Intestinal CREBH prevents lithogenic diet-induced hypercholesterolemia by decreasing *Npc1l1* expression

小腸の CREBH は *Npc1l1* の発現を抑制し、高コレステロール食による高コレステロール血症を抑制する

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Abstract

CREBH (encorded by *Creb3l3*) is a liver and intestine-specific transcription factor. Recent papers reported that hepatic CREBH plays a key role in energy metabolism such as anti-obesity and anti-diabetes effects via increasing fatty acid lipolysis and gluconeogenesis during starvation. However, the function of intestinal CREBH remains to be determined. We generated transgenic mice that highly expressed the nuclear form of *CREBH* in the intestine, but weakly in the liver (*CREBH*-Tg mice). We fed *CREBH*-Tg mice with a lithogenic diet (LD). LD-fed CREBH-Tg mice showed decreased plasma cholesterol levels as well as prevention of fatty liver and cholesterol crystal formations in the gallbladder. *CREBH*-Tg mice increased fecal cholesterol output. In addition, we found that the expression of *Npc1l1*, a rate-limiting transporter mediating intestinal cholesterol absorption, was decreased in *CREBH*-Tg mice. Intestinal *Srb1*, *Abca1*, and *Abcg5/8* expression were also reduced in *CREBH*-Tg mice. With luciferase assay, we showed that CREBH decreased mouse Npc1l1 promoter activity. These data suggest that the intestinal cholesterol absorption in *CREBH*-Tg mice is decreased. Collectively, these results support that intestinal CREBH prevents LD-induced hypercholesterolemia by decreasing expression of intestinal cholesterol transporters.

Chapter 1

General Introduction

1-1. *Cholesterol metabolism*

In mammals, cholesterol is one of the essential structural components of the cell membrane, as well as a precursor molecule for steroid hormones and bile acids. Cholesterol is abundant in the blood stream and cell membrane [1]. Lathosterolosis, the diseases with cholesterol biosynthesis defect, show the importance of cholesterol in normal embryonic development. This disorder is characterized by low cholesterol levels in the tissues and blood, accompanied with multiple congenital anomalies, such as progressive cholestasis and microcephaly [2]. People who have high blood cholesterol levels have a greater risk of metabolic diseases such as cardiovascular diseases, NASH and gallstones. Increase cholesterol is estimated to cause 2.6 million deaths globally (4.5% of the total number of deaths) and 29.7 million disability adjusted life years [3]. A 10% reduction in plasma cholesterol in men aged 40 is considered to lead to a 50% reduction in heart disease within 5 years [4]. However, the picture of cholesterol metabolism is still incomplete. This study identified a novel mechanism to modulate cholesterol homeostasis.

Cholesterol homeostasis is determined mainly by hepatic *de novo* synthesis, conversion into bile acids, and intestinal absorption. Dietary and biliary cholesterol is absorbed from the proximal part of small intestine [4]. About 80% of body cholesterol is derived form endogenous synthesis. HMGCR catalyzes the rate-limiting step [5]. About 23% of body cholesterol is in the brain, and is mainly synthesized *in situ*. Dietary cholesterol does not cross blood-brain barrier [2].

Lipoproteins are mainly synthesized in the liver and intestine. Lipoproteins play important roles in regulating the cholesterol transport to and from tissues through the bloodstream. After ingestion of a meal, dietary fat are absorbed into small intestine and form chylomicrons. Chylomicrons are particles that consists of triglycerides (85–92%), phospholipids (6–12%), cholesterol (1–3%) and proteins (1–2%) [6]. Pre-chylomicron formation is followed by cellular lipid re-esterification and lipid-packaging with apoprotein B48. The pre-chylomicron is synthesized in the endoplasmic reticulum and transported to the Golgi. Fusion with the Golgi complex is required to form mature chylomicron particles. Mature chylomicrons contain ApoAI, ApoAIV, and ApoB48, and are secreted from the basolateral membrane of enterocytes. In the blood and lymph, lipoprotein lipase (LPL) mediates hydrolysis of triglycerides in chylomicrons. Free fatty acids are incorporated into muscle for energy production, brown adipose tissue for heat production and white adipose tissue for storage.

Chylomicron remnants are rapidly cleared by the liver. Intrahepatic lipids are repackaged and secreted as VLDL, which is hydrolyzed by LPL and converted to VLDL remnants (IDL), which is hydrolyzed by hepatic lipase, producing LDL. LDL transports cholesterol from the liver to the peripheral tissues [5] (Fig. 1-1).

1-2. *Excessive cholesterol and related diseases*

Excessive cholesterol level is one of the major risk factors leading to non-alcoholic fatty liver, cholesterol gallstone, and cardiovascular diseases. Cholesterol taken up by macrophages is oxidized. Oxycholesterol enhances cytokine production and yield of inflammatory cells. LDL-accumulated macrophages become foam cells on the arterial wall, which progress to atherogenic plaques, resulting in cardiovascular diseases such as coronary heart disease or stroke [5]. Cardiovascular diseases are a major cause of world-wide death. The current treatment guidelines describes that the patients who is at risk for these diseases should meet the defined targets for LDL-cholesterol levels. Consequently, a high number of individuals need to receive cholesterol-lowering therapy.

Concentrated bile, which is converted from cholesterol, is stored in the gallbladder. Gallstone disease is one of the most common biliary tract diseases in the world. The disease is sometimes accompanied with pain and inflammation of the

gallbladder, leading to receive considerable financial and social burdens [7]. The occurrence is due to the following five factors; genetic alteration of lithogenic genes, hepatic hypersecretion of bile and cholesterol, gallbladder hypomotility, accelerated phase transitions of cholesterol, and increased amount of cholesterol of intestinal origin due to high efficiency of cholesterol absorption [7]. Statin, a drug that inhibits hepatic cholesterol synthesis, reduces the cholesterol content in bile and prevents cholesterol crystals formation [8]. In addition, Ezetimibe, an intestinal cholesterol absorption inhibitor, has been shown to reduce bile cholesterol levels in gallstone patients by decreasing expression of NPC1L1, an intestinal cholesterol transporter [9].

Lipid accumulation drives the development of progressive hepatic inflammation and fibrosis. Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease that is characterized by excessive lipid deposition in the liver. NAFLD is one of the most common liver disease and observed among 15-46% of adults in the USA[10], and the progressive state is defined as Non-alcoholic steatohepatitis (NASH). It is well known that dyslipidemia, obesity, diabetes and insulin resistance are the risk factors for the development of inflammation and steatosis in the liver [11].

1-3 *Intestinal cholesterol absorption*

Intestinal cholesterol absorption and excretion are important components of

cholesterol homeostasis. Bile salt micelles facilitate the transfer of cholesterol across the brush border membrane. Intestinal cholesterol absorption is a selective multistep process, which is regulated by multiple sterol transporter genes. NPC1L1 has been identified as a critical player for the uptake of cholesterol across the plasma membrane of intestinal enterocytes [12]. NPC1L1 undergoes clathrin-mediated internalization following sterol binding. The absorbed sterols are delivered to the endoplasmic reticulum (ER) for esterification. ACAT2 functions to convert cellular cholesterol into cholesterol ester [13]. Plant sterols are less effective substrates for ACAT2 and are secreted back into the intestinal lumen. SRB1 is a cholesterol transporter, which is involved in intestinal chylomicron production [14,15]. ATP binding cassette protein ABCA1 in the intestine forms HDL-cholesterol and transport from the epithelial cells into bloodstream [16]. Two hemi-ABC transporters, ABCG5 and ABCG8, dimerize to form a complete transporter to efflux cholesterol and plant sterols back into the lumen for fecal disposal [17,18]. These cholesterol transporters are highly expressed in the jejunum, where the nutrients are absorbed. Intestinal cholesterol ester forms chylomicron, and triglycerides are incorporated into chylomicron by microsomal triglyceride transfer protein (MTTP). The mature chylomicron enters the lymphatic circulation (Fig. 1-2).

1-4 *The roles of CREBH in lipid and cholesterol metabolism*

CREBH (encorded by *Creb3l3*) is a basic leucine zipper domain transcriptional factor of the CREB/activating transcription factor (ATF) family [19]. CREBH is highly and selectively expressed in gastrointestinal tract tissues including the liver, pyloric stomach, duodenum, and ileum [20]. CREBH is localized in the endoplasmic reticulum membrane. CREBH is activated and/or induced under various kinds of stresses including ER stress, pro-inflammatory cytokines, bacterial endotoxin LPS. CREBH is cleaved by Golgi-resident site-1 protease and site-2 protease, the same enzymes responsible for the cleavage of SREBPs, the regulators of lipid biosynthesis [21].

Our previous work showed that hepatic overexpression of nuclear *CREBH* led to therapeutic effects on diabetes and obesity. CREBH induced systemic lipolysis, hepatic ketogenesis and insulin sensitivity with increased energy expenditure. Hepatic CREBH induces anti-metabolic disorder hormones including FGF21 and IGFBP2 [22]. In addition, several papers have been reported a hepatic function of CREBH in triglyceride metabolism [23,24]. Hepatic CREBH induces the expression of LPL coactivaters such as ApoA4, ApoA5 and ApoC3, which reduces plasma triglyceride levels [23]. In an atherogenic diet-fed state, hepatic CREBH is involved in lipogenesis, FA oxidation, and lipolysis. However, the roles of intestinal CREBH remain to be

determined. In the present study, we revealed that intestinal CREBH decreased cholesterol transporter expression and prevents high cholesterol diet-induced hypercholesterolemia.

1-5. Figures

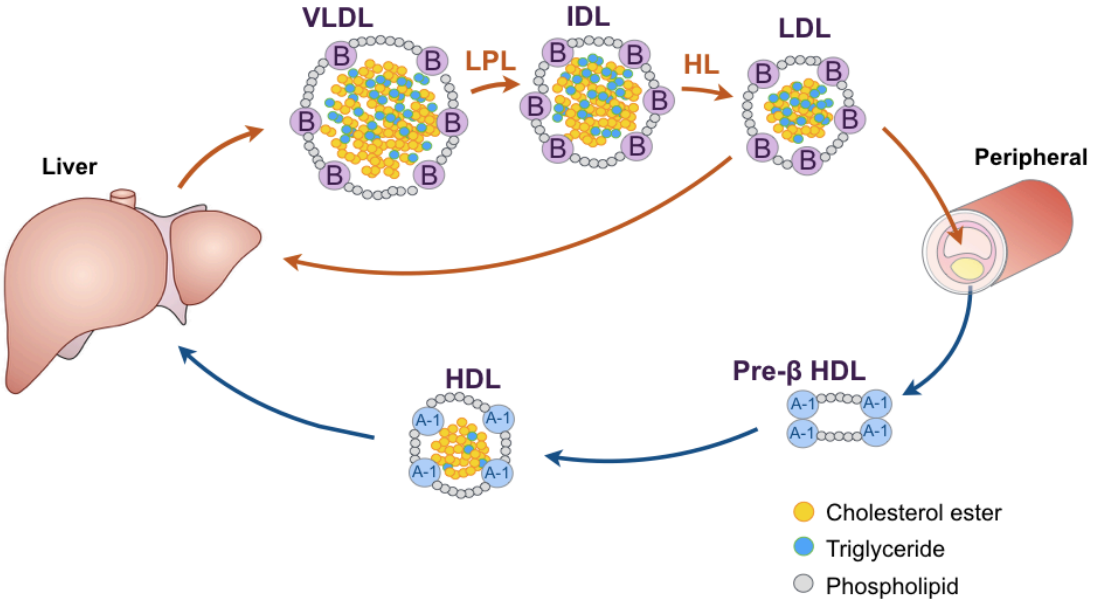

Figure 1-1. Lipoprotein metabolism

Chylomicron remnants are rapidly cleared by the liver. Intrahepatic lipids are repackaged and secreted as VLDL, which is hydrolyzed by lipoprotein lipase (LPL) and converted to VLDL remnants (IDL), which is hydrolyzed by hepatic lipase (HL), producing LDL. LDL transports cholesterol from the liver to the peripheral tissues. HDL acquires cholesterol from peripheral tissues and transports the cholesterol back to the liver.

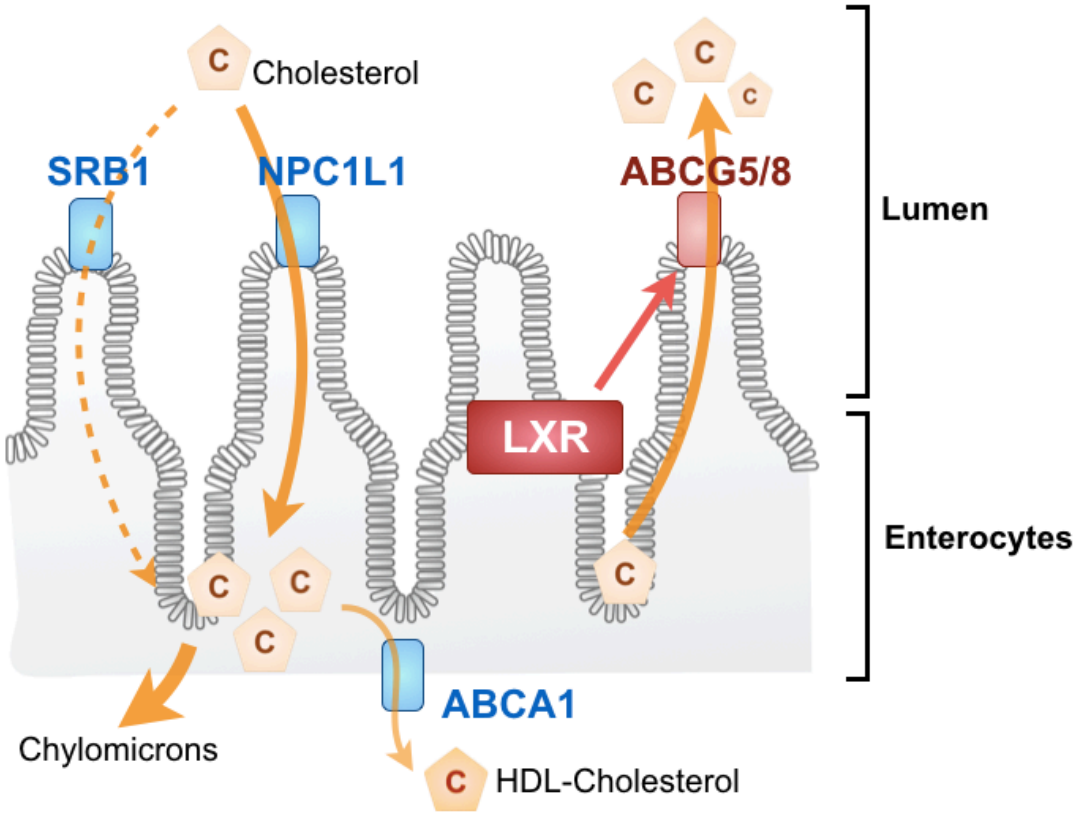

Figure 1-2. The roles of intestinal cholesterol transporters

NPC1L1 is a rate limiting cholesterol transporter in the intestine. SRB1 is involved in chylomicron synthesis and cholesterol absorption. ABCA1 is involved in HDL-cholesterol synthesis. ABCG5/8 functions for reverse cholesterol transport from enterocytes to the lumen. LXR up-regulates the intestinal expression of ABCG5/8.

Chapter 2

Analysis of metabolic phenotype of LD-fed *CREBH*-Tg mice

2-1 *Introduction*

As mentioned above, the function of intestinal CREBH is poorly understood. Intestinal *CREBH* is highly expressed in the epithelial cells of jejunum. This part of intestine is important for nutrient absorption. Several cholesterol transporters, such as *NPC1L1* and *SRB1,* are expressed in similar parts of intestine as CREBH. In this study, we investigated the effect of *CREBH* overexpression on cholesterol homeostasis and gene expression changes of intestinal cholesterol transporters.

2-2 *Materials and Methods*

Animals and diets

C57/BL6J (wild type [WT]) mice were obtained from CLEA Japan. To generate the active form of human *CREBH* transgenic mice, cDNA encoding the rat PCK1 promoter, human CREBH (1-320 amino acid residues), and the 3' polyadenylation signal of human growth hormone was microinjected into C57BL6J eggs. Mice were housed in a pathogen-free barrier facility with a 12 h light/dark cycle

and were given free access to water. Mice were fed with a normal chow diet (MF: Oriental Yeast, Tokyo, Japan) or a lithogenic diet (LD) (F2HFD1: Oriental Yeast, Tokyo, Japan) for 2 weeks. Age-matched males were used for each experiment. The mice were sacrificed during the light phase. Eight-week old mice were fed with an LD. After 2-week feeding, they were killed in a fed state. Plasma samples were collected from the post-orbital plexus. All animal husbandry and animal experiments were consistent with the University of Tsukuba's Regulations of Animal Experiments and were approved by the Animal Experiment Committee of the University of Tsukuba.

Plasma, hepatic, and gallbladder bile lipid and gallstone analysis

Plasma samples were collected from the post-orbital plexus using heparinized capillaries, and were examined for total cholesterol, triglyceride, bile acids, NEFA, glucose, ALT and AST. Hepatic lipids were extracted from liver tissues using Folch solution (chloroform-methanol, 2:1 v/v), dried and dissolved in 2-propanol. The hepatic lipid extracts were assayed for hepatic cholesterol and triglyceride levels. Gallbladder bile were collected from the gallbladder, diluted with water. The parameters were determined by the commercial assay kits (Cholesterol C-Test Wako, Triglyceride G-Test Wako, Total Bile Acids-Test Wako, Transaminase CII-Test Wako, Wako Pure Chemicals, Tokyo, Japan). The bile of the gallbladder was examined for monohydrate

cholesterol crystals under polarizing light microscopy.

Fecal cholesterol and bile acids output

Mice were fed an LD for 2 weeks, and fresh feces were collected and dried. 100 mg feces powders were mixed with 2 ml of Folch solution, which was also used for hepatic cholesterol measurement. The feces were incubated at 60ºC in a water bath over shaking for 1 hour; the tubes were centrifuged at 3000 rpm for 5 minutes, the supernatants were collected into a glass tube, dried up at 60Cº in the heat block, dissolved with 400 μl of isopropanol, and examined for cholesterol contents. For the measurement of fecal bile acids levels, 50 mg feces powders were mixed with 2.5 ml of 99.5% EtOH in a glass tube with cap. The mixture was incubated at 65Cº in a water bath with shaking for 1 hour, and centrifuged at 3000 rpm for 5 minutes; the supernatant was transferred into a glass tube, and dried up completely in the heat block. The dried pellet was dissolved with 1 ml of 90% EtOH, and examined for bile acid contents The cholesterol and bile acid levels in the solutions were determined by Total Bile Acids-Test Wako (Wako Pure Chemicals, Tokyo, Japan).

Analysis of gene expression

RNA was extracted from frozen mouse tissues using Sepasol (Nakarai Tesque, Kyoto, Japan). For the analysis of jejunal gene expression, the fragment of the first 15% of the relative distance from pyloric sphincter to rectum was used. Total RNA was reverse transcribed using the PrimeScript RT Master kit (Takara, Bio Inc., Shiga, Japan). Real-time PCR was performed using ABI Prism 7300 System with LightCycler-DNA Master SYBR Green I Mix (Roche DIagnostics Ltd, Lewes, UK). mRNA expression was normalized to cyclophilin mRNA content and expressed as fold change compared to WT mice using the ΔΔCT method. Microarray analysis was performed by TAKARA Bio Inc. using Agilent Expression Array. Heat maps were generated in R (v3.3.0) and bioconductor.

Statistical analysis

All results are presented as means ±SEM. Data were analyzed using Student's t-test, and considered statistically significant if $p < 0.05$.

2-3 *Results*

Generation of *CREBH*-Tg mice

To investigate the function of *CREBH*, we generated three lines of *CREBH*-Tg mice. In *CREBH*-Tg mice, the active form of human CREBH was overexpressed by the *Pck1* promoter. One of the three lines of *CREBH*-Tg mice expressed the transgene highly in the liver. The line of *CREBH*-Tg mice used in this study expressed the transgene highly in the intestine, but scarcely in the liver (Fig. 2-1). In those mice, the hepatic expression of *fgf21,* the target gene of CREBH in the liver, tends to increase but did not change significantly. Thus, there was a low effect of the transgene in the liver of *CREBH*-Tg mice (Fig. 2-2). In this study, we used the line of *CREBH*-Tg mice to analyze the intestinal CREBH function rather than hepatic CREBH. The mice were fed an LD to check the effect of intestinal *CREBH* overexpression on cholesterol metabolism. There was no significant difference in BW, food intake, liver weight or WAT weight (Fig. 2-3).

Intestinal CREBH protects against LD-induced hypercholesterolemia

Plasma lipid levels were measured to determine the effect of *CREBH* overexpression on LD-induced hypercholesterolemia. LD-feeding increased plasma cholesterol in WT mice and interestingly LD-fed *CREBH*-Tg mice showed lower

plasma cholesterol levels than WT mice (Fig. 2-4). It is known that NPC1L1 inhibitor improves LD-induced hypercholesterolemia not only in a fed state, but also in a fasted state [25] when the effect of biliary cholesterol re-absorption is well observed. Plasma triglyceride levels were not changed between chow-fed WT and *CREBH*-Tg mice (Fig. 2-4). NEFA levels were lower in *CREBH*-Tg mice than WT mice on a chow diet (Fig. 2-5). It has been reported that CREBH regulates lipolysis via inducing ApoA4 and ApoC2 expression in the liver and intestine [11], which might lower triglyceride and NEFA levels in *CREBH*-Tg mice. LD-feeding decreased plasma NEFA levels in both of WT and *CREBH*-Tg mice, therefore, LD-feeding might blunt the significant difference between WT and *CREBH*-Tg mice. Plasma bile acids levels were not changed significantly, neither in chow-fed or in LD-fed state (Fig. 2-5). Lipoprotein particle distributions were determined by HPLC analysis. This result showed that *CREBH*-Tg mice had higher plasma cholesterol levels at any lipoprotein fractions. Plasma ALT levels tended to be lower in *CREBH*-Tg mice than WT mice (Fig. 2-6). Collectively, these results showed that intestinal CREBH prevents LD-induced hypercholesterolemia.

We checked the liver histology and hepatic lipid contents in WT and *CREBH*-Tg mice fed an LD. *CREBH*-Tg mice had more reddish liver than WT mice when fed an LD (Fig. 2-7). This data indicated improved LD-induced fatty liver in *CREBH*-Tg mice. H&E staining of liver sections showed *CREBH* overexpression

decreased hepatic lipid accumulation (Fig. 2-7). Liver weight was not significantly changed between WT and *CREBH*-Tg mice. Liver cholesterol contents were lower in *CREBH*-Tg mice fed a LD (Fig. 2-8). Hepatic bile acids levels were not significantly changed (Fig. 2-8). These results show that intestinal CREBH prevents LD-induced cholesterol accumulation in the liver.

Several papers reported that NPC1L1 inhibition prevents gallbladder crystal formation under LD-feeding conditions [25,26]. We next checked gallbladder bile crystal formation in *CREBH*-Tg mice. Representative pictures of gallbladders of WT and *CREBH*-Tg mice fed with a LD. These pictures showed that gallbladder stones were accumulated in WT mice but scarcely in *CREBH*-Tg mice (Fig. 2-9). Gallbladder bile was checked by the light polarizing microscopy for the presence of cholesterol monohydrate crystals and sandy stones (Fig. 2-9). The average crystal stone size was decreased in *CREBH*-Tg mice compared to WT mice (Fig. 2-10). Cholesterol and bile acid levels in the gallbladder of *CREBH*-Tg mice were decreased (Fig. 2-10). Gallbladder phospholipid levels were not changed between groups (data not shown). These data suggested that intestinal CREBH plays an important role in gallbladder stone prevention.

Intestinal cholesterol contents were lower in LD-fed *CREBH*-Tg mice than in WT mice (Fig. 2-11). LD-fed *CREBH*-Tg mice increased fecal cholesterol output (Fig.

2-12). Fecal bile acids output was not significantly changed, compared to WT mice (Fig. 2-12). It has been reported that inhibition of intestinal cholesterol absorption by ezetimibe treatment led to decreased intestinal cholesterol content and increased fecal cholesterol output in LD-fed mice [9,27]. Thus, these data suggested that the intestinal cholesterol absorption was reduced in LD-fed *CREBH*-Tg mice compared to WT mice.

Intestinal cholesterol transporter expression was decreased in HCD-fed *CREBH*-Tg mice

We next investigated the mechanisms underlying the effect of intestinal CREBH on cholesterol homeostasis. Initially, we performed microarray analysis to compare the intestinal gene expression between WT and *CREBH*-Tg mice under chow- and LD-feeding, respectively. Interestingly, the data suggested that *Npc1l1, Srb1* and *Abca1* expression were decreased in the intestine of *CREBH*-Tg mice, on chow or LD diet. It has been well reported that NPC1L1 plays a critical role to transport cholesterol from the lumen to enterocyte [12,28]. Although there are conflicting reportsm, *Srb1* and *Abca1* have been also suggested to be involved in intestinal cholesterol absorption [29,30]. Next, we performed real time-PCR with intestinal mRNA of LD-fed WT and *CREBH*-Tg mice. We identified reductions of *Npc1l1, Srb1, and Abca1* expression in the intestine of LD-fed *CREBH*-Tg mice, as well as of *Abcg5*, *Abcg8* and *Lxr* (Fig.

2-13). It is known that NPC1L1 inhibition by ezetimibe treatment leads to decreased Abcg5/8 expression [8,26], which was consistent with the gene expression changes observed in the intestine of LD-fed *CREBH*-Tg mice. The reduction of intestinal *Lxrβ* is possibly due to reduced systemic cholesterol levels in LD-fed *CREBH*-Tg mice. Collectively, these results suggested that decreased *Npc1l1* expression is one of the reason that *CREBH*-Tg mice showed anti-hypercholesterolemic phenotype when subjected to LD-feeding. To check the NPC1L1 protein levels in the intestine of chow-fed WT and *CREBH*-Tg mice, Western blotting was performed. We found that CREBH decreased Npc1l1 protein levels in the intestine (Fig. 2-14)

Cholesterol homeostasis is maintained as the balance of intestinal absorption, hepatic synthesis and hepatic conversion into bile acids. In order to check whether the metabolic phenotype of LD-fed *CREBH*-Tg mice was derived from the liver, we determined the expression of hepatic cholesterol synthesis and conversion genes. We performed real time-PCR with liver mRNA of LD-fed WT and *CREBH*-Tg mice (Fig. 2-15). Hepatic lipid synthesis genes were not significantly changed in *CREBH*-Tg mice. FXR-SHP-Cyp7a1 pathway for bile acids synthesis was leaned toward decreased hepatic conversion of cholesterol in *CREBH*-Tg mice.

2-4 *Discussion*

We have demonstrated that the LD-induced hypercholesterolemia is prevented in *CREBH*-Tg mice. *CREBH* overexpression reduced intestinal cholesterol transporters expression, including *Npc1l1*, *Abca1*, *Srb1* and *Abcg5/8*. LD-fed *CREBH*-Tg mice attenuated systemic hypercholesterolemia, accompanied with increased fecal cholesterol output.

NPC1L1 is a rate-limiting transporter of cholesterol absorption in small intestine and a key modulator of systemic cholesterol homeostasis [12]. Intestinal ABCA1 contributes to HDL biogenesis and plays an important role in intestinal cholesterol absorption [29,30]. Several papers suggest that SRB1 is involved in intestinal chylomicron production [31,32] and is responsible for the uptake of cholesterol from the lumen of the proximal small intestine into the brush border membrane [33,34], although the initial reports including the studies of global *Srb1* knockout mice have yielded conflicting result [35,36]. Intestinal *Abcg5* and *Abcg8*, on the other hand, promote to excrete cholesterol from enterocytes into lumen [17,37]. We observed reduced expression of *Npc1l1*, *Srb1*, *Abca1* and *Abcg5/8* in the intestine of LD-fed *CREBH*-Tg mice. This gene expression change may lead to reduced intestinal cholesterol absorption, considering that intestinal cholesterol absorption was decreased in *Npc1l1-Abcg5-Abcg8* triple knockout mice [38].

Npc1l1, *Srb1* and *Abca1* expressions were decreased in both of chow-fed and

LD-fed *CREBH*-Tg mice. On the other hand, *Abcg*5/8 expression was reduced in LD-fed *CREBH*-Tg mice, but not in chow-fed mice. We though that the decreased *Abcg*5/8 expression in LD-fed *CREBH*-Tg mice was secondary to the decreased systemic cholesterol levels by decreased *Npc1l1*, *Srb1*, and *Abca1* expression. LXR might not be activated in LD-fed *CREBH*-Tg mice as much as in WT mice, because systemic cholesterol levels were lower than in WT mice, which consequently led to lower expression of *Abcg5/8*, the LXR downstream genes.

2-5 *Conclusion*

LD-fed *CREBH*-Tg mice showed reduced systemic cholesterol levels compared to WT mice. We found increased fecal cholesterol output in LD-fed *CREBH*-Tg, accompanied with decreased *Npc1l1* expression in the intestine. Thus, these results suggest that LD-fed *CREBH*-Tg mice decreased intestinal cholesterol absorption possibly by reducing *Npc1l1* expression.

2-6. Figures

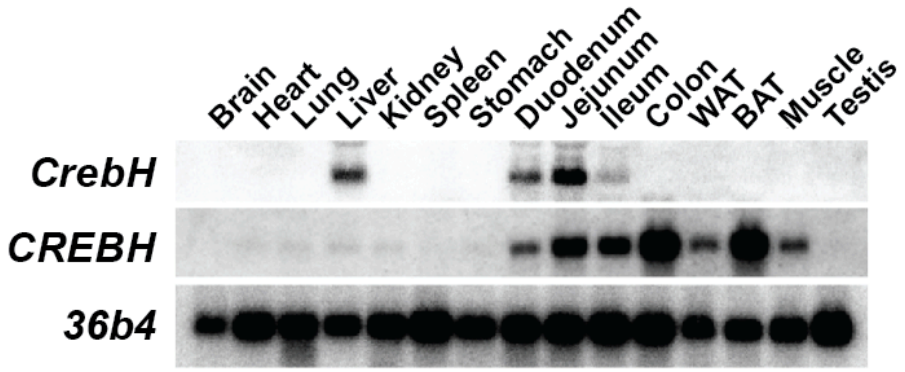

Figure 2-1. Tissue distribution of *CrebH* and *CREBH* expression

Northern blotting was performed to examine the expression of *CrebH* and *CREBH*, using mRNAs extracted from *CREBH*-Tg mice.

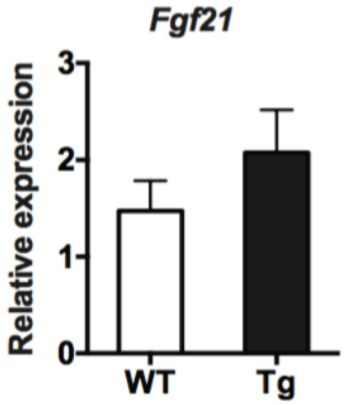

Figure 2-2. Hepatic expression of *Fgf21* is not significantly changed in *CREBH*-Tg mice

Hepatic *Fgf21* expression of chow-fed WT and *CREBH*-Tg mice was determined by real time-PCR (n = 3/group). Results are presented as means ±SEM.

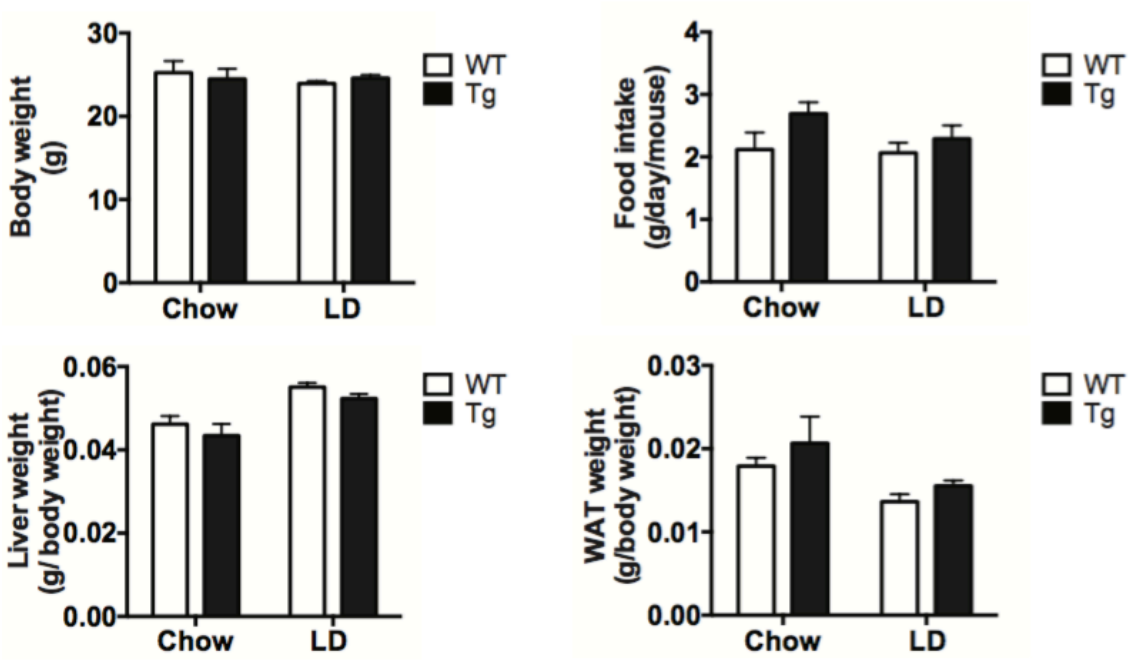

Figure 2-3. Body and tissue weights of *CREBH*-Tg mice.

Body weight, food intake, liver weight, WAT weight and food intake were measured in WT and CREBH-Tg mice fed on chow or LD fed states. Results are presented as means ±SEM.

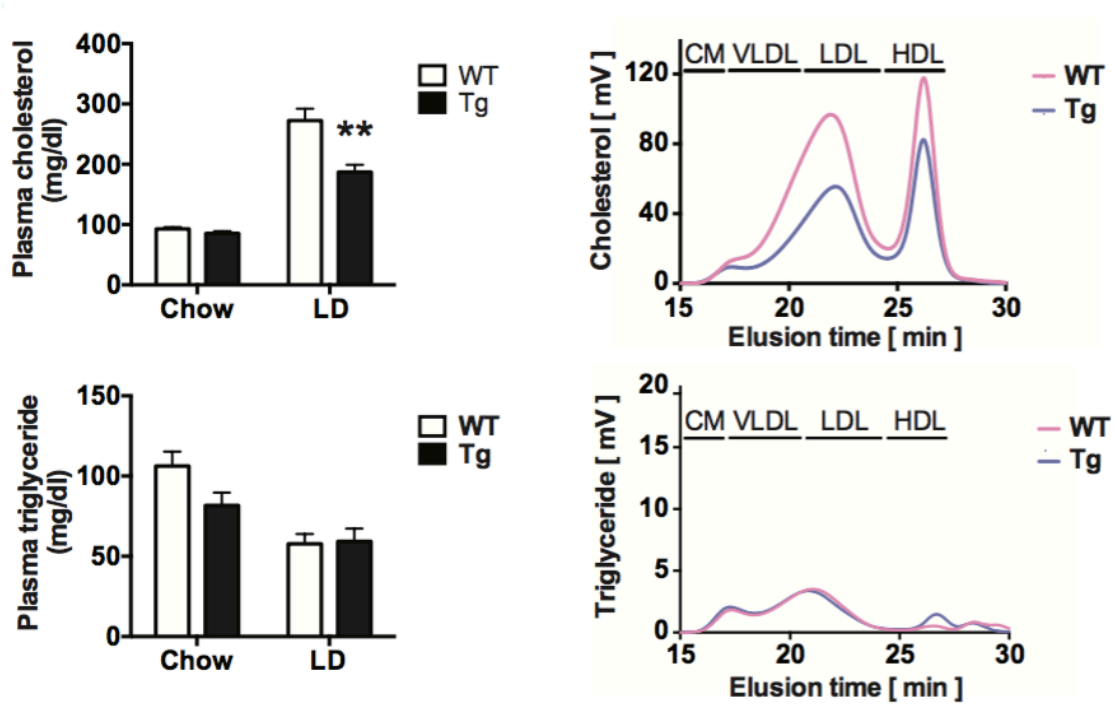

Figure 2-4. Plasma cholesterol and triglyceride levels in *CREBH*-Tg mice.

Plasma cholesterol and triglyceride levels were measured in WT and *CREBH*-Tg mice fed with a normal chow diet and an LD for 2 weeks (plasma cholesterol, n = 19-31, plasma triglyceride, n = 10-26/group). Results are presented as means ±SEM. Differences between genotypes on either nutrient state, **$p < 0.01$. Lipoprotein particle distributions were determined by HPLC analysis.

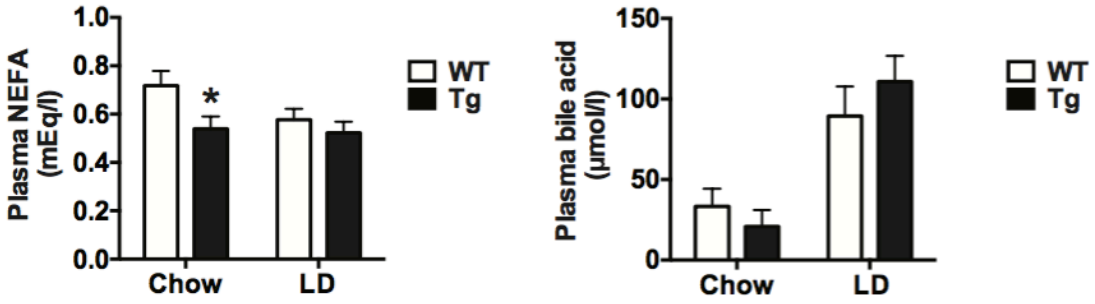

Figure 2-5. Plasma NEFA and bile acid levels in *CREBH*-Tg mice.

Plasma NEFA and bile acid levels were measured in WT and *CREBH*-Tg mice fed with a normal chow diet or an LD for 2 weeks (plasma bile acid n = 9-46/group, NEFA, n = 14-23/group). Results are presented as means ±SEM. Differences between genotypes on either nutrient state, *$p < 0.05$.

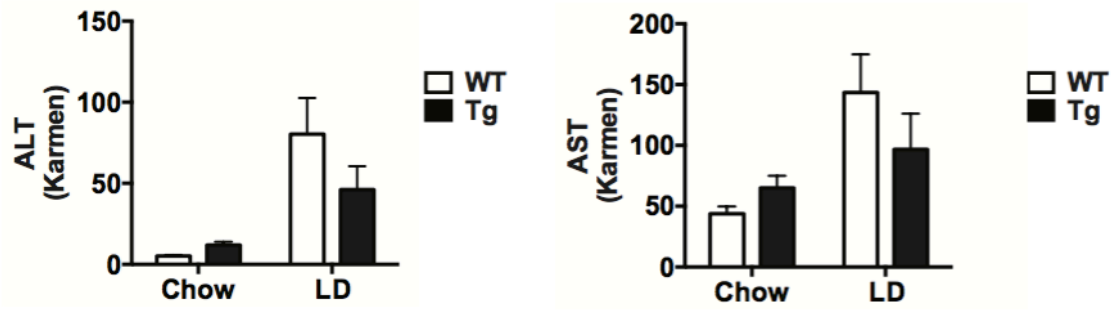

Figure 2-6. Plasma ALT and AST levels in *CREBH*-Tg mice.

Plasma AST and ALT levels were measured in WT and *CREBH*-Tg mice fed with a normal chow diet or an LD for 2 weeks (n = 15-27/group). Results are presented as means ±SEM. Differences between genotypes on either nutrient state.

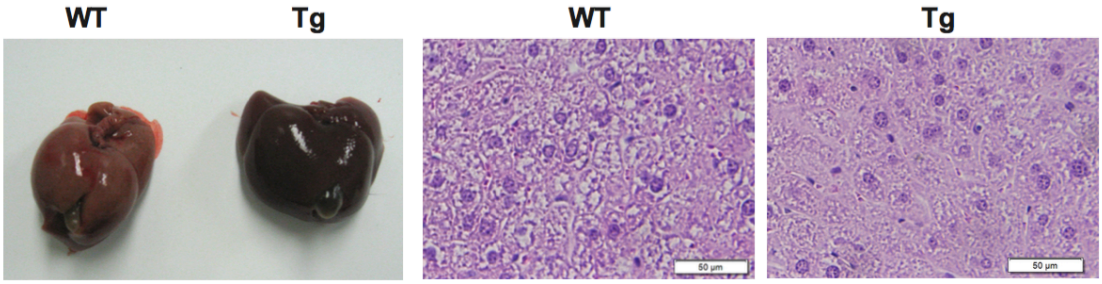

Figure 2-7. Lower lipid accumulation contents in *CREBH*-Tg mice on an LD.

The representative pictures of the livers collected from WT and *CREBH*-Tg mice fed an LD. Hematoxylin and Eosin (H&E) staining was performed with the liver sections of LD-fed WT and CREBH-Tg mice.

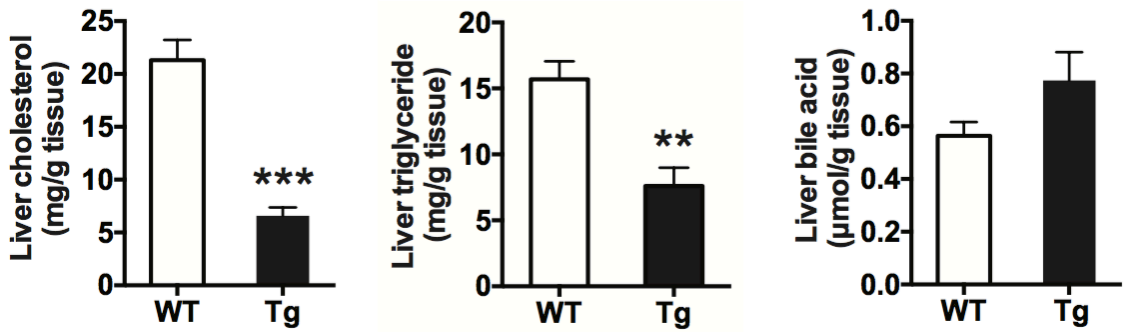

Figure 2-8. Lower cholesterol contents in the liver of *CREBH*-Tg mice on a LD.

Liver cholesterol, triglyceride and bile acid levels were measured in *CREBH*-Tg mice fed an LD (n = 8-15/group). Results are presented as means ±SEM. ***$p < 0.001$, **$p < 0.01$.

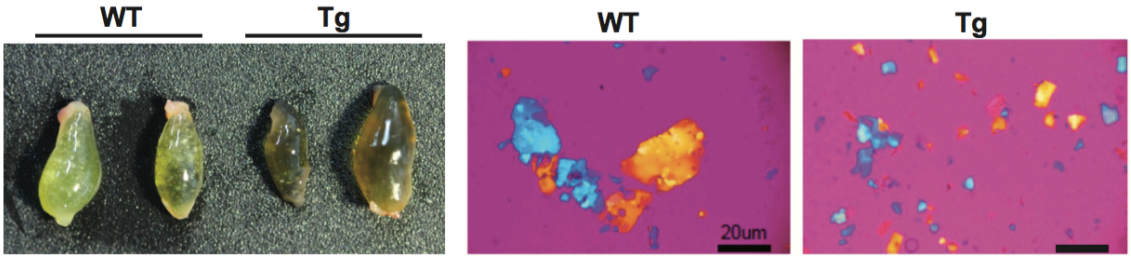

Figure 2-9. Gallbladder crystal formation is prevented in the gallbladder of *CREBH*-Tg mice on an LD.

Gallbladders were collected from LD-fed WT and *CREBH*-Tg mice. Representative pictures of the gallbladders are shown. Images of the gallbladder bile were analyzed by the light polarizing microscopy for the presence of cholesterol monohydrate crystal and sandy stones. The birefringence, or double refraction, was obtained as the decomposition of a ray of light into two rays when it passes through certain anisotropic materials. The blue color and yellow color in the picture stand for the opposite direction of reflection.

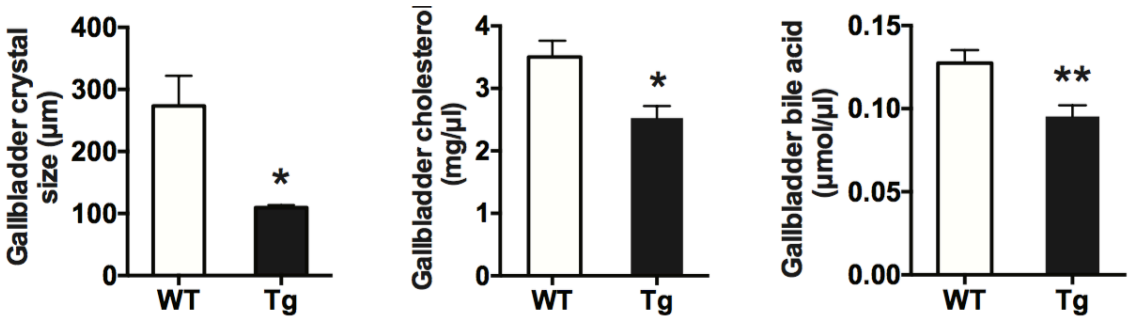

Figure 2-10. Lower cholesterol contents in the gallbladder of *CREBH*-Tg mice on an LD.

Cholesterol, triglyceride and bile acid levels were measured in the gallbladder of *CREBH*-Tg mice fed with an LD (n = 8-15). Results are presented as means ±SEM. **p < 0.01, *p < 0.05.

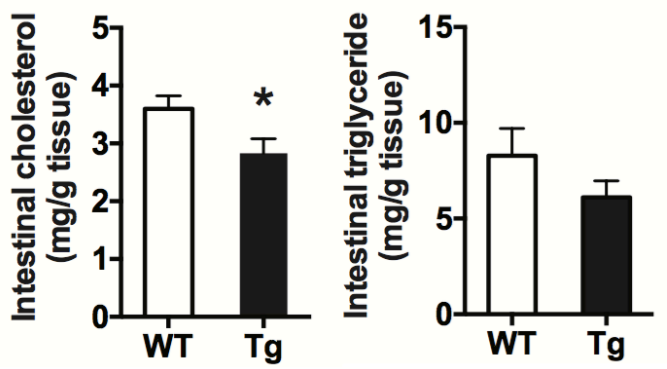

Figure 2-11. Lower cholesterol levels in the intestine of *CREBH*-Tg mice on an LD.

Intestinal cholesterol (n = 6/group) and triglyceride (n = 6/group) levels were measured from WT and *CREBH*-Tg mice fed an LD. Results are presented as means ±SEM. *p < 0.05.

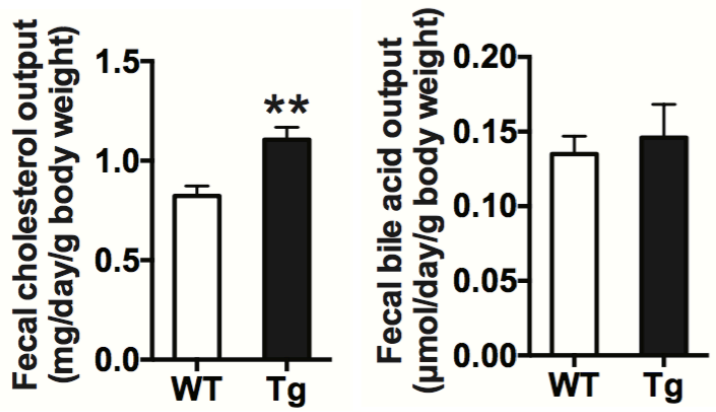

Figure 2-12. Increased fecal cholesterol output in *CREBH*-Tg mice on an LD.

Fecal cholesterol (n = 6/group) and bile acid output (n = 7-9/group) were measured from WT and *CREBH*-Tg mice fed an LD. Results are presented as means ±SEM. **p < 0.01.

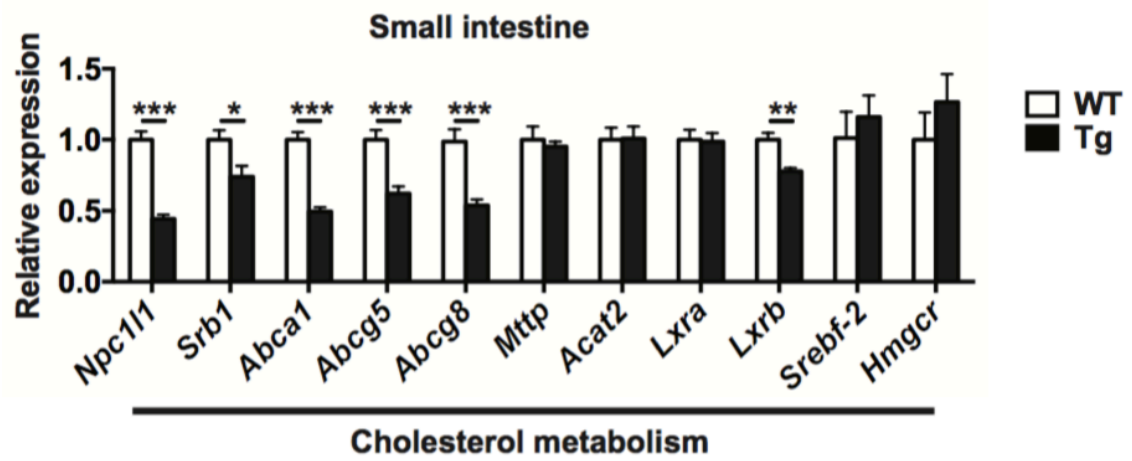

Figure 2-13. Reduced intestinal cholesterol transporter expression in CREBH-Tg mice.

Intestinal mRNA were extracted from WT and *CREBH*-Tg mice fed an LD. Real time-PCR was performed to determine the expression of intestinal cholesterol transporters (n = 6-14/group). Results are presented as means ±SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

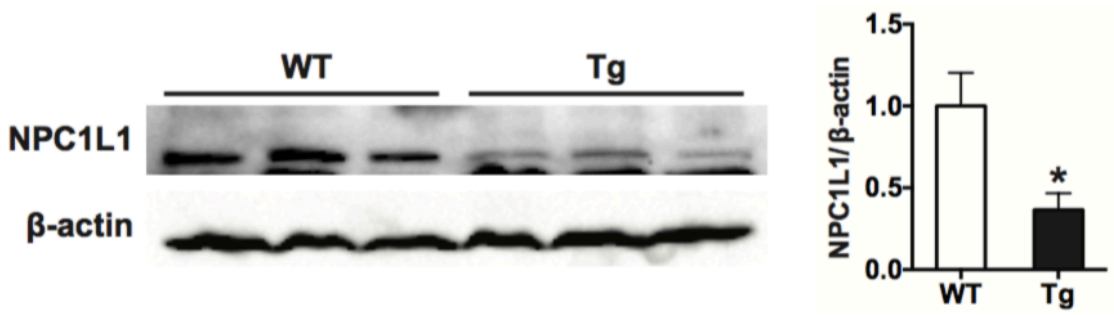

Figure 2-14. Reduced intestinal *NPC1L1* levels in *CREBH*-Tg mice.

Intestinal protein was extracted from WT and *CREBH*-Tg mice. Western blotting was performed to determine Npc111 protein levels. Results are presented as means ±SEM. *$p < 0.05$.

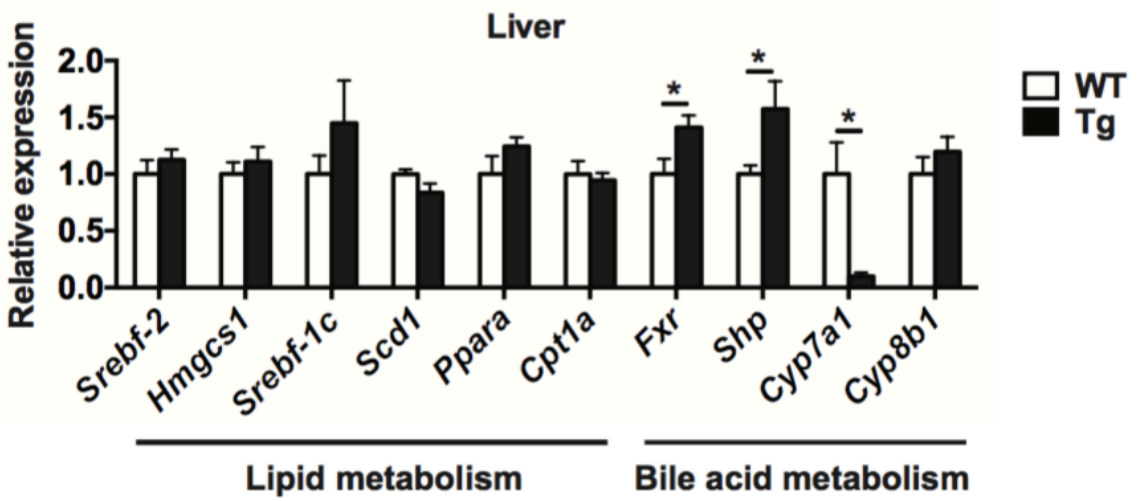

Figure 2-15. Hepatic genes involved in lipid synthesis and cholesterol conversion in *CREBH*-Tg mice.

Real time-PCR was performed to check hepatic genes involved in lipid synthesis and cholesterol conversion (n = 6-7/group). Results are presented as means ±SEM. *p < 0.05.

Chapter 3

Mechanism of intestinal CREBH in cholesterol homeostasis

3-1 *Introduction*

CREBH is a transcriptional factor, which binds to the promoter region of its target genes and regulates the expressions. It has been reported that hepatic CREBH binds to the promoter region of target genes such as PEPCK, G6PC [39] and ApoA4 [40]. Since NPC1L1 is known to be a rate limiting-cholesterol transporter in the jejunum [12,28] and *Npc1l1* expression was reduced in the *CREBH*-Tg mice, we investigated whether intestinal CREBH binds to the Npc1l1 promoter region and regulates the promoter activity.

3-2 *Material and methods*

Cell culture, transfection, and luciferase assay

Caco-2 cells were grown routinely in flasks at 37ºC in a 5% CO2-95% air environment. Caco-2 cells were seeded at a density of 4 x 10^5 cells into 24-well plates. Twenty four hours later, 0.3 kbp-mouse Npc1l1 promoter-luciferase constructs and expression vector for CREBH and/or SREBP-2 were transfected using lipofectamine

3000 (Invitrogen, Grand Island, NY, USA). Renilla expression construct was co-transfected as an internal control for transfection efficiency. 48 hours after transfection, firefly luciferase activity was measured with a luciferase assay system (PicaGene, Toyo-Inki, Tokyo, Japan), and renilla activity was examined with renilla reporter assay system (Promega, Madison, WI, USA). The promoter activity was described as a ratio of luciferase to renilla activity in each sample.

Electrophoretic mobility shift assay

We generated the HA-tagged active form of CREBH from an expression vector using an in vitro reticulocyte transcriptionetranslation system (Promega). We used the following sequences in the electrophoretic mobility shift assays (EMSAs): 5'-ggaagttgacctcagaaggaggagatggaatggca-3' for -106 to -69 of the Npc1l1 promoter; 5'-ggcaccatctgatgtaagggaagagaaaataaattattaa-3' for -75 to -33 of the Npc1l1 promoter; 5'-gagaaaataaattattaaccagtacgg-3' for -53 to -23 of the Npc1l1 promoter; and 5'-gtacggcccagtcctattggccccatgacagacgagg-3' for -32 to +5 of the Npc1l1 promoter. 5'-gtacggcccagtcctattggccccTAATATAacgagg-3' for -32 to +5 of mutant Npc1l1 promoter. We incubated the in vitro-translated protein lysates and anti-HA antibodies (12CA5, Santa Cruz) in a reaction mixture as previously described and resolved the DNA protein complexes on a 4% polyacrylamide gel.

3-3 *Results*

CREBH suppressed the transcriptional activity of NPC1L1

To determine whether CREBH regulates the Npc1l1 promoter activity, luciferase assay was performed using Caco-2 cells. The 300 bp Npc1l1 promoter was inserted into a luciferase vector. We found that CREBH reduced mouse Npc1l1 promoter activity in Caco-2 cells (Fig. 3-1). It has been reported that intestinal SREBP-2 increased Npc1l1 promoter activity [14]. In this study, we observed that CREBH reduced SREBP-2-induced the Npc1l1 promoter activity (Fig. 3-2). These data suggested that CREBH suppresses the Npc1l1 promoter activity, including in the condition, in which SREBP2 induces *Npc1l1* expression, such as upon LD-feeding.

CREBH binds to the mouse Npc1l1 promoter

To confirm the binding site of CREBH in the mouse Npc1l1 promoter, we performed an EMSA assay. In vitro translated CREBH proteins bound to the -32 and +5 bp region of the Npc1l1 promoter, but not to other regions (Fig. 3-3). Competing with the mutant probe reduced the binding of CREBH to the Npc1l1 promoter (Fig. 3-4). In the mutant probe, -7 to -2 bp was mutated in the -32 to +5 sequence of the Npc1l1 promoter. The -7 to -2 bp sequence of the Npc1l1 promoter is similar to the CREBH

responsive element of PEPCK [38].

3-4 *Discussion*

These data demonstrated that CREBH directly binds to the Npc1l1 promoter region and reduces Npc1l1 promoter activity. Several papers reported that intestinal *NPC1L1* expression is affected by PPARα, PPARδ, and SREBP-2 [41-44]. With the luciferase assay using a 300 bp-mouse *Npc1l1* promoter, we observed that SREBP-2 increased Npc1l1 promoter activity and CREBH reduced SREBP-2-induced NPC1L1 promoter activity. This finding suggested that intestinal SREBP-2 and CREBH are coordinately involved in the process that ensures balanced systemic cholesterol levels.

From the data of EMSA, we showed that CREBH binds to the -32 to +5 bp sequence of the Npc1l1 promoter. Moreover, a -7 to -2 bp sequence of mutant Npc1l1 promoter probe reduced the binding of CREBH to Npc1l1 promoter. The sequence is similar to the CREBH responsive element of PEPCK [39]. Therefore, the -7 to -2 bp sequence was considered as the CREBH responsive element in the Npc1l1 promoter.

3-5 *Conclusion*

These *in vitro* studies using Caco-2 cells indicate that CREBH directly binds to the mouse Npc1l1 promoter and reduced the transcriptional activity. Moreover,

EMSA data suggested that the -7 to -2 bp sequence of Npc1l1 promoter is the CREBH binding element. NPC1L1 is a rate-limiting intestinal cholesterol transporter. Therefore, CREBH may prevent LD-induced hypercholesterolemia by directly reducing *Npc1l1* expression.

3-6. Figures

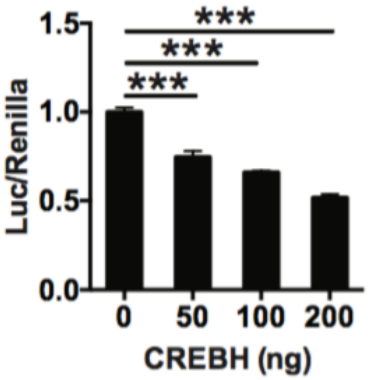

Figure 3-1. CREBH reduces Npc1l1 promoter activity.

Caco-2 cells were transiently transfected with mouse Npc1l1 promoter vector along with empty vector or nuclear form of CREBH vector in a dose-dependent manner (n = 3/group). Results are presented as means ±SEM. ***$p < 0.001$.

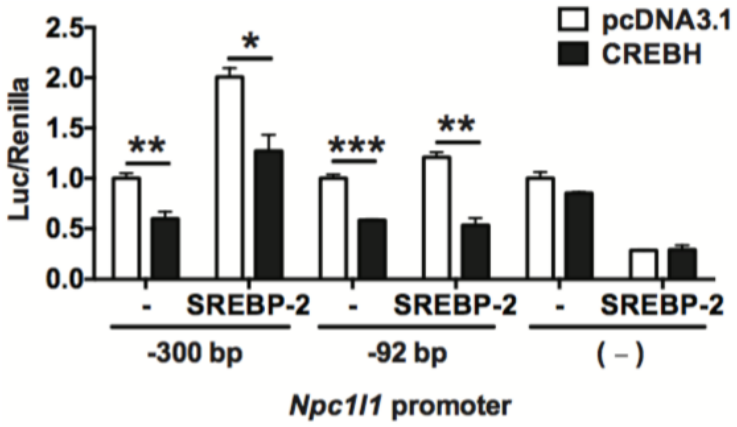

Figure 3-2. CREBH reduces SREBP-2-induced Npc1l1 promoter activity.

The effects of CREBH and SREBP-2 on a series of *Npc1l1* promoter luc vectors were determined (n = 3/group). Results are presented as means ±SEM. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$.

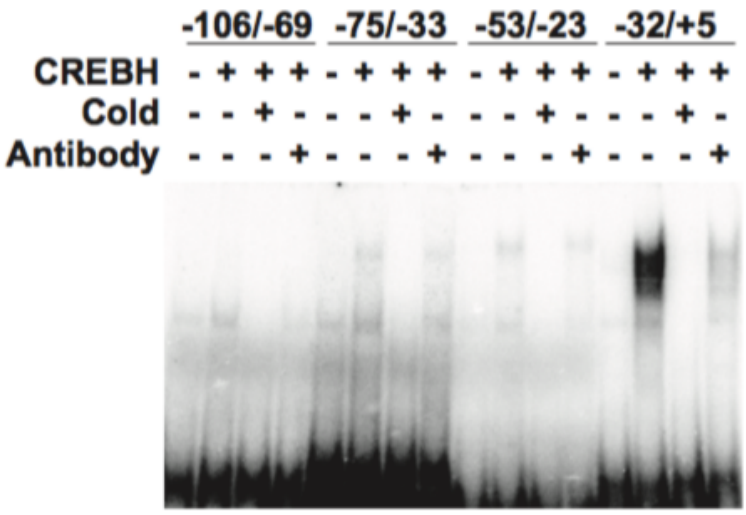

Figure 3-3. CREBH binds to the Npc1l1 promoter.

An EMSA assay indicated that CREBH directly bound to the region from -32 to +5 bp of Npc1l1 promoter.

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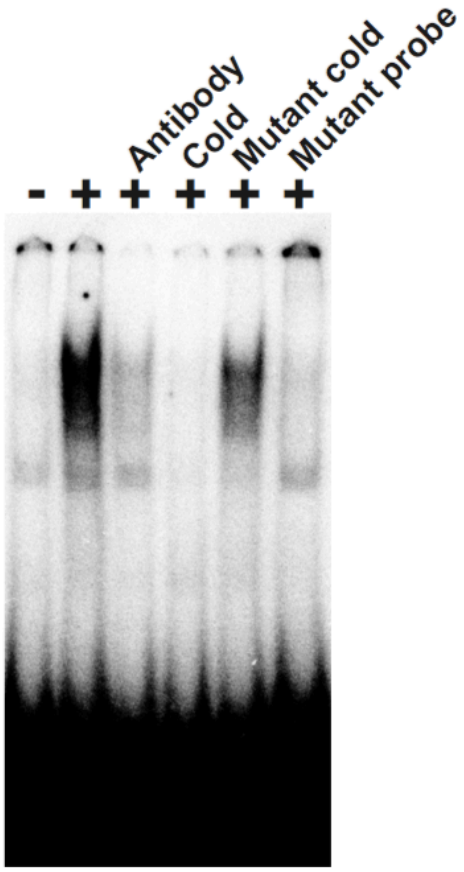

Figure 3-4. A mutant probe reduces the binding of CREBH to the Npc1l1 promoter.

A mutant probe of Npc1l1 promoter was used as the compepitor to examine whether CREBH binds to the Npc1l1 promoter. The mutant probe was an oligonucleotide corresponding to the -32 to +5 bp sequence of Npc1l1 promoter, in which -7 to -2 bp was mutated.

Chapter 4

Analysis of metabolic phenotype of LD-fed *CrebH* null mice

4-1. *Introduction*

Since we showed that *CrebH* overexpression reduces intestinal *Npc1l1* expression and prevented LD-induced hypercholesterolemia, we next examined the effect of *CrebH* depletion on cholesterol homeostasis. Previous studies identified severe hypertriglycemia in *CrebH* null mice [22-24], however, the effects of *CrebH* depletion on systemic cholesterol levels were not fully elucidated. In this study, we used *CrebH* null mice and examine the metabolic phenotype when the mice were fed with a LD.

4-2. *Material and methods*

Animals and diets

Creb3l3$^{tm1.1Sad}$*/J* (*CrebH* null) mice were purchased from The Jackson Laboratory. *CrebH* null were housed as *CREBH*-Tg mice. All animal husbandry and animal experiments were consistent with the University of Tsukuba's Regulations of Animal Experiments and were approved by the Animal Experiment Committee of the University of Tsukuba.

Plasma, hepatic, and gallbladder bile lipid and gallstone analysis

The experimental procedure was described in chapter 2-2 .

Fecal cholesterol and bile acids output

The experimental procedure was described in chapter 2-2 .

Analysis of gene expression

The experimental procedure was described in chapter 2-2.

Statistical analysis

All results are presented as means ±SEM. Data were analyzed using Student's t-test, and considered statistically significant if $p < 0.05$.

4-3 *Results*

LD-fed *CrebH* null mice showed severe hypercholesterolemia.

To determine the effect of *CrebH* depletion on cholesterol metabolism, *CrebH* null mice were fed with an LD. The plasma, liver, and intestinal cholesterol

levels were measured. On contrary to the phenotype of LD-fed *CREBH* Tg mice, *CrebH* null mice showed higher levels of plasma, liver, and intestinal cholesterol, compared to the WT mice (Fig. 4-1,2,3). The representative pictures of the liver and H&E staining of liver section from WT and KO mice are shown. Severe fatty liver was observed in LD-*CrebH* null mice (Fig. 4-4).

LD-fed *CrebH* null mice showed increased fecal output and a tendency of increased intestinal *Npc1l1* expression.

CrebH null mice had lower fecal cholesterol output (Fig. 4-5). Real time-PCR was performed to assess jejunal gene expression in LD-fed *CrebH* null mice. Jejunal *Npc1l1* expression was increased in a chow-fed *CrebH* null mice, however, there was no clear difference between WT and *CrebH* null mice under LD-feeding (Fig. 4-6). It is known that increased systemic cholesterol levels suppress *Npc1l1* expression. Thus, severe hypercholesterolemia might inhibit the *Npc1l1* expression in LD-fed *CrebH* null mice. It is still expected that increased jejunal *Npc1l1* expression in initial stage of LD feeding contributed to the metabolic changes of *CrebH* null mice fed with a LD for 2 weeks. It should also be mentioned that hepatic *CrebH* null depletion would also lead to the hypercholesterolemic phenotype, as we previously reported that *CrebH* null affects hepatic *Srebf2* and the expression of its target genes [45].

4-4 *Discussion*

These results showed severe hypercholesterolemia in LD-fed *CrebH* null mice, compared to WT mice. This is the opposite outcome compared to LD-fed *CREBH*-Tg mice. In *CrebH* null mice, both hepatic and intestinal *CrebH* expression were depleted, thus, it can not be determined whether the metabolic phenotype was derived from hepatic and/or intestinal *CrebH*. We observed that intestinal *Npc1l1* expression was significantly increased in chow-fed *CrebH* null mice and tended to increase in LD-fed *CrebH* null mice. Thus, the increase of intestinal cholesterol transport may partially explain the severe hypercholesterolemia in *CrebH* null mice.

We previously reported that liver-specific *CrebH* null mice fed with a chow diet showed higher plasma cholesterol, triglyceride and NEFA levels compared to control mice. Hepatic cholesterol synthesis gene expression is altered in the liver of liver-specific *CrebH* null mice, which causes increased plasma cholesterol levels [22]. A recent paper also mentioned that hepatic CREBH decreased plasma cholesterol levels in *Ldlr*$^{-/-}$ mice [46]. Therefore, both intestinal and hepatic CREBH are involved in cholesterol metabolism. Interestingly, we observed decreased plasma cholesterol levels in chow-fed CREBH null mice, which is consistent with the data from an earlier paper [47]. These observations suggest that multiple hepatic genes may be related to the

alteration of cholesterol metabolism in *CrebH* null mice. Another paper reported that plasma cholesterol levels was not significantly changed in *CrebH* null mice fed with a diet containing 1.25% and 0.5% cholic acids for 6 months [22], although we observed increased plasma cholesterol levels in *CrebH* null mice fed with a LD for 2 weeks. A high cholesterol diet suppresses intestinal *Npc1l1* expression, therefore, is difficult to observe the difference of plasma cholesterol levels between WT and *CrebH* null mice under a long term feeding of a high cholesterol diet.

4-5 *Conclusion*

CREBH depletion led to severe hypercholesterolemia in mice fed with an LD. In addition, CREBH depletion increased cholesterol output and tendency of increased intestinal cholesterol *Npc1l1* expression. Our observations suggest that intestinal CREBH plays an important role in cholesterol homeostasis.

4-6. Figures

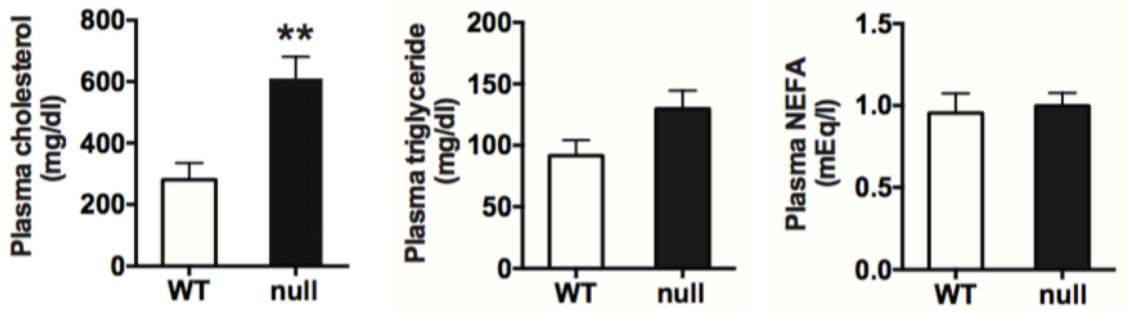

Figure 4-1. Hypercholesterolemia in LD-fed *CrebH* null mice.

Plasma cholesterol (n = 6-11/group), triglyceride (n = 10/group) and NEFA (n = 10/group) levels in LD-fed WT and *CrebH* null mice. Results are presented as means ±SEM. **$p < 0.01$.

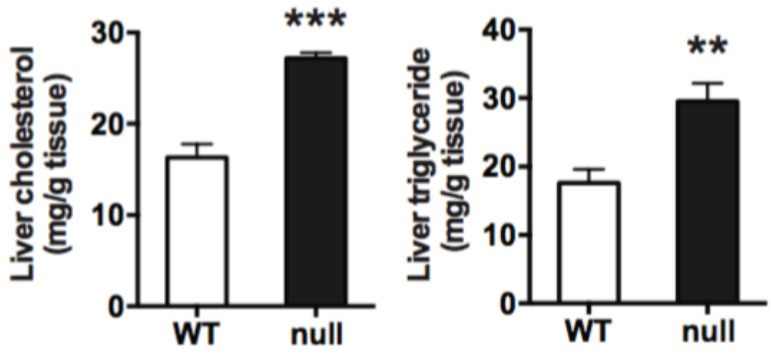

Figure 4-2. Increased cholesterol and triglyceride content in LD-fed *CrebH* null mice.

Liver cholesterol and triglyceride levels in LD-fed WT and *CrebH* null mice were measured (n = 6-10/group). Results are presented as means ±SEM. ***p < 0.001, **p < 0.01.

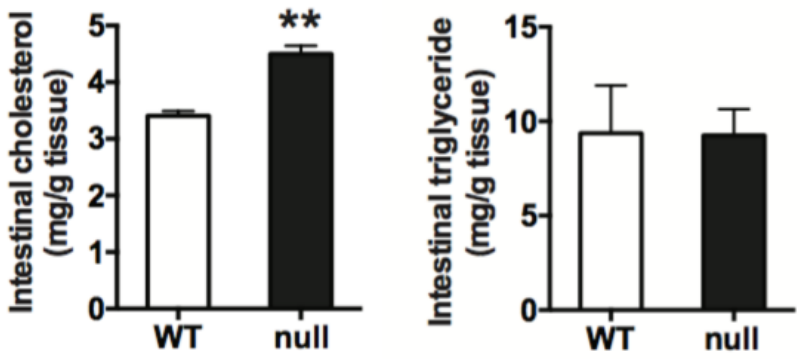

Figure 4-3. Increased intestinal cholesterol levels in LD-fed *CrebH* null mice.

Fecal cholesterol output was examined in WT and *CrebH* null mice (n = 4-5/group). Results are presented as means ±SEM. **p < 0.01.

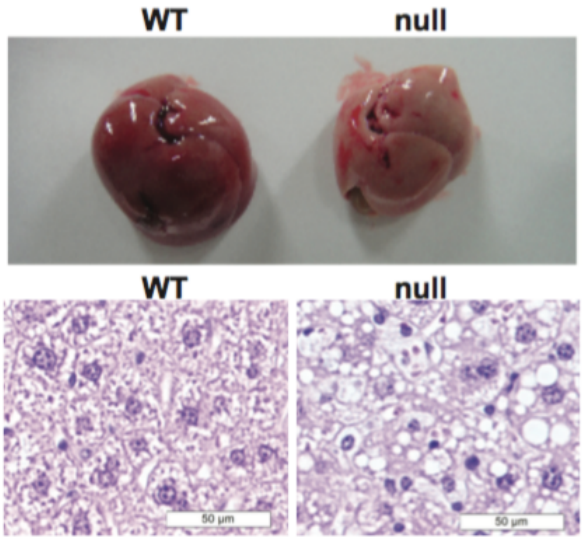

Figure 4-4. Severe lipid accumulation was observed in the liver of LD-fed *CrebH* null mice.

Representative pictures of liver from LD-fed WT and *CrebH* null mice are shown, as well as H&E stainings of liver sections from LD-fed WT and *CrebH* null mice.

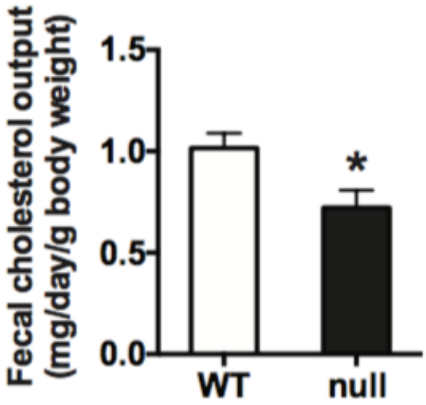

Figure 4-5. Reduced fecal cholesterol output in LD-fed *CrebH* null mice.

Fecal cholesterol output was examined in WT and *CrebH* null mice (n = 4-5/group). Results are presented as means ±SEM. *$p < 0.05$.

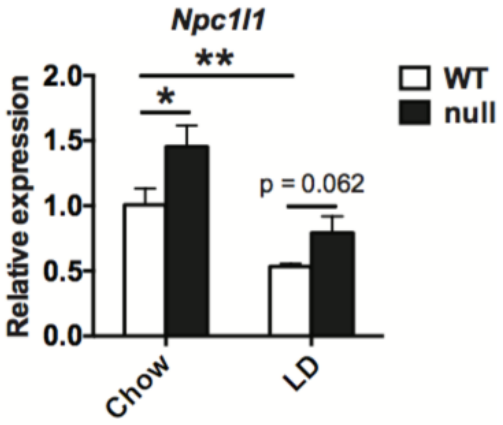

Figure 4-6. Intestinal expression of *Npc1l1* in chow-fed and LD-fed WT and *CrebH* null mice

Intestinal *Npc1l1* expression of chow fed and LD-fed WT and *CrebH* null mice were determined by real time-PC. Results are presented as means ±SEM. **p < 0.01, *p < 0.05.

Chapter 5

Conclusions

CREBH is a transcription factor expressed selectively in the liver and intestine. Its metabolic effect has recently been investigated. Although the mechanism underlying anti-hypertriglyceridemic effects of hepatic CREBH was reported, the roles of intestinal CREBH remains to be determined. The present study demonstrated an anti-hypercholesterolemic effect of intestinal CREBH in the mice fed with a LD. *CREBH* overexpression reduced the expression of intestinal cholesterol transporters, including NPC1L1, ABCA1, SRB1 and ABCG5/8. LD-fed *CREBH*-Tg mice attenuated systemic hypercholesterolemia, accompanied with increased fecal cholesterol output. These findings suggests decreased intestinal cholesterol absorption in LD-fed *CREBH*-Tg mice. In addition, we found that CREBH decreased NPC1L1 promoter activity with *in vitro* experiments using Caco-2 cells. Collectively, These results suggest that intestinal CREBH reduces intestinal absorption of dietary cholesterol at least via regulating *Npc1l1* expression.

There was no significant change of systemic cholesterol levels in chow-fed *CREBH*-Tg mice. Considering that *Npc1l1* depletion did not reduce plasma cholesterol

levels in chow-fed mice [3], a plasma cholesterol-lowering effect by intestinal *CREBH* overexpression may be observed in a LD-fed state, but not in a chow-fed state.

As recent studies demonstrated that CREBH regulates ApoA4 and ApoC2 expression [46-48], we found the increased expression of ApoA4 and ApoC2 in the intestine of *CREBH*-Tg mice while decreased expression of ApoA4 and ApoC2 in *CrebH* null mice. ApoA4 and ApoC2 are involved in intestinal chylomicrons secretion. However, we found that fecal triglyceride output was not significantly changed by *CREBH* overexpression. This is consistent with the result of a previous paper showing that *CrebH* depletion did not affect dietary fat absorption [39].

This work suggests that intestinal CREBH functions as a metabolic regulator to prevent diet-induced hypercholesterolemia and cholelithiasis, at least via affecting expression of *Npc1l1*. This may indicate that the combination of therapeutic effects of hepatic and intestinal CREBH could be a future therapeutic target in the treatment of dyslipidemia and its related metabolic diseases.

5. Figure

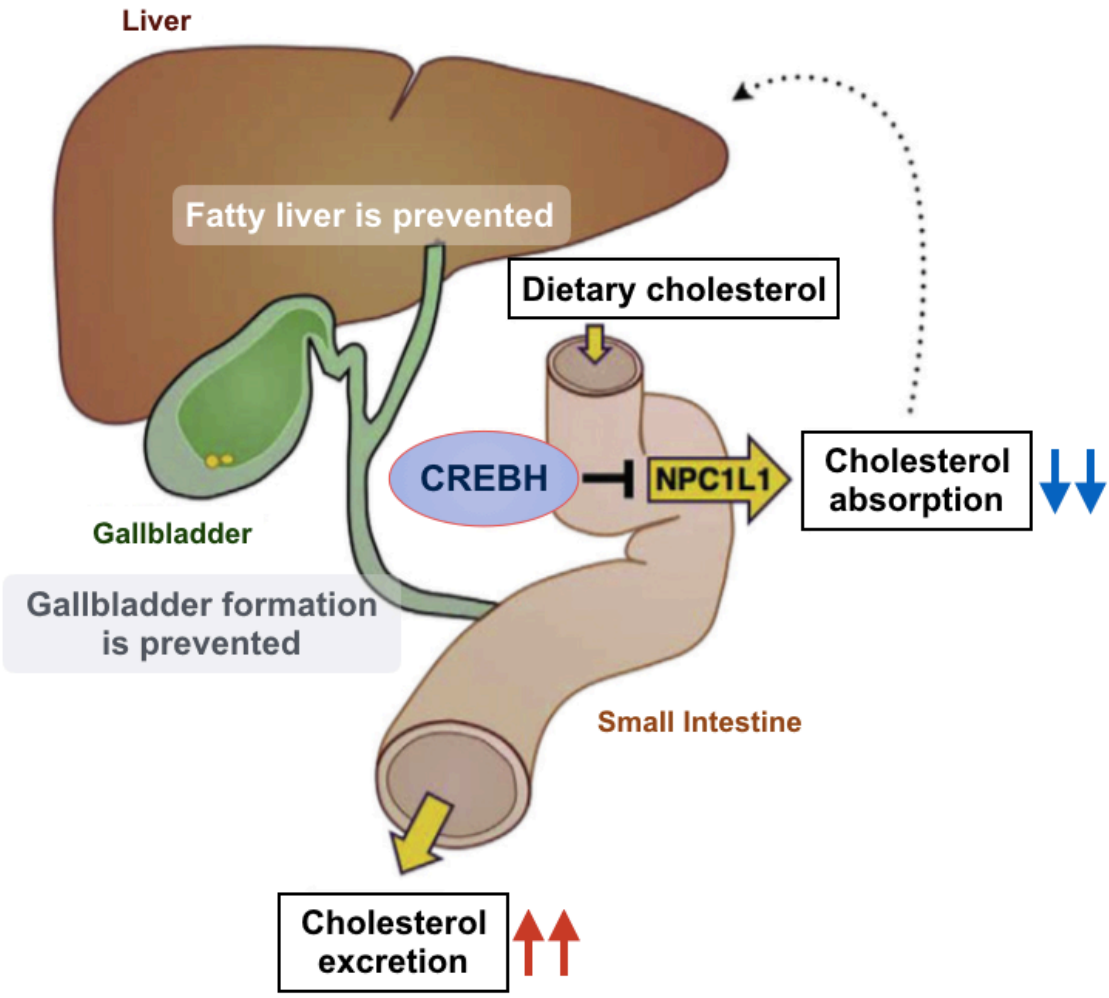

Figure 5. Intestinal CREBH reduces intestinal expression of NPC1L1 and prevents LD-induced systemic hypercholesterolemia and cholelithiasis

List of abbreviations

ABCA1	ATP-binding cassette transporter A1
ABCG5/8	ATP-binding cassette transporter G5/8
ACAT2	Acyl cholesterol acyltransferase-2
ALT	Alanine aminotransferase
AST	Aspartate aminotransferase
CREBH	Cyclic AMP-responsive element-binding protein 3-like 3
FGF21	Fibroblast growth factor 21
HL	Hepatic lipase
HMGCR	3-hydroxy-3-methylglutaryl coenzyme A reductase
HPLC	High performance liquid chromatography
IGFBP2	Insulin growth factor binding protein 2
LD	Lithogenic diet
LDL	Low-density lipoprotein
LDLR	Low-density lipoprotein receptor
LPL	Lipoprotein lipase
LPS	Lipopolysaccharide
NAFLD	Non-alcoholic fatty liver disease
NASH	Non-alcoholic steatohepatitis

NEFA	Non-esterified fatty acids
NPC1L1	Niemann-Pick C1-like 1
PPAR	Peroxisome proliferator-activated receptor
SREBP	Sterol response element-binding proteins
SRB1	Scavenger receptor B1
VLDL	Very low-density lipoproteins
WAT	White adipose tissue

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