnant rats a CD diet impaired immune function in dams (Dellschaft et al., 2015) and lymphocyte development in pups (Lewis et al., 2016). Our data suggest that a CD diet impairs intestinal immune homeostasis and allows uncontrolled proliferation of *C. rodentium*. Furthermore, *in vitro* experiments show that the PC content of membranes in intestinal epithelial cells influences the ability of the cell to block pro-inflammatory signaling cascades (Treede et al., 2007). Consistent with an inability to control inflammatory signaling and high levels of luminal *C. rodentium* in mice fed the CD diet, there was enhanced production of pro-inflammatory cytokines/chemokines in the colon of mice from the CD group. Elevated colonic levels of Eotaxin-1, MIP-2, and MCP-1 have been reported previously in mice infected with *C. rodentium* and represent increased immune cell recruitment (including neutrophils) to the injured colon mucosa (Khan et al., 2006, Coburn et al., 2013). Therefore, in addition to impairing the ability of mice to control *C. rodentium* infection, a CD diet exacerbates inflammatory signaling and colonic damage relative to diet containing adequate levels of choline.

Evidence from germ-free mice and antibiotic-treated mice suggests that gut commensal microbes influence the integrity of the gut mucus barrier (Wlodarska et al., 2011, Johansson et al., 2015). Dietary choline did not impact the structure or the diversity of the microbial communities in the ileum, caecum or colon, suggesting that changes to microbial structure are likely not a primary driver of enhanced colonic inflammation in mice fed the CD diet. However, by comparing microbial profiles across the three dietary groups, we identified certain bacterial taxa that

responded to dietary choline levels. The genera *Allobaculum* and *Turicibacter* (both belonging to the class of Erysipelotrichia) were significantly increased in response to a CD diet. The abundance of Erysipelotrichia has been linked to risk of fatty liver development with a choline-depleted diet in humans, suggesting that Erysipelotrichia is a biomarker for choline deficiency in both mice and humans (Spencer et al., 2011). In addition, the genus of *Oscillospira* was reduced in the colonic contents of mice fed the CD diet compared to mice fed the CS or CE diets. Interestingly, it has been shown that *Oscillospira* is a butyrate producer that is suppressed in the setting of inflammatory disease, consistent with its decrease in mice fed the CD diet relative to mice fed the CS or CE diets (Konikoff and Gophna, 2016, Gophna et al., 2017). These results suggest that choline availability influences the niche for specific gut microbes and these gut microbes might subsequently influence the gut mucosal homeostasis.

5.5 Conclusions

In conclusion, this study shows that mice fed a CD diet have exacerbated severity of *C. rodentium*-induced colitis compared to mice fed a CS or CE diet. In addition, a CD diet induces changes to speific microbial species in the gut without impacting overall microbial structure. Our results suggests that dietary choline supply is a major determinent of gut health in the setting of C. rodentium-induced inflammtion.

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CHAPTER 6: Overall discussion

6.1 Overall discussion

Mammals store most of their metabolic energy as hydrocarbon-rich TGs in adipose tissue (Frühbeck et al., 2001). Since the intestine absorbs and distributes much of this metabolic fuel, understanding the mechanisms involved in dietary lipid uptake will provide important insight into how mammals regulate whole-body energy balance. A major aim of this thesis was to determine the precise role of intestinal *de novo* PC synthesis in dietary lipid absorption and chylomicron secretion. In addition to defining a critical role for intestinal CTα activity in fatty acid and cholesterol uptake, our experiments revealed an important role for dietary choline supply and intestinal *de novo* PC synthesis in protecting against intestinal inflammation. Taken together, the findings presented in this thesis show that intestinal *de novo* PC synthesis is important for the maintenance of intestinal metabolic function and mucosal integrity.

Chapter 3 identifies CTα as a key enzyme involved in intestinal lipid uptake and shows that, despite large amounts of PC arriving in the intestine in bile (~20mg/day or the equivalent of the liver's entire PC content (Kuipers et al., 1997)), mammals have a specific requirement for 'new' intestinally-formed PC. Our initial hypothesis was that loss of CTα would cause TG accumulation in enterocytes due to insufficient supply of PC for chylomicron formation. This hypothesis was based on evidence that loss of *de novo* PC synthesis in the liver causes TG accumulation in hepatocytes, at least partly due to impaired VLDL secretion (Jacobs et al., 2004). Unexpectedly, when fed a chow diet, $CT\alpha^{IKO}$ mice have normal fat absorption, suggesting that biliary PC can fully support chylomicron secretion under these conditions. However, when acutely fed a HFD, $CT\alpha^{IKO}$ mice have impaired uptake of fatty acids from the intestinal lumen into enterocytes. Interestingly, it has recently been reported that knockdown of CTα in Caco2 cells, an

intestinal epithelial cell line, causes intracellular TG accumulation due to impaired TG secretion (Lee and Ridgway, 2018), as was initially predicted in CTα^{IKO} mice. A potential reason for this difference between models is that CTα-deficient Caco2 cells have detectable CTβ protein levels which might compensate for loss of CTα, while minimal CT activity was detected in the intestines of CTα^{IKO} mice. Alternatively, the difference in TG accumulation in the intestines of CTα^{IKO} mice and CTα-deficient Caco2 cells could indicate that intestinal epithelial cell-extrinsic factors (e.g. delayed gastric emptying) influence fatty acid uptake from the intestinal lumen into enterocytes in CTα^{IKO} mice. To address whether delayed gastric emptying contributes to the lower postprandial plasma TG concentrations, we recently measured solid phase gastric emptying in control and CTα^{IKO} mice using a standard protocol (Barrachina et al., 1997, Maida et al., 2008). Briefly, individually housed mice were fasted overnight before being fed a pre-weighed food 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. Surprisingly, the rate of gastric emptying was 3 times higher in CTα^{IKO} mice compared to control mice. Furthermore, higher lipid concentrations in the feces of CTα^{IKO} mice compared to controls suggests that impaired lipid absorption is the primary reason for low postprandial TG concentrations in CTα^{IKO} mice. Additionally, the observed amplification of GLP-1 and PYY secretion in CTα^{IKO} mice after a meal suggests that higher concentrations of fatty acids are reaching the distal intestine due to malabsorption in the proximal intestine. Therefore, lower postprandial plasma TG concentrations observed in CTα^{IKO} mice is not due to delayed gastric emptying. Instead, our data suggest that impaired fat absorption in CTα^{IKO} mice is mechanistically linked to the repression of genes involved in lipid uptake, including *Cd36* and *Npc1l1*. While germline deletion of *Cd36* in mice does to impair uptake of fatty acids from the intestinal lumen

into enterocytes (Drover et al., 2005), it is conceivable that widespread repression of a program of genes linked to lipid absorption (as observed in CTα^{IKO} mice by microarray analysis), impairs fat uptake. Nevertheless, other intestinal epithelial cell-extrinsic factors, including imbalances to gut water and ion transport (Tsai et al., 2017) large changes to circulating hormone concentrations (e.g. GLP-1 and PYY; Chapter 3), changes to bile acid metabolism (Chapter 3), altered food intake patterns, and gut microbes could influence fat absorption in CTα^{IKO} mice and warrant future research. To exclude the effects of intestinal epithelial cell-extrinsic factors on fat absorption in CTα^{IKO} mice, future studies should aim to isolate primary enterocytes from control mice and CTα^{IKO} mice to compare their rates of radiolabeled fatty acids and cholesterol uptake.

In Chapter 3 we report an unexpected role for intestinal *de novo* PC synthesis in maintaining normal enterohepatic circulation of bile acids and the spatial expression of metabolic genes in the small intestine. We undertook extensive experiments to gain insight into the reason for enhanced bile flow in CTα^{IKO} mice relative to controls: our analysis of hepatic cholesterol uptake and secretion, the analysis of fecal bile output, and measurement of expression of bile acid-synthetic genes showed that the mechanism promoting enterohepatic bile cycling in CTα^{IKO} mice is not increased hepatic bile acid synthesis. Instead, our data suggest that a shift in expression of bile acid transporters and bile acid-regulated genes towards more proximal parts of the small intestine is the mechanism mediating crosstalk between the intestine and liver in CTα^{IKO} mice. A change in the spatial expression of bile-acid responsive genes to the proximal gut has been reported previously in mice with intestine-specific deletion of a transcription factor that controls jejunal-ileal identity, GATA4 (Bosse et al., 2006, Battle et al., 2008). The shift in expression of bile acid-responsive genes to the proximal intestine of GATA4-deficient mice is also linked to enhanced bile flow (Beuling et al., 2010, Out et al., 2015) and impaired lipid uptake (Battle et al., 2008).

Interestingly, it has recently been shown that microbes influence the expression of GATA4 and its target genes in the intestine (Out et al., 2015) and so it will be interesting in future to determine whether enhanced microbial interaction with the intestinal epithelium influences bile acid metabolism in CTα^{IKO} mice. Consistent with a link between CTα and maintenance of normal intestinal gene expression patterns, chapter 4 shows that the colons of CTα^{IKO} mice have transcriptional repression of the goblet cell maturation program, which is associated with lower abundance of mature goblet cells compared to control mice. Furthermore, microarray analysis showed that PPARα-responsive genes are repressed in the intestines of CTα^{IKO} mice, and that SREBP-1c-responsive genes are activated. These findings are consistent with evidence suggesting that endogenous PC synthesis regulates PPARα and SREBP-1c in mouse liver (Chakravarthy et al., 2009, Walker et al., 2011). The changes to intestinal gene expression patterns in CTα^{IKO} mice (including the repression of PPARα and HNF4α and the induction of transcripts linked to the intestinal defense response) are highly comparable to those observed in germ-free mice colonized with microbes for the first time (Camp et al., 2014, Davison et al., 2017, El Aidy et al., 2013). Therefore, it should be investigated in future whether increased microbial interaction with epithelial cells accounts for some of the changes to gene expression observed in the intestines of CTα^{IKO} mice.

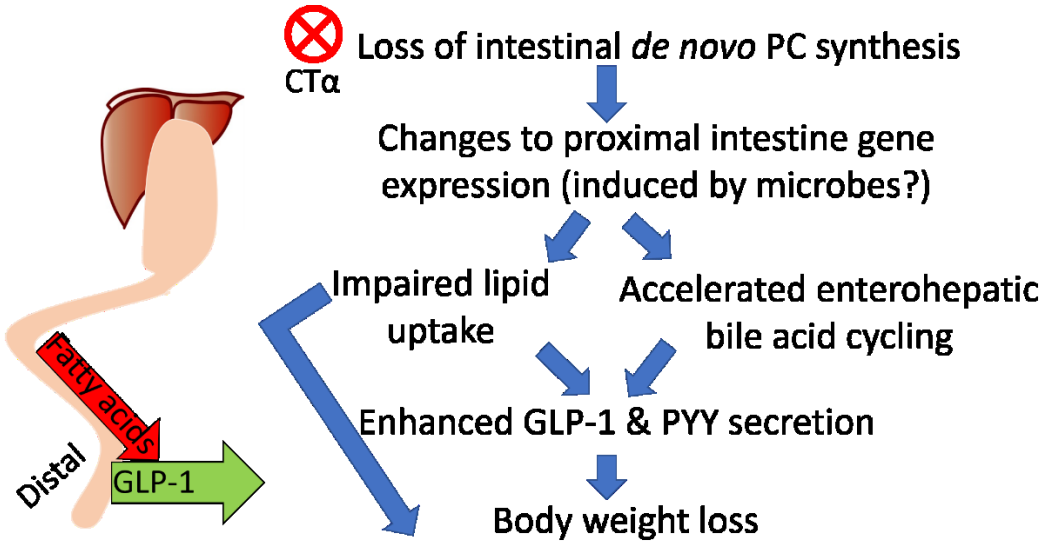

Figure 6.1 Consequences of loss of *de novo* PC synthesis in the small intestinal epithelium. Loss of CTα in the small intestinal epithelium induces changes to proximal intestine gene expression profiles, including induction of transcripts linked to bile acid metabolism and repression of transcripts linked to lipid uptake. Invasion of the intestinal epithelium by microbes might contribute to changes to intestinal gene expression. These changes to proximal intestine gene expression are associated with impaired lipid absorption, accelerated enterohepatic bile acid cycling, enhanced enteroendocrine hormone secretion, and body weight loss.

In chapter 4, we used gene expression profiling to establish a link between CTα and intestinal inflammation. IBD is driven by a shift from intestinal immune homeostasis to a state of uncontrolled cytokine and chemokine production (Khor et al., 2011). Evidence suggests that immune system activation leading to IBD occurs due to: (1) breakdown of the mucosal barrier, (2) increase exposure of the intestinal immune system to luminal antigens, and (3) an unrestrained immune response (Khor et al., 2011). We report that *de novo* PC synthesis is an important factor that mediates the interaction between the intestinal epithelium and gut microbes. Like $CT\alpha^{IKO}$ mice, 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). The similarity in response to loss of intestinal CTα and depletion of IgA, an established component of the mucosal barrier, suggests that intestinal PC synthesis plays a role in maintaining host-microbe commensalism. Identification of *de novo* PC synthesis as a factor that influences microbial interaction with the intestinal epithelium and underlying immune system can provide a basis for future research aimed at preventing or treating IBD and other diseases that involve host-microbe interactions. Indeed, clinical trials have shown beneficial effects of supplying exogenous PC to the gut mucosa of ulcerative colitis patients (Karner et al., 2014). Our data provide insight into some mechanisms by which PC might exert anti-inflammatory effects on the gut epithelium (e.g. by preventing excessive ER stress in intestinal epithelial cells), which could help to guide next-generation therapeutic strategies.

Future studies should aim to understand the contribution of microbial invasion of the intestinal epithelium to the phenotype of intestinal inflammation in $CT\alpha^{IKO}$ mice. While antibiotic treatment partially ameliorated inflammation in the colons of $CT\alpha^{IKO}$ mice, they still experienced extensive loss of goblet cells and higher abundance of several inflammatory markers relative to control mice. Antibiotics will reduce a subset of bacteria in the gut but will not eradicate other potential microbial contributors to intestinal inflammation including viruses. In order to fully understand the role of gut microbes in the intestines of $CT\alpha^{IKO}$ mice, germ-free mice lacking CTα in intestinal epithelial cells should be generated.

Does restoring PC concentrations in the ER of intestinal epithelial cells prevent or reverse inflammation and associated metabolic complications in $CT\alpha^{IKO}$ mice? Since $CT\alpha^{IKO}$ mice have impaired lipid uptake and small intestinal hyperproliferation despite enhanced delivery of PC to the small intestine in bile, it is unlikely that additional dietary PC (which would constitute a small amount of PC relative to the large and continuous supply of PC from bile) will reverse these

complications. However, providing CTα^{IKO} mice with injections of CDP-choline (the product of CTα), might allow sufficient endogenous PC production at the ER of intestinal epithelial cells to prevent complications. Under normal physiological conditions, PC metabolism is fundamentally different in the colon compared to the small intestine because the colon does not receive substantial amounts of PC in bile (likely making the colon more dependent on endogenous PC synthesis). Interestingly, the colons of CTα^{IKO} mice appeared more overtly damaged than their small intestines upon histological examination. Therefore, if supplementary PC could be delivered to the large intestine (for example in a pH-dependent delayed-release capsule), it might ameliorate some of the complications (e.g. colonic ER stress and loss of goblet cells) observed in CTα^{IKO} mice.

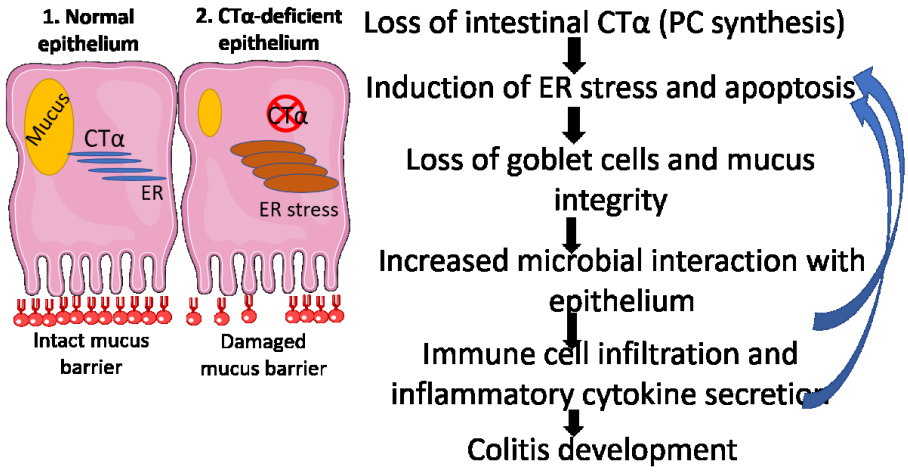

Figure 6.2. Consequences of loss of *de novo* PC synthesis in the colon. (1) In the normal colon epithelium, homeostatic systems exist to prevent excessive ER stress and there is a protective mucus barrier over the epithelium. (2) After loss of CTα in the colon epithelium, there is induction of ER stress and apoptosis which is associated with loss of goblet cells and the mucus barrier. Loss of mucus integrity allows microbes to invade the epithelium, causing enhanced inflammatory cytokine secretion. Enhanced inflammatory cytokine may further exacerbate ER stress and loss of goblet cells. These combined pathologies are linked to spontaneous colitis, suggesting that CTα is critical for colonic protection.

Inflammatory disorders of the gastrointestinal tract are frequently accompanied by metabolic perturbations including fat malabsorption (Tsai et al., 2017, Shulzhenko et al., 2011). A similar interplay between metabolic function and inflammation occurs in many tissues, including muscle and adipose tissue, where inflammatory cytokine secretion induced by nutrient excess can promote insulin resistance (Hotamisligil, 2017). Enteric infection of mice with *Citrobacter rodentium* has been shown to induce diarrhea by upregulation of Claudin-2 to promote water efflux and pathogen clearance (Tsai et al., 2017). Interestingly, microarray analysis revealed a significant increase in Claudin-2 mRNA in the intestines of $CT\alpha^{IKO}$ mice compared to control mice. Furthermore, rapid gastric emptying observed in $CT\alpha^{IKO}$ mice could be an attempt to accelerate pathogen clearance (Tsai et al., 2017). Therefore, it is conceivable that fat malabsorption observed in $CT\alpha^{IKO}$ mice is related to gut inflammation induced by loss of intestinal CTα. Furthermore, the presence of the microbiota and invasion of the epithelium by bacteria could be another potential reason for the difference between $CT\alpha^{IKO}$ mice and CTα-deficient Caco2 cells. It will be interesting in future to determine whether restoring gut immune homeostasis (potentially by restoring intestinal phospholipid homeostasis) in $CT\alpha^{IKO}$ mice or other models of intestinal immune dysfunction can normalize intestinal nutrient absorption and metabolic homeostasis.

One of the most striking phenotypic effects of loss of intestinal CTα is the massive amplification of GLP-1 and PYY secretion relative to controls after a high fat meal. Both GLP-1 and PYY can reduce food intake at high concentrations, as was observed in $CT\alpha^{IKO}$ mice (Cummings and Overduin, 2007). GLP-1 and PYY are released into portal circulation when fatty acids in the distal intestinal lumen interact with L cells (Hirasawa et al., 2005). Therefore, it is likely that impaired fatty acid uptake in $CT\alpha^{IKO}$ mice contributes to high plasma postprandial GLP-1 and PYY levels. Future work should determine whether enhanced GLP-1 receptor activation is

the primary driver of reduced food intake and body weight in CTα^{IKO} mice relative to controls. This could be achieved by treating CTα^{IKO} mice and control mice with the GLP-1 receptor antagonist exendin-9 before assessing food intake and body weight. There is also evidence that intestinal inflammation, bacterial products, and cytokines can act as physiological triggers for GLP-1 secretion (Ellingsgaard et al., 2011, Nguyen et al., 2014, Kahles et al., 2014, Lebrun et al., 2017). In chapter 4 we show that disruption of intestinal CTα promotes inflammatory cytokine secretion, increases bacterial interaction with the intestinal epithelium, and a colitis-like phenotype in mice. Therefore, both inflammatory and metabolic signals in the intestines of CTα^{IKO} mice could trigger supra-physiological GLP-1 secretion. Additionally, it will be important to determine whether other hormonal factors (e.g. GLP-2, CCK, somatostatin etc.) influence intestinal function in CTα^{IKO} mice.

Based on the results reported in chapter 4 which show that disruption of the CDP-choline pathway specifically in intestinal epithelial cells induces a colitis-like phenotype in mice, we hypothesized that insufficient dietary choline supply would predispose mice to exacerbated colitis severity. In chapter 5 we show that insufficient dietary choline aggravates the severity of *Citrobacter rodentium*-induced colitis in mice. Intestinal damage in mice fed the choline devoid diet was characterized by greater loss of goblet cells relative to mice fed sufficient dietary choline. These findings are consistent with observations from CTα^{IKO} mice, which have loss of goblet cell mucus granules. Taken together, chapter 4 and chapter 5 demonstrate an important role for choline metabolism in protecting against intestinal damage induced by luminal antigens. These findings provide a basis for future investigations into phospholipids as a potential therapeutic tool for IBD.

6.2 Conclusions

The research presented in this thesis shows that *de novo* PC synthesis plays a duel metabolic and anti-inflammatory role in the intestine. Loss of CTα in intestinal epithelial cells induces fatty acid and cholesterol malabsorption in the setting of a HFD, while leaving the intestinal epithelium susceptible to invasion by luminal microbes. A role for choline metabolism in intestinal barrier function was further supported by experiments showing that insufficient dietary choline increases susceptibility to *Citrobacter rodentium*-induced colitis in mice. An understanding of the molecular factors that maintain metabolic and immune function in the gut can provide a basis for future work aimed at preventing or treating intestinal disorders.

6.3 Future experiments

Our data support a working model in which loss of intestinal CTα leads to ER stress in intestinal epithelial cells due to lipid disequilibrium. This ER stress results in loss of goblet cells, which are especially susceptible to ER-stress driven apoptosis due to their high protein-folding and secretory demands (Heazlewood et al., 2008, Kaser et al., 2008). Furthermore, there is a failure to replace goblet cells lost to apoptosis due to transcriptional repression of the goblet cell maturation program. Loss of goblet cells allows microbes to access the epithelium, which induces inflammation and widespread changes to gene expression profiles. Some of the metabolic consequences resulting from breakdown of the mucosal barrier include fat malabsorption and body weight loss. Given this background, it will important in future to address the following research questions:

- What contribution does microbial interaction with the intestinal epithelium make to the phenotype of $CT\alpha^{IKO}$ mice? We hypothesize that eliminating microbes in $CT\alpha^{IKO}$ mice will ameliorate inflammatory cytokine secretion and alterations to intestinal gene expression

profiles. Furthermore, we hypothesize that fat malabsorption in CTα^{IKO} mice will be prevented in the absence of gut microbes. These hypotheses can be tested by generating germ-free CTα^{IKO} and control mice.

- How do CTα^{IKO} mice support enhanced intestinal epithelial cell proliferation in the absence of *de novo* PC synthesis? PC is thought to be a key factor in cell cycle progression (Cui et al., 1996, Cornell and Ridgway, 2015); however, CTα^{IKO} mice have enhanced proliferation in the intestinal epithelium. We hypothesize that this enhanced proliferation is supported by augmented uptake of PC from circulating lipoproteins and from bile. Alternatively, enhanced production of PE (Chapter 3) might be sufficient to support intestinal proliferation in CTα^{IKO} mice.
- Does PC supplementation reverse any of the metabolic or inflammatory complications observed in CTα^{IKO} mice? We hypothesize that delivery of PC to the distal intestine will reduce colitis severity in CTα^{IKO} mice.
- Is *de novo* PC synthesis required for normal transcription factor activity in the small intestine? PPARα-responsive genes are repressed in the intestines of CTα^{IKO} mice. Therefore, we hypothesize that a PPARα agonist will prevent some of the metabolic complications observed in CTα^{IKO} mice.
- Does resolution of ER stress reduce fat malabsorption in CTα^{IKO} mice? We hypothesize that PBA or tauroursodeoxycholate (TUDCA) will partially rescue fat malabsorption in CTα^{IKO} mice.
- Do gut hormones other than GLP-1 and PYY influence fat absorption, food intake and body weight loss in CTα^{IKO} mice?

- Do people with loss-of-function mutations in PCYT1A (Payne et al., 2014) have gastrointestinal abnormalities comparable to those observed in CTα^{IKO} mice?
- Does impaired production of PC-derived lipid metabolites (e.g. sphingomyelin) contribute to the phenotype of CTα^{IKO} mice**?** A recent study reported that impaired production of sphingomyelin induces inflammatory bowel disease in mice (Li et al., 2018), suggesting that impaired production of this lipid might be involved in the pathogenesis of inflammation after loss of intestinal CTα.

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Appendix 1 Gene symbol, description, fold change and ANOVA P Value for genes found to be changed in the small intestines of CTα^{IKO} mice relative to control mice by microarray

Gene Symbol	Description	Fold Change (linear) (Fasted KO vs. Fasted Control)	ANOVA p-value (Fasted KO vs. Fasted Control)
Igkv6-23	immunoglobulin kappa variable 6-23	8.29	0.00694
Igkv6-25	immunoglobulin kappa chain variable 6-25	8.22	0.04462
Igkv14-100	immunoglobulin kappa chain variable 14-100	5.97	0.04549
Ighv5-17; Ighg; Igh-V7183	immunoglobulin heavy variable 5-17; Immunoglobulin heavy chain (gamma polypeptide); immunoglobulin heavy chain (V7183 family)	4.83	0.03081
Scd2; Mir5114	stearoyl-Coenzyme A desaturase 2; microRNA 5114	4.64	0.00083
Ifi44	interferon-induced protein 44	3.94	0.00257
Igkv1-117	immunoglobulin kappa variable 1-117	3.9	0.03409
Igkv5-45; Igkv5-43	immunoglobulin kappa chain variable 5-45; immunoglobulin kappa chain variable 5-43	3.79	0.03736
Ighv6-3	immunoglobulin heavy variable 6-3	3.61	0.00634
Igk-V1; Igkv1-110; Igk-V5	immunoglobulin kappa chain variable 1 (V1); immunoglobulin kappa variable 1-110; immunoglobulin kappa chain variable 5 (V5 family)	3.47	0.03292
Igkv4-57	immunoglobulin kappa variable 4-57	3.25	0.03676
Mboat1	membrane bound O-acyltransferase domain containing 1	3.22	0.00881
Igkv4-61; Igkv4-68	immunoglobulin kappa chain variable 4-61; immunoglobulin kappa variable 4-68	3.22	0.02264
Igkv6-17	immunoglobulin kappa variable 6-17	3.21	0.04032
Igkv6-15; Igkv6-14	immunoglobulin kappa variable 6-15; immunoglobulin kappa variable 6-14	3.11	0.04972
Igkv14-111	immunoglobulin kappa variable 14-111	3.03	0.02698
Igkv4-78	immunoglobulin kappa variable 4-78	3.02	0.04096
Igkv6-20	immunoglobulin kappa variable 6-20	2.98	0.01978
Igkv4-73	immunoglobulin kappa variable 4-73	2.83	0.03746

Igkv4-70	immunoglobulin kappa chain variable 4-70	2.82	0.03215
Clca3b	chloride channel accessory 3B	2.78	0.00788
Igkv12-41	immunoglobulin kappa chain variable 12-41	2.77	0.03937
Il1rl1	interleukin 1 receptor-like 1	2.73	0.0028
Igkv4-68	immunoglobulin kappa variable 4-68	2.73	0.03286
Reg3g	regenerating islet-derived 3 gamma	2.72	0.03486
Ddah1	dimethylarginine dimethylaminohydrolase 1	2.71	0.04713
Oas1g	2-5 oligoadenylate synthetase 1G	2.67	0.00165
Igkv4-69	immunoglobulin kappa variable 4-69	2.66	0.0297
Bmp4	bone morphogenetic protein 4	2.65	0.01594
Ighv3-2	immunoglobulin heavy variable 3-2	2.64	0.02518
Ighv1-20	immunoglobulin heavy variable V1-20	2.5	0.03521
Aqp4	aquaporin 4	2.45	0.00202
Clca3a1; Clca1	chloride channel accessory 3A1; chloride channel accessory 1	2.42	0.00888
Ccna2	cyclin A2	2.4	0.0083
Asf1b	anti-silencing function 1B histone chaperone	2.37	0.00749
Igkv4-50	immunoglobulin kappa variable 4-50	2.37	0.04356
Ighv5-9-1	immunoglobulin heavy variable 5-9-1	2.36	0.02995
Ccnb1	cyclin B1	2.35	0.00258
Psat1	phosphoserine aminotransferase 1	2.35	0.00564
Ighv14-4	immunoglobulin heavy variable 14-4	2.35	0.00716
Bgn	biglycan	2.35	0.01489
Igkv17-121	immunoglobulin kappa variable 17-121	2.32	0.0291
Cldn2	claudin 2	2.3	0.00036
AI506816	expressed sequence AI506816	2.3	0.00308
Hist1h2ab	histone cluster 1, H2ab	2.29	0.04287
Hist1h1b	histone cluster 1, H1b	2.28	0.00982
Ighv5-6; Igh-V7183	immunoglobulin heavy variable 5-6; immunoglobulin heavy chain (V7183 family)	2.27	0.02672
Mmrn1	multimerin 1	2.24	0.00227

Igh-VJ558; Ighv14-3	immunoglobulin heavy chain (J558 family); immunoglobulin heavy variable V14-3	2.24	0.03766
Igkv12-38	immunoglobulin kappa chain variable 12-38	2.23	0.01653
Plk1	polo-like kinase 1	2.22	0.00693
Hist1h2ag	histone cluster 1, H2ag	2.22	0.02316
Nipal2	NIPA-like domain containing 2	2.21	0.00016
Ednrb	endothelin receptor type B	2.2	0.0103
Nuf2	NUF2, NDC80 kinetochore complex component, homolog (S. cerevisiae)	2.19	0.0018
Rrm1	ribonucleotide reductase M1	2.16	0.00668
Ube2c	ubiquitin-conjugating enzyme E2C	2.16	0.0104
Akr1b8	aldo-keto reductase family 1, member B8	2.15	0.00138
Scd1	stearoyl-Coenzyme A desaturase 1	2.15	0.00536
Dbf4	DBF4 homolog (S. cerevisiae)	2.14	0.00182
Gm13139	predicted gene 13139	2.13	0.00765
Ighv1-76	immunoglobulin heavy variable 1-76	2.13	0.04859
Gm5154	predicted gene 5154	2.12	0.00062
Pbk	PDZ binding kinase	2.11	0.0112
Hist2h2ac	histone cluster 2, H2ac	2.1	0.00835
Tubb5	tubulin, beta 5 class I	2.1	0.01187
Trip13	thyroid hormone receptor interactor 13	2.07	0.00875
Dmbt1	deleted in malignant brain tumors 1	2.07	0.04388
Bub1	budding uninhibited by benzimidazoles 1 homolog (S. cerevisiae)	2.06	0.00548
Mir1949	microRNA 1949	2.06	0.02379
Hmmr	hyaluronan mediated motility receptor (RHAMM)	2.04	0.00299
Pole	polymerase (DNA directed), epsilon	2.04	0.02759
Oas2	2-5 oligoadenylate synthetase 2	2.03	0.00373
Kif11	kinesin family member 11	2.01	0.00296
Melk	maternal embryonic leucine zipper kinase	2.01	0.00425
Ighv3-4; Ighv3-5	immunoglobulin heavy variable V3-4; immunoglobulin heavy variable 3-5	2.01	0.00643

Hist1h2bk	histone cluster 1, H2bk	2	0.00265
Aurka	aurora kinase A	2	0.00646
Mecom	MDS1 and EVI1 complex locus	2	0.00759
Mcm5	minichromosome maintenance deficient 5, cell division cycle 46 (S. cerevisiae)	2	0.03227
Shmt2	serine hydroxymethyltransferase 2 (mitochondrial)	1.99	0.00385
Ly6c2	lymphocyte antigen 6 complex, locus C2	1.99	0.00577
Snord93	small nucleolar RNA, C/D box 93	1.97	0.00016
Nrg1	neuregulin 1	1.97	0.00369
Kif20b	kinesin family member 20B	1.96	0.00164
Myef2	myelin basic protein expression factor 2, repressor	1.96	0.01255
Eng	endoglin	1.95	0.00978
Igkv4-79	immunoglobulin kappa variable 4-79	1.95	0.03491
Hist1h4i	histone cluster 1, H4i	1.95	0.04466
Fads1	fatty acid desaturase 1	1.95	0.05
Birc5	baculoviral IAP repeat-containing 5	1.94	0.01222
Ly6a	lymphocyte antigen 6 complex, locus A	1.94	0.01634
Prmt1	protein arginine N-methyltransferase 1	1.94	0.0199
Ndc1	NDC1 transmembrane nucleoporin	1.92	0.01232
Cdk1	cyclin-dependent kinase 1	1.92	0.03241
Kif15	kinesin family member 15	1.9	0.00069
Hist1h2af	histone cluster 1, H2af	1.9	0.04056
Tpx2	TPX2, microtubule-associated protein homolog (Xenopus laevis)	1.89	0.0019
Nmur1	neuromedin U receptor 1	1.89	0.02628
Reg3b	regenerating islet-derived 3 beta	1.89	0.03618
Rrm2	ribonucleotide reductase M2	1.88	0.01003
Gm16494	predicted gene 16494 [Source:MGI Symbol;Acc:MGI:3641930]	1.88	0.02243
Nup43	nucleoporin 43	1.87	0.00039
Shcbp1	Shc SH2-domain binding protein 1	1.87	0.00582
Dna2	DNA replication helicase 2 homolog (yeast)	1.87	0.01522
Nsl1	NSL1, MIND kinetochore complex component, homolog (S. cerevisiae)	1.87	0.01977

Col3a1	collagen, type III, alpha 1	1.87	0.02412
Ttk	Ttk protein kinase	1.86	0.00641
Zwilch	zwilch kinetochore protein	1.86	0.00669
Kif20a	kinesin family member 20A	1.86	0.00885
Prc1	protein regulator of cytokinesis 1	1.85	0.00183
Tacc3	transforming, acidic coiled-coil containing protein 3	1.85	0.00549
Pcsk9	proprotein convertase subtilisin/kexin type 9	1.85	0.0057
Adamdec1	ADAM-like, decysin 1	1.85	0.02338
Duox2	dual oxidase 2	1.85	0.04602
Htr4	5 hydroxytryptamine (serotonin) receptor 4	1.84	0.0097
Racgap1	Rac GTPase-activating protein 1	1.84	0.01575
Tmem176a	transmembrane protein 176A	1.84	0.02075
Aurkb	aurora kinase B	1.84	0.03151
Nop56	NOP56 ribonucleoprotein	1.84	0.0411
Ccnb2	cyclin B2	1.84	0.04442
Utp14a	UTP14, U3 small nucleolar ribonucleoprotein, homolog A (yeast)	1.83	0.00504
Ncapg	non-SMC condensin I complex, subunit G	1.83	0.00783
Ska3	spindle and kinetochore associated complex subunit 3	1.83	0.00897
Fads3	fatty acid desaturase 3	1.83	0.01665
Brca1	breast cancer 1, early onset	1.83	0.02111
Ipo5	importin 5	1.83	0.0226
n-R5s85	nuclear encoded rRNA 5S 85 [Source:MGI Symbol;Acc:MGI:4421933]	1.82	0.00373
Ticrr	TOPBP1-interacting checkpoint and replication regulator	1.82	0.0108
Fam57a	family with sequence similarity 57, member A	1.82	0.01899
Tspan1	tetraspanin 1	1.82	0.02144
Ccl9	chemokine (C-C motif) ligand 9	1.82	0.02343
Chaf1b	chromatin assembly factor 1, subunit B (p60)	1.82	0.03847
Pik3r3	phosphatidylinositol 3 kinase, regulatory subunit, polypeptide 3 (p55)	1.81	0.00228
Gm5593	predicted gene 5593	1.81	0.00346
Kif14	kinesin family member 14	1.81	0.00458
Mad2l1	MAD2 mitotic arrest deficient-like 1	1.81	0.00473

Nusap1	nucleolar and spindle associated protein 1	1.81	0.01615
Taf1d	TATA box binding protein (Tbp)-associated factor, RNA polymerase I, D	1.8	0.00173
Iqgap3	IQ motif containing GTPase activating protein 3	1.8	0.00533
1810065E05Rik	RIKEN cDNA 1810065E05 gene	1.8	0.00786
Ighv5-9	immunoglobulin heavy variable 5-9	1.8	0.013
Lipg	lipase, endothelial	1.8	0.01317
Rad18	RAD18 E3 ubiquitin protein ligase	1.79	0.00093
Fdps	farnesyl diphosphate synthetase	1.79	0.00147
Wdr75	WD repeat domain 75	1.79	0.00365
LOC105244345; Gm14277	small nuclear ribonucleoprotein Sm D1-like; predicted gene 14277 [Source:MGI Symbol;Acc:MGI:3650350]	1.79	0.00436
Parpbp	PARP1 binding protein	1.79	0.0057
Tfrc	transferrin receptor	1.79	0.01122
Dtl	denticleless homolog (Drosophila)	1.79	0.01929
Hist2h2ab	histone cluster 2, H2ab	1.79	0.03485
Snora2b	small nucleolar RNA, H/ACA box 2B	1.78	0.00491
Ncaph	non-SMC condensin I complex, subunit H	1.78	0.01822
Kif2c	kinesin family member 2C	1.78	0.02396
Mybbp1a	MYB binding protein (P160) 1a	1.78	0.0277
Slc7a5	solute carrier family 7 (cationic amino acid transporter, y+ system), member 5	1.78	0.04043
Kif23	kinesin family member 23	1.77	0.00527
Anln	anillin, actin binding protein	1.77	0.01793
Snora20	small nucleolar RNA, H/ACA box 20	1.77	0.03315
Top2a	topoisomerase (DNA) II alpha	1.77	0.03595
Cd44	CD44 antigen	1.76	0.00143
Rpf2	ribosome production factor 2 homolog (S. cerevisiae)	1.76	0.00441
Oasl2	2-5 oligoadenylate synthetase-like 2	1.76	0.0046
Ripk3	receptor-interacting serine-threonine kinase 3	1.76	0.00471
Cenpu	centromere protein U	1.76	0.00641
Mcm3	minichromosome maintenance deficient 3 (S. cerevisiae)	1.76	0.00729
Bcap29	B cell receptor associated protein 29	1.76	0.01559

Rad54l	RAD54 like (S. cerevisiae)	1.76	0.01845
Npm1	nucleophosmin 1	1.76	0.01963
Hhip	Hedgehog-interacting protein	1.76	0.03553
Ccnd1	cyclin D1	1.76	0.03987
Nudcd2	NudC domain containing 2	1.75	0.00164
Sgol1	shugoshin-like 1 (S. pombe)	1.75	0.00492
Hist1h2bf	histone cluster 1, H2bf	1.75	0.00622
Rad51	RAD51 homolog	1.75	0.0068
Pla2g4a	phospholipase A2, group IVA (cytosolic, calcium-dependent)	1.75	0.00932
Foxm1	forkhead box M1	1.75	0.01706
Mmp10	matrix metallopeptidase 10	1.75	0.02833
Cenpe	centromere protein E	1.74	0.00266
Trim37	tripartite motif-containing 37	1.74	0.00397
Slc7a1	solute carrier family 7 (cationic amino acid transporter, y+ system), member 1	1.74	0.00813
Rfc4	replication factor C (activator 1) 4	1.74	0.02065
Gtpbp4	GTP binding protein 4	1.73	0.00093
Lamb1	laminin B1	1.73	0.01492
Fignl1	fidgetin-like 1	1.73	0.0269
Mcm4	minichromosome maintenance deficient 4 homolog (S. cerevisiae)	1.73	0.02958
Cenpp	centromere protein P	1.72	0.00922
C1qbp	complement component 1, q subcomponent binding protein	1.72	0.01357
Gm5611	predicted gene 5611 [Source:MGI Symbol;Acc:MGI:3647121]	1.72	0.01958
Car12	carbonic anyhydrase 12	1.72	0.0347
Gm1987; Ccl21a	predicted gene 1987; chemokine (C-C motif) ligand 21A (serine)	1.72	0.03969
Mastl	microtubule associated serine/threonine kinase-like	1.71	0.00261
D17H6S56E-5	DNA segment, Chr 17, human D6S56E 5	1.71	0.0044
Cep55	centrosomal protein 55	1.71	0.00974
Exosc1	exosome component 1	1.71	0.02501
Igkj1; Igk-V28	immunoglobulin kappa joining 1; immunoglobulin kappa chain variable 28 (V28)	1.71	0.03002
H2afx	H2A histone family, member X	1.71	0.03183
Gm17430	predicted gene, 17430 [Source:MGI Symbol;Acc:MGI:4937064]	1.71	0.03611

Smc2	structural maintenance of chromosomes 2	1.7	0.00096
Cenpi	centromere protein I	1.7	0.00363
Kif22	kinesin family member 22	1.7	0.00405
Hells	helicase, lymphoid specific	1.7	0.00639
Isg15	ISG15 ubiquitin-like modifier	1.7	0.03111
Fbn1	fibrillin 1	1.7	0.04032
Mthfd2	methylenetetrahydrofolate dehydrogenase (NAD+ dependent), methenyltetrahydrofolate cyclohydrolase	1.7	0.04545
Ddx60	DEAD (Asp-Glu-Ala-Asp) box polypeptide 60	1.7	0.04936
Mif	macrophage migration inhibitory factor	1.69	0.00869
Pno1	partner of NOB1 homolog (S. cerevisiae)	1.69	0.0087
Ddx21	DEAD (Asp-Glu-Ala-Asp) box polypeptide 21	1.69	0.01836
Cdca5	cell division cycle associated 5	1.69	0.0218
Gnl3	guanine nucleotide binding protein-like 3 (nucleolar)	1.69	0.02379
Ecm1	extracellular matrix protein 1	1.69	0.02652
C330027C09Rik	RIKEN cDNA C330027C09 gene	1.68	0.00441
Dlgap5	discs, large (Drosophila) homolog-associated protein 5	1.68	0.00447
Lyar	Ly1 antibody reactive clone	1.68	0.00999
Bub1b	budding uninhibited by benzimidazoles 1 homolog, beta (S. cerevisiae)	1.68	0.0121
Cdkn3	cyclin-dependent kinase inhibitor 3	1.68	0.01394
Mis18bp1	MIS18 binding protein 1	1.68	0.02639
Ddx20	DEAD (Asp-Glu-Ala-Asp) box polypeptide 20	1.67	0.00368
Timm9	translocase of inner mitochondrial membrane 9	1.67	0.00636
Cdca8	cell division cycle associated 8	1.67	0.00934
Mki67	antigen identified by monoclonal antibody Ki 67	1.67	0.01584
Gm5263	predicted gene 5263 [Source:MGI Symbol;Acc:MGI:3647343]	1.66	0.00018
Kif4	kinesin family member 4	1.66	0.00164
Steap2	six transmembrane epithelial antigen of prostate 2	1.66	0.00418
Mcm7	minichromosome maintenance deficient 7 (S. cerevisiae)	1.66	0.00445
Blm	Bloom syndrome, RecQ helicase-like	1.66	0.00714
Gm17783	predicted gene, 17783	1.66	0.0295

Lrrc32	leucine rich repeat containing 32	1.66	0.03335
Incenp	inner centromere protein	1.65	0.00171
Cks2	CDC28 protein kinase regulatory subunit 2	1.65	0.00565
Gm12891	predicted gene 12891 [Source:MGI Symbol;Acc:MGI:3649646]	1.65	0.00565
Cks2	CDC28 protein kinase regulatory subunit 2	1.65	0.00565
Mmp2	matrix metallopeptidase 2	1.65	0.00925
Rbmxl1	RNA binding motif protein, X-linked like-1	1.65	0.00943
E2f2	E2F transcription factor 2	1.65	0.01257
Slc16a12	solute carrier family 16 (monocarboxylic acid transporters), member 12	1.65	0.01291
Fabp4	fatty acid binding protein 4, adipocyte	1.65	0.02033
Kif18a	kinesin family member 18A	1.65	0.0254
Sox4	SRY (sex determining region Y)-box 4	1.65	0.02963
Agpat4	1-acylglycerol-3-phosphate O-acyltransferase 4 (lysophosphatidic acid acyltransferase, delta)	1.65	0.03164
Ndc80	NDC80 homolog, kinetochore complex component (S. cerevisiae)	1.64	0.00201
Ammecr1	Alport syndrome, mental retardation, midface hypoplasia and elliptocytosis chromosomal region gene 1	1.64	0.0041
Nol8	nucleolar protein 8	1.64	0.02091
Plvap	plasmalemma vesicle associated protein	1.64	0.02107
C1qb	complement component 1, q subcomponent, beta polypeptide	1.64	0.03379
Ect2	ect2 oncogene	1.63	0.00312
Wdr55	WD repeat domain 55	1.63	0.00503
Isyna1	myo-inositol 1-phosphate synthase A1	1.63	0.01602
Prim2	DNA primase, p58 subunit	1.63	0.02169
Pla2g5	phospholipase A2, group V	1.63	0.02392
Uhrf1	ubiquitin-like, containing PHD and RING finger domains, 1	1.63	0.02526
Ran	RAN, member RAS oncogene family	1.63	0.02584
Nifk	nucleolar protein interacting with the FHA domain of MKI67	1.63	0.03304
Trav16d-dv11	T cell receptor alpha variable 16D-DV11	1.63	0.04691
Atr	ataxia telangiectasia and Rad3 related	1.62	0.0155
Snhg6	small nucleolar RNA host gene 6	1.62	0.02368

Dkc1	dyskeratosis congenita 1, dyskerin	1.62	0.02837
Gart	phosphoribosylglycinamide formyltransferase	1.62	0.03606
Gm9312	predicted gene 9312 [Source:MGI Symbol;Acc:MGI:3647775]	1.61	0.00261
Chek1	checkpoint kinase 1	1.61	0.00357
Suv39h2	suppressor of variegation 3-9 homolog 2 (Drosophila)	1.61	0.00376
Haus6	HAUS augmin-like complex, subunit 6	1.61	0.00426
Gar1	GAR1 ribonucleoprotein homolog (yeast)	1.61	0.0077
Casc5	cancer susceptibility candidate 5	1.61	0.0083
Dtymk	deoxythymidylate kinase	1.61	0.01517
Cdc25c	cell division cycle 25C	1.61	0.02318
Shmt1	serine hydroxymethyltransferase 1 (soluble)	1.61	0.02443
Slitrk6	SLIT and NTRK-like family, member 6	1.61	0.03379
Gm22973	predicted gene, 22973 [Source:MGI Symbol;Acc:MGI:5452750]	1.61	0.04905
Rcc2	regulator of chromosome condensation 2	1.61	0.04937
Gusb	glucuronidase, beta	1.6	0.00044
Gm9797	predicted pseudogene 9797 [Source:MGI Symbol;Acc:MGI:3704349]	1.6	0.00791
Rbbp7	retinoblastoma binding protein 7	1.6	0.0088
Ccdc34	coiled-coil domain containing 34	1.6	0.01121
Exo1	exonuclease 1	1.6	0.0163
Dnmt1	DNA methyltransferase (cytosine-5) 1	1.6	0.0193
Diaph3	diaphanous related formin 3	1.6	0.02866
Mcm6	minichromosome maintenance deficient 6 (MIS5 homolog, S. pombe) (S. cerevisiae)	1.6	0.03772
Fads2	fatty acid desaturase 2	1.6	0.04321
Fcgr4	Fc receptor, IgG, low affinity IV	1.6	0.04648
Rad51b	RAD51 homolog B	1.59	0.00016
Arhgap11a	Rho GTPase activating protein 11A	1.59	0.00243
Hpse2	heparanase 2	1.59	0.00273
Igkv1-35	immunoglobulin kappa variable 1-35	1.59	0.0028
2810417H13Rik	RIKEN cDNA 2810417H13 gene	1.59	0.00907
Gm24588	predicted gene, 24588 [Source:MGI Symbol;Acc:MGI:5454365]	1.59	0.02402

Havcr2	hepatitis A virus cellular receptor 2	1.59	0.02805
Cyp51	cytochrome P450, family 51	1.59	0.02813
1700025G04Rik	RIKEN cDNA 1700025G04 gene	1.59	0.02817
I830077J02Rik	RIKEN cDNA I830077J02 gene	1.59	0.04682
Abcc4	ATP-binding cassette, sub-family C (CFTR/MRP), member 4	1.58	5.1E-05
Nr2c2ap	nuclear receptor 2C2-associated protein	1.58	0.00508
Oas1a	2-5 oligoadenylate synthetase 1A	1.58	0.00752
Gtse1	G two S phase expressed protein 1	1.58	0.00787
Suv39h1	suppressor of variegation 3-9 homolog 1 (Drosophila)	1.58	0.00956
Atad2	ATPase family, AAA domain containing 2	1.58	0.01389
Zgrf1	zinc finger, GRF-type containing 1	1.58	0.01391
Alg8	asparagine-linked glycosylation 8 (alpha-1,3-glucosyltransferase)	1.58	0.01464
Nup133	nucleoporin 133	1.58	0.01637
Apobec3	apolipoprotein B mRNA editing enzyme, catalytic polypeptide 3	1.58	0.01997
Wdr12	WD repeat domain 12	1.58	0.02421
Col6a3	collagen, type VI, alpha 3	1.58	0.02443
Ncapg2	non-SMC condensin II complex, subunit G2	1.58	0.03011
Anp32b-ps1	Bacidic (leucine-rich) nuclear phosphoprotein 32 family, member B, pseudogene 1	1.58	0.03279
Cdc20	cell division cycle 20	1.58	0.04007
Tipin	timeless interacting protein	1.58	0.04429
Cd4	CD4 antigen	1.58	0.04727
Atic	5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase	1.58	0.04924
Ccnjl	cyclin J-like	1.57	0.00306
Hist4h4	histone cluster 4, H4	1.57	0.01199
2610318N02Rik	RIKEN cDNA 2610318N02 gene	1.57	0.02346
Rsl1d1	ribosomal L1 domain containing 1	1.57	0.02719
LOC105243690; Gm14681	nucleophosmin-like; predicted gene 14681 [Source:MGI Symbol;Acc:MGI:3705734]	1.57	0.03045
Phlda2	pleckstrin homology-like domain, family A, member 2	1.57	0.03265
5730507C01Rik	RIKEN cDNA 5730507C01 gene	1.57	0.03344

Dis3	DIS3 mitotic control homolog (S. cerevisiae)	1.57	0.03764
Lbr	lamin B receptor	1.57	0.04003
Rtp4	receptor transporter protein 4	1.57	0.04248
Col14a1	collagen, type XIV, alpha 1	1.57	0.04974
Pdia5	protein disulfide isomerase associated 5	1.56	0.00146
Nupl2	nucleoporin like 2	1.56	0.00579
Oit1	oncoprotein induced transcript 1	1.56	0.00737
Oxct1	3-oxoacid CoA transferase 1	1.56	0.01617
Kif18b	kinesin family member 18B	1.56	0.01676
Gm6736	predicted gene 6736 [Source:MGI Symbol;Acc:MGI:3643048]	1.56	0.02079
Lsm2	LSM2 homolog, U6 small nuclear RNA associated (S. cerevisiae)	1.56	0.02098
Snord52	small nucleolar RNA, C/D box 52	1.56	0.02129
Acot1	acyl-CoA thioesterase 1	1.56	0.02169
Eif4ebp1	eukaryotic translation initiation factor 4E binding protein 1	1.56	0.0246
Gm25432	predicted gene, 25432	1.56	0.02964
Mb21d1	Mab-21 domain containing 1	1.56	0.03185
Hmga1-rs1	high mobility group AT-hook I, related sequence 1	1.56	0.03236
Plk4	polo-like kinase 4	1.56	0.03297
E2f8	E2F transcription factor 8	1.56	0.0354
Mrto4	mRNA turnover 4, homolog (S. cerevisiae)	1.56	0.03544
Nutf2-ps1; Nutf2-ps2	nuclear transport factor 2, pseudogene 1; nuclear transport factor 2, pseudogene 2	1.56	0.03609
Asns	asparagine synthetase	1.56	0.03761
Nop58	NOP58 ribonucleoprotein	1.56	0.0397
Mcm2	minichromosome maintenance deficient 2 mitotin (S. cerevisiae)	1.55	0.00211
1500012F01Rik	RIKEN cDNA 1500012F01 gene	1.55	0.00422
Fanca	Fanconi anemia, complementation group A	1.55	0.00719
Nasp	nuclear autoantigenic sperm protein (histone-binding)	1.55	0.00978
Cdh5	cadherin 5	1.55	0.01322
Stil	Scl/Tal1 interrupting locus	1.55	0.01837
Igkv8-30	immunoglobulin kappa chain variable 8-30	1.55	0.04747

Nudcd1	NudC domain containing 1	1.54	0.00149
Gm10384	predicted gene 10384	1.54	0.00498
Tmem209	transmembrane protein 209	1.54	0.00516
Erh	enhancer of rudimentary homolog (Drosophila)	1.54	0.00571
Lsm5	LSM5 homolog, U6 small nuclear RNA associated (S. cerevisiae)	1.54	0.00692
Cad	carbamoyl-phosphate synthetase 2, aspartate transcarbamylase, and dihydroorotase	1.54	0.00826
Ola1	Obg-like ATPase 1	1.54	0.00867
Utp15	UTP15, U3 small nucleolar ribonucleoprotein, homolog (yeast)	1.54	0.01296
Plpp3	phospholipid phosphatase 3	1.54	0.01777
Ddx18	DEAD (Asp-Glu-Ala-Asp) box polypeptide 18	1.54	0.02087
Cdk4	cyclin-dependent kinase 4	1.54	0.0317
9930014A18Rik	RIKEN cDNA 9930014A18 gene	1.54	0.03178
Lmnb1	lamin B1	1.54	0.03535
Dpy19l1	dpy-19-like 1 (C. elegans)	1.53	0.00048
Cep192	centrosomal protein 192	1.53	0.00286
Haus7	HAUS augmin-like complex, subunit 7	1.53	0.00426
BC055324	cDNA sequence BC055324	1.53	0.0045
Arntl	aryl hydrocarbon receptor nuclear translocator-like	1.53	0.00678
Tdp1	tyrosyl-DNA phosphodiesterase 1	1.53	0.01152
Ftsj3	FtsJ homolog 3 (E. coli)	1.53	0.01201
Nde1	nuclear distribution gene E homolog 1 (A nidulans)	1.53	0.01268
Chek2	checkpoint kinase 2	1.53	0.01668
Exosc8	exosome component 8	1.53	0.01687
Olfr917	olfactory receptor 917	1.53	0.01722
Fkbp3	FK506 binding protein 3	1.53	0.01892
Ifi27l2a	interferon, alpha-inducible protein 27 like 2A	1.53	0.02259
Spc24	SPC24, NDC80 kinetochore complex component, homolog (S. cerevisiae)	1.53	0.03095
Ppt1	palmitoyl-protein thioesterase 1	1.53	0.03132
Ncl	nucleolin	1.53	0.03186
Amd1	S-adenosylmethionine decarboxylase 1	1.53	0.03295

Dph6	diphthamine biosynthesis 6	1.53	0.04263
Nubp2	nucleotide binding protein 2	1.52	0.00121
Usp14	ubiquitin specific peptidase 14	1.52	0.00131
Papln	papilin, proteoglycan-like sulfated glycoprotein	1.52	0.00213
Mtbp	Mdm2, transformed 3T3 cell double minute p53 binding protein	1.52	0.00239
Hspd1	heat shock protein 1 (chaperonin)	1.52	0.00763
Gmds	GDP-mannose 4, 6-dehydratase	1.52	0.01062
Rsad2	radical S-adenosyl methionine domain containing 2	1.52	0.01284
Cirh1a	cirrhosis, autosomal recessive 1A (human)	1.52	0.02053
Snhg1	small nucleolar RNA host gene 1	1.52	0.02423
Ephb2	Eph receptor B2	1.52	0.02656
Vars	valyl-tRNA synthetase	1.52	0.02715
Ranbp1	RAN binding protein 1	1.52	0.03517
Pgap1	post-GPI attachment to proteins 1	1.51	0.00065
Lyve1	lymphatic vessel endothelial hyaluronan receptor 1	1.51	0.00094
Gm25203	predicted gene, 25203 [Source:MGI Symbol;Acc:MGI:5454980]	1.51	0.00331
Cps1	carbamoyl-phosphate synthetase 1	1.51	0.00344
Thg1l	tRNA-histidine guanylyltransferase 1-like (S. cerevisiae)	1.51	0.00376
Mbnl3	muscleblind-like 3 (Drosophila)	1.51	0.00417
Alg3	asparagine-linked glycosylation 3 (alpha-1,3-mannosyltransferase)	1.51	0.00429
Nutf2-ps2	nuclear transport factor 2, pseudogene 2	1.51	0.00439
Fam206a	family with sequence similarity 206, member A	1.51	0.00934
Vrk1	vaccinia related kinase 1	1.51	0.01091
n-R5s71	nuclear encoded rRNA 5S 71 [Source:MGI Symbol;Acc:MGI:4421916]	1.51	0.01976
Nhp2	NHP2 ribonucleoprotein	1.51	0.02014
Adgrl4	adhesion G protein-coupled receptor L4	1.51	0.0215
Prmt5	protein arginine N-methyltransferase 5	1.51	0.02471
Nop2	NOP2 nucleolar protein	1.51	0.02632
Ighv2-6-8	immunoglobulin heavy variable 2-6-8	1.51	0.03007
Col1a1	collagen, type I, alpha 1	1.51	0.03755
Hist1h1a	histone cluster 1, H1a	1.51	0.03876

Cd34	CD34 antigen	1.51	0.0468
Snhg5	small nucleolar RNA host gene 5	1.51	0.04728
Pramef25	PRAME family member 25	-1.51	0.00016
Sh2d6	SH2 domain containing 6	-1.51	0.00208
Bhlhe40	basic helix-loop-helix family, member e40	-1.51	0.00418
Gm22009	predicted gene, 22009 [Source:MGI Symbol;Acc:MGI:5451786]	-1.51	0.00518
Gm11346	X-linked lymphocyte-regulated 5 pseudogene	-1.51	0.0095
Stmn3	stathmin-like 3	-1.51	0.00962
Capn9	calpain 9	-1.52	0.00372
Atf7ip	activating transcription factor 7 interacting protein	-1.52	0.00462
Olfr777	olfactory receptor 777	-1.52	0.00912
Gm5441	predicted gene 5441	-1.52	0.00948
Gm24806	predicted gene, 24806 [Source:MGI Symbol;Acc:MGI:5454583]	-1.52	0.00986
Mfhas1	malignant fibrous histiocytoma amplified sequence 1	-1.52	0.01106
4930443O20Rik	RIKEN cDNA 4930443O20 gene	-1.52	0.01177
Lrp12	low density lipoprotein-related protein 12	-1.52	0.02335
Ptpn21	protein tyrosine phosphatase, non-receptor type 21	-1.52	0.03814
Otud1	OTU domain containing 1	-1.53	0.0046
Olfr170	olfactory receptor 170	-1.53	0.03195
Srd5a2	steroid 5 alpha-reductase 2	-1.53	0.03477
Gm14147	predicted gene 14147	-1.53	0.03692
Scarna10; Ncapd2	small Cajal body-specific RNA 10; non-SMC condensin I complex, subunit D2	-1.54	1.7E-05
Olfr1018	olfactory receptor 1018	-1.54	0.00289
Cpm	carboxypeptidase M	-1.54	0.00321
Gm23226	predicted gene, 23226 [Source:MGI Symbol;Acc:MGI:5453003]	-1.54	0.00446
Lpar3	lysophosphatidic acid receptor 3	-1.54	0.00837
Cish	cytokine inducible SH2-containing protein	-1.54	0.01552
Trav6-4	T cell receptor alpha variable 6-4	-1.54	0.01733
Gm23977	predicted gene, 23977 [Source:MGI Symbol;Acc:MGI:5453754]	-1.54	0.01793
Gm26065	predicted gene, 26065 [Source:MGI Symbol;Acc:MGI:5455842]	-1.54	0.02051
Zfp467	zinc finger protein 467	-1.54	0.02555

Smad7	SMAD family member 7	-1.54	0.02907
Gm13763	predicted gene 13763 [Source:MGI Symbol;Acc:MGI:3650763]	-1.54	0.03226
Rb1	retinoblastoma 1	-1.54	0.03787
Olfr1446	olfactory receptor 1446	-1.55	0.00688
Gm23738	predicted gene, 23738 [Source:MGI Symbol;Acc:MGI:5453515]	-1.55	0.00791
Lrrn1	leucine rich repeat protein 1, neuronal	-1.55	0.02547
Gm26437	predicted gene, 26437 [Source:MGI Symbol;Acc:MGI:5456214]	-1.55	0.02954
Gm24128	predicted gene, 24128 [Source:MGI Symbol;Acc:MGI:5453905]	-1.55	0.03018
Gm22485	predicted gene, 22485 [Source:MGI Symbol;Acc:MGI:5452262]	-1.55	0.03517
Ces2a	carboxylesterase 2A	-1.56	0.00107
Gm25860	predicted gene, 25860 [Source:MGI Symbol;Acc:MGI:5455637]	-1.56	0.002
Gm25185	predicted gene, 25185 [Source:MGI Symbol;Acc:MGI:5454962]	-1.56	0.00714
Dusp23	dual specificity phosphatase 23	-1.56	0.01265
Stxbp5l	syntaxin binding protein 5-like	-1.56	0.01728
Rab6b	RAB6B, member RAS oncogene family	-1.56	0.02303
Olfr1230	olfactory receptor 1230	-1.56	0.02399
St3gal1	ST3 beta-galactoside alpha-2,3-sialyltransferase 1	-1.56	0.02906
Gm25429	predicted gene, 25429 [Source:MGI Symbol;Acc:MGI:5455206]	-1.56	0.04295
Snord89	small nucleolar RNA, C/D box 89	-1.57	8.1E-05
Olfr1126	olfactory receptor 1126	-1.57	0.00652
Ces1d	carboxylesterase 1D	-1.57	0.01965
Gm23803	predicted gene, 23803 [Source:MGI Symbol;Acc:MGI:5453580]	-1.57	0.02533
Gm24796	predicted gene, 24796 [Source:MGI Symbol;Acc:MGI:5454573]	-1.57	0.04952
Olfr846	olfactory receptor 846	-1.58	0.00037
Olfr1504	olfactory receptor 1504	-1.58	0.01195
Gas6	growth arrest specific 6	-1.59	0.00044
Gm26226	predicted gene, 26226 [Source:MGI Symbol;Acc:MGI:5456003]	-1.59	0.01961
Rpph1	ribonuclease P RNA component H1	-1.59	0.02494
Gm25334	predicted gene, 25334 [Source:MGI Symbol;Acc:MGI:5455111]	-1.6	0.00594
Tpsg1	tryptase gamma 1	-1.6	0.01248
Gm22850	predicted gene, 22850 [Source:MGI Symbol;Acc:MGI:5452627]	-1.6	0.0232

Ube2dnl2	ubiquitin-conjugating enzyme E2D N-terminal like 2	-1.6	0.02783
Cyp2b10	cytochrome P450, family 2, subfamily b, polypeptide 10	-1.6	0.03945
Gm25804	predicted gene, 25804 [Source:MGI Symbol;Acc:MGI:5455581]	-1.6	0.04414
Gm22975	predicted gene, 22975 [Source:MGI Symbol;Acc:MGI:5452752]	-1.61	0.00662
Bend5	BEN domain containing 5	-1.61	0.02027
LOC101055995; Gm5819	putative E3 ubiquitin-protein ligase UNKL pseudogene; predicted gene 5819 [Source:MGI Symbol;Acc:MGI:3643122]	-1.61	0.02282
Gm25637	predicted gene, 25637 [Source:MGI Symbol;Acc:MGI:5455414]	-1.61	0.02571
Bmp1	bone morphogenetic protein 1	-1.61	0.04968
Gm22284	predicted gene, 22284 [Source:MGI Symbol;Acc:MGI:5452061]	-1.62	0.00087
Plekhb1	pleckstrin homology domain containing, family B (evectins) member 1	-1.62	0.01514
Naip5	NLR family, apoptosis inhibitory protein 5	-1.62	0.02671
Gm23860	predicted gene, 23860 [Source:MGI Symbol;Acc:MGI:5453637]	-1.63	4.6E-05
Trav6-3; Traj4	T cell receptor alpha variable 6-3; T cell receptor alpha joining 4	-1.63	0.00079
Igkv1-115	immunoglobulin kappa variable 1-115	-1.63	0.0336
Dusp1	dual specificity phosphatase 1	-1.64	0.04418
Ghr	growth hormone receptor	-1.65	0.00035
Gm25121	predicted gene, 25121 [Source:MGI Symbol;Acc:MGI:5454898]	-1.65	0.00108
Gm22019	predicted gene, 22019 [Source:MGI Symbol;Acc:MGI:5451796]	-1.65	0.00594
Olfr1109	olfactory receptor 1109	-1.65	0.01032
Lama3	laminin, alpha 3	-1.65	0.01275
Nrg4	neuregulin 4	-1.65	0.02181
Gm5705	predicted gene 5705 [Source:MGI Symbol;Acc:MGI:3643224]	-1.65	0.02252
Rdh19	retinol dehydrogenase 19	-1.65	0.02285
Slc25a23	solute carrier family 25 (mitochondrial carrier; phosphate carrier), member 23	-1.65	0.03512
Gm25602	predicted gene, 25602 [Source:MGI Symbol;Acc:MGI:5455379]	-1.65	0.04566
Gm11300	predicted gene 11300 [Source:MGI Symbol;Acc:MGI:3651398]	-1.66	0.02026
Traj29	T cell receptor alpha joining 29	-1.66	0.03226
Gm26038	predicted gene, 26038 [Source:MGI Symbol;Acc:MGI:5455815]	-1.67	0.00022
Gm23899	predicted gene, 23899 [Source:MGI Symbol;Acc:MGI:5453676]	-1.68	0.00209
Scg3	secretogranin III	-1.68	0.00598

Bmp3	bone morphogenetic protein 3	-1.68	0.00959
Jun	jun proto-oncogene	-1.69	0.00456
Olfr165	olfactory receptor 165	-1.69	0.03097
Ano7	anoctamin 7	-1.7	0.00084
Snord116l1; Snord116l2	small nucleolar RNA, C/D box 116-like 1; small nucleolar RNA, C/D box 116-like 2	-1.7	0.01176
Dao	D-amino acid oxidase	-1.7	0.01189
Gm22500	predicted gene, 22500 [Source:MGI Symbol;Acc:MGI:5452277]	-1.7	0.01365
Zfp677	zinc finger protein 677	-1.7	0.01961
Olfr390	olfactory receptor 390	-1.72	9.8E-05
Nrn1	neuritin 1	-1.72	0.00735
BC094916	cDNA sequence BC094916	-1.72	0.01281
Hlf	hepatic leukemia factor	-1.73	0.00633
Calcb	calcitonin-related polypeptide, beta	-1.73	0.01112
Gm25520	predicted gene, 25520 [Source:MGI Symbol;Acc:MGI:5455297]	-1.73	0.03916
Pcdh19	protocadherin 19	-1.75	0.00363
Gm13270	predicted gene 13270	-1.76	0.00625
Gm24960	predicted gene, 24960 [Source:MGI Symbol;Acc:MGI:5454737]	-1.76	0.02626
Gm11360	predicted gene 11360 [Source:MGI Symbol;Acc:MGI:3649916]	-1.76	0.04116
1700016G22Rik	RIKEN cDNA 1700016G22 gene	-1.77	0.01395
Gip	gastric inhibitory polypeptide	-1.78	0.00045
Fer1l6	fer-1-like 6 (C. elegans)	-1.78	0.00959
Crp	C-reactive protein, pentraxin-related	-1.78	0.04224
Gm24805	predicted gene, 24805 [Source:MGI Symbol;Acc:MGI:5454582]	-1.78	0.04632
Cldn34c3	claudin 34C3	-1.79	0.00196
Ntn4	netrin 4	-1.79	0.0029
Gm27177	predicted gene 27177	-1.79	0.01499
Tns4	tensin 4	-1.8	0.02262
Phxr4	per-hexamer repeat gene 4	-1.8	0.03268
Dio1	deiodinase, iodothyronine, type I	-1.81	0.00331

Per2; 9830107B12Rik	period circadian clock 2; RIKEN cDNA 9830107B12 gene	-1.81	0.00407
Gm26611	predicted gene, 26611 [Source:MGI Symbol;Acc:MGI:5477105]	-1.81	0.03638
Gm24465	predicted gene, 24465 [Source:MGI Symbol;Acc:MGI:5454242]	-1.82	0.00404
Cyp2j9	cytochrome P450, family 2, subfamily j, polypeptide 9	-1.82	0.01028
Klkb1; Cyp4v3	kallikrein B, plasma 1; cytochrome P450, family 4, subfamily v, polypeptide 3	-1.82	0.03018
A930033H14Rik	RIKEN cDNA A930033H14 gene	-1.83	0.0053
Acsm5	acyl-CoA synthetase medium-chain family member 5	-1.84	0.01402
Akr1c14	aldo-keto reductase family 1, member C14	-1.85	0.02745
Cym	chymosin	-1.85	0.03335
Vmn1r227	vomeronasal 1 receptor 227	-1.86	0.00371
Igkv1-132	immunoglobulin kappa variable 1-132	-1.86	0.01409
Spib	Spi-B transcription factor (Spi-1/PU.1 related)	-1.87	0.01273
Gm27177; Gm21464	predicted gene 27177; predicted gene, 21464	-1.88	0.00911
Gm23523	predicted gene, 23523 [Source:MGI Symbol;Acc:MGI:5453300]	-1.88	0.02747
Mir669a-3	microRNA 669a-3	-1.88	0.03648
Kcnu1	potassium channel, subfamily U, member 1	-1.89	0.00012
Lect2	leukocyte cell-derived chemotaxin 2	-1.92	0.00304
Iyd	iodotyrosine deiodinase	-1.92	0.03984
Gm17268	predicted gene, 17268 [Source:MGI Symbol;Acc:MGI:4936902]	-1.96	0.01091
Gm23260	predicted gene, 23260 [Source:MGI Symbol;Acc:MGI:5453037]	-1.99	0.04054
Per3	period circadian clock 3	-2.02	0.01336
Cubn	cubilin (intrinsic factor-cobalamin receptor)	-2.07	0.01104
Ido1	indoleamine 2,3-dioxygenase 1	-2.08	0.00184
Gm26081	predicted gene, 26081 [Source:MGI Symbol;Acc:MGI:5455858]	-2.09	0.00506
Gm38463; Gm27177	predicted gene, 38463; predicted gene 27177	-2.09	0.00732
Gm5423	predicted gene 5423 [Source:MGI Symbol;Acc:MGI:3643175]	-2.14	0.03456
Gm24170	predicted gene, 24170 [Source:MGI Symbol;Acc:MGI:5453947]	-2.17	0.02411

Sst	somatostatin	-2.27	4.87E-07
Gm26034	predicted gene, 26034 [Source:MGI Symbol;Acc:MGI:5455811]	-2.27	0.00663
Bco1	beta-carotene oxygenase 1	-2.3	0.00014
Cyp2c66	cytochrome P450, family 2, subfamily c, polypeptide 66	-2.35	0.02392
Plscr4	phospholipid scramblase 4	-2.4	0.02097
Gm15963	predicted gene 15963 [Source:MGI Symbol;Acc:MGI:3802140]	-2.46	0.00906
Clu	clusterin	-2.57	0.00502
Fras1	Fraser syndrome 1 homolog (human)	-2.69	0.00258
Dbp	D site albumin promoter binding protein	-2.86	0.02974
Pcyt1a	phosphate cytidylyltransferase 1, choline, alpha isoform	-3.99	5.55E-07