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Original Article

MOLECULAR METABOLISM

Intestinal CREBH overexpression prevents high-cholesterol diet-induced hypercholesterolemia by reducing *Npc1l1* expression

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ABSTRACT

Objective: The transcription factor cyclic AMP-responsive element-binding protein H (CREBH, encoded by *Creb3l3*) is highly expressed in the liver and small intestine. Hepatic CREBH contributes to glucose and triglyceride metabolism by regulating fibroblast growth factor 21 (*Fgf21*) expression. However, the intestinal CREBH function remains unknown.

Methods: To investigate the influence of intestinal CREBH on cholesterol metabolism, we compared plasma, bile, fecal, and tissue cholesterol levels between wild-type (WT) mice and mice overexpressing active human CREBH mainly in the small intestine (CREBH Tg mice) under different dietary conditions.

Results: Plasma cholesterol, hepatic lipid, and cholesterol crystal formation in the gallbladder were lower in CREBH Tg mice fed a lithogenic diet (LD) than in LD-fed WTs, while fecal cholesterol output was higher in the former. These results suggest that intestinal CREBH overexpression suppresses cholesterol absorption, leading to reduced plasma cholesterol, limited hepatic supply, and greater excretion. The expression of Niemann—Pick C1-like 1 (*Npc1l1*), a rate-limiting transporter mediating intestinal cholesterol absorption, was reduced in the small intestine of CREBH Tg mice. Adenosine triphosphate-binding cassette transporter A1 (*Abca1*), *Abcg5/8*, and scavenger receptor class B, member 1 (*Srb1*) expression levels were also reduced in CREBH Tg mice. Promoter assays revealed that CREBH directly regulates *Npc1l1* expression. Conversely, CREBH null mice exhibited higher intestinal *Npc1l1* expression, elevated plasma and hepatic cholesterol, and lower fecal output.

Conclusion: Intestinal CREBH regulates dietary cholesterol flow from the small intestine by controlling the expression of multiple intestinal transporters. We propose that intestinal CREBH could be a therapeutic target for hypercholesterolemia.

Keywords CREBH; *Npc1l1*; Cholesterol; Intestine

1. INTRODUCTION

Cholesterol absorption in the gut has been studied extensively because of its significant positive correlation with plasma cholesterol concentration, which, in turn, is a major risk factor for atherosclerosis [1,2]. Cholesterol absorption depends on transport from the intestinal lumen across enterocytes into the plasma [3]. Stimulation of reverse cholesterol transport (RCT), which disposes endogenous cholesterol through feces, can inhibit the development of atherosclerosis. Although the hepato-biliary system is considered the dominant route for RCT, it

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Abbreviations: Abca1, ATP-binding cassette, sub-family A1; ABCG5/8, adenosine triphosphate-binding cassette transporter G5/G8; ALT, alanine aminotransferase; Apoa4, apolipoprotein A-IV; AST, aspartate aminotransferase; CREBH, cyclic AMP-responsive element-binding protein H; Cpt1a, carnitine palmitoyltransferase 1a, liver; Cyp7a1, cytochrome P450, family 7, subfamily a, polypeptide 1; ER, endoplasmic reticulum; FGF21, fibroblast growth factor 21; FXR, Farnesoid X receptor; LD, lithogenic diet; LPL, lipoprotein lipase; LXR, liver X receptor; NEFA, non-esterified fatty acids; NPC1L1, Nieman Pick C1-like 1; PPARα, proliferator activated receptor alpha; RCT, reverse cholesterol transport; Shp, small heterodimer partner; Srb1, scavenger receptor class B, member 1; Srebf, sterol regulatory element-binding factor; SREBP, sterol regulatory element-binding protein; TG, triglyceride; WT, wild type

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has been demonstrated that the small intestine also excretes large amounts of endogenous cholesterol [4,5]. Cholesterol uptake from the lumen by enterocytes is the rate-limiting step in cholesterol absorption [6], and NPC1L1 plays a pivotal role in this process [7,8]. NPC1L1 is exclusively expressed in enterocytes of the proximal small intestine [8]. The clinical anti-hypercholesterolemia drug ezetimibe (Zetia) lowers plasma cholesterol levels by inhibiting NPC1L1 [8,9]. Ezetimibe treatment in mice increased fecal neutral sterol excretion without altering hepato-biliary cholesterol disposal into the bile [10]. ATP binding cassette subfamily G isoforms G5 and G8 are expressed in both the liver and small intestine where they heterodimerize into a functional transporter [11] to promote sterol secretion, with a preference for plant sterols over cholesterol [12]. In the liver, they are expressed on the apical membrane of hepatocytes [13] and secrete both cholesterol and plant sterols into the bile [14,15]. In the small intestine, they are presumed to be expressed on the apical brush border membrane of enterocytes and secrete cholesterol and plant sterols into the intestinal lumen [16].

CREBH is a basic leucine zipper domain transcriptional factor of the CREB/activating transcription factor family [17]. *CrebH* is highly and selectively expressed in gastrointestinal tract tissues, including the liver, pyloric stomach, duodenum, and ileum [18]. Hepatic *CrebH* mRNA expression is regulated by fasting and re-feeding, with nuclear levels of active CREBH increasing in times of starvation [19]. Translated CREBH protein localizes to the endoplasmic reticulum (ER) before transfer to the Golgi apparatus, where the transcriptionally active N-terminal region is cleaved prior to translocation to the nucleus [19]. CREBH and peroxisome proliferator activated receptor alpha (PPARα) synergistically activate hepatic fibroblast growth factor 21 (*Fgf21*) expression and exert effects on energy metabolism through the modulation of plasma FGF21 levels [20,21]. CREBH reduces plasma triglyceride (TG) levels by increasing hepatic gene expression of apolipoproteins such as apolipoprotein A-IV (*Apoa4*), *Apoa5*, and *Apoc2*. These apolipoproteins activate plasma lipoprotein lipase (LPL), resulting in reduced plasma TG levels. However, the function of intestinal CREBH remains unclear.

To investigate the functions of intestinal CREBH, we generated CREBH Tg mice and examined effects of CREBH overexpression on cholesterol metabolism.

2. MATERIALS AND METHODS

2.1. Animals and diets

Wild-type (WT) C57/BL6J mice were obtained from CLEA Japan. To generate CREBH Tg mice, cDNAs encoding the rat *Pck1* promoter, active human CREBH (amino acids 1−320), and the 3′ polyadenylation signal of human growth hormone were microinjected into C57BL6J eggs [21]. *Creb3l3*$^{tm1.1Sad}$*/J* (CREBH null) mice [18] were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). All mice were housed in a pathogen-free barrier facility under a 12-h light/dark cycle and given free access to water. Mice were fed a normal chow diet (MF: Oriental Yeast, Tokyo, Japan) or a lithogenic diet (LD) (16.5% fat, 1.25% cholesterol, 0.5% cholic acid (CA); F2HFD1; Oriental Yeast) (F2HFD1: Oriental Yeast, Tokyo, Japan) [22] for 2 weeks. All animal husbandry and experimental protocols conformed to the University of Tsukuba Regulations of Animal Experiments and were approved by the Animal Experiment Committee of the University of Tsukuba.

2.2. RNA purification and real time-PCR

Total RNA was extracted from frozen mouse tissues using Sepasol (Nakarai Tesque, Kyoto, Japan). For the analysis of jejunal gene expressions, the first 15% length from the pyloric sphincter to the 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 the 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 control mice using the ΔΔCT method.

2.3. Immunoblotting

Immunoblotting of whole cell lysates was performed as described previously [23]. Protein expression levels of NPC1L1 were analyzed in small intestine lysates using a rabbit anti-NPC1L1 antibody (Novus Biologicals cat. NB400-127).

2.4. Histological analysis

The liver was fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E).

2.5. Cell culture, transfection, and luciferase assay

Caco-2 cells were maintained in DMEM supplemented with 10% fetal bovine serum (FBS) and penicillin-streptomycin at 37 °C in a humidified 5% CO_2/95% air environment. Mouse *Npc1l1* promoter-luciferase constructs and expression vectors for CREBH and/or SREBP-2 were co-transfected into Caco-2 cells using lipofectamine 3000 (Invitrogen, Grand Island, NY, USA). A renilla expression construct was co-transfected as an internal control for transfection efficiency. At 48 h after transfection, luciferase and renilla activities were measured using commercial assay systems (Luciferase: PicaGene, Toyo-Inki, Tokyo, Japan; Renilla: Promega, Madison, WI, USA). The promoter activity was expressed as the ratio of luciferase to renilla activities in each sample.

2.6. Electrophoretic mobility shift assay

We generated the HA-tagged active form of CREBH from an expression vector using an *in vitro* reticulocyte transcription−translation 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. We incubated the *in vitro*-translated protein lysate and anti-HA antibodies (12CA5, Santa Cruz) in a reaction mixture as previously described [23] and resolved the DNA−protein complexes on a 4% polyacrylamide gel.

2.7. Plasma, hepatic, and gallbladder bile lipid and gallstone analysis

Plasma and liver parameters [23], hepatic bile acids [22], and gallbladder bile [22] were analyzed as described previously. Gallbladders bile was examined for monohydrate cholesterol crystals under polarizing light microscopy.

2.8. Fecal cholesterol and bile acids output

Fecal cholesterol and bile acid outputs were measured as described [24,25]. Briefly, after 2 weeks of normal or LD feeding, mice were individually housed for fecal collection. The feces were dried, weighed, and crushed into powder. Fecal bile acids were extracted from powdered feces with 90% ethanol [24] and concentrations determined

enzymatically using a total bile acids kit (Wako). Fecal cholesterol and triglycerides were extracted from powdered feces with chloroform/methanol (2:1 vol/vol) [25] and concentrations determined enzymatically using a total cholesterol kit (Wako).

2.9. Statistical analysis

All results are presented as mean ± SEM. Treatment group means were compared by unpaired sample Student's t-test and differences were considered statistically significant at $p < 0.05$.

3. RESULTS

3.1. CREBH Tg mice showed lower plasma cholesterol levels than WT mice when fed a LD

Endogenous *CrebH* was broadly expressed in the small intestine, but expression was higher in jejunum than other subregions (Supplementary Figure 1A). As the *Pck1* promoter is active in the liver, kidney, brown adipose, and small intestine [26], CREBH Tg mice overexpressed the active form of human CREBH in small intestine, colon, adipose tissues, and muscle (Supplementary Figure 1A). We confirmed that CREBH Tg mice had 3 copies of transgene in genome. It was speculated that the position effects of the transgenic cassette inserted into the genome happened not to express ectopic CREBH expression in the liver. To confirm the effects of transgene in the liver, we determined *Fgf21* expression, which is the target of CREBH [20,21], in the liver. There was a trend to increase *Fgf21* expression in CREBH Tg mice, but it was not significant (Supplementary Figure 1A). Thus, there was a low effect of transgene in the liver of CREBH Tg mice. To explore the effects of CREBH on cholesterol metabolism, numerous metabolic indices were first compared between WT and CREBH Tg mice fed either a normal chow diet or a LD for 2 weeks. There were no differences in body weight, liver weight, white adipose tissue (WAT) weight, or food intake between WT and CREBH Tg mice fed normal chow or LD (Figure 1A). There was also no difference in plasma cholesterol between genotypes when fed a normal chow diet. However, LD-fed CREBH Tg mice showed 33% lower plasma cholesterol levels ($p < 0.002$) compared to LD-fed WT mice (Figure 1B). HPLC analysis revealed that CREBH Tg mice had lower plasma cholesterol levels in all lipoprotein fractions, while there were no differences in plasma triglyceride levels between genotypes (Figure 1B). Plasma TG, non-esterified fatty acids (NEFA), and bile acid levels did not differ between genotypes when fed a LD (Figure 1B). When fed a normal chow diet, plasma NEFA levels were reduced in CREBH Tg mice, but other parameters (cholesterol, TG, NEFA, and bile acids) did not differ (Figure 1B). An LD-induced plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in CREBH Tg mice has a trend to decrease, but this was not significant (Figure 1B).

3.2. CREBH Tg prevented high cholesterol-diet induced hypercholesterolemia

WT mice fed with LD for 2 weeks had a white liver, but CREBH Tg mice did not (Figure 2A). Histological analysis of liver sections showed that CREBH overexpression decreased lipid droplet accumulation compared to WT mice (Figure 2A). Liver cholesterol and triglyceride levels were significant lower in LD-fed CREBH Tg mice than LD-fed WT mice (Figure 2B). Liver bile acid levels did not differ significantly between genotypes following a LD (Figure 2B). The mean gallbladder size of CREBH Tg mice was not different from WTs (Figure 2C). In this study, we used a LD diet containing 0.5% colic acid, which induces the gallstone formation [27,28]. Gallbladder bile samples from LD-fed WT and CREBH Tg mice were inspected by light polarizing microscopy to determine the presence and appearance of cholesterol monohydrate crystals and sandy stones (Figure 2C). Average crystal stone size was reduced in CREBH Tg mice compared to WT mice following the LD (Figure 2D). Cholesterol and bile acids levels in the gallbladder were also significantly lower in CREBH Tg mice (Figure 2D). CREBH Tg mice had significantly lower intestinal cholesterol levels than WT mice as well (Figure 2E). Conversely, CREBH Tg mice exhibited significantly higher fecal cholesterol output than WT mice, while fecal bile acid output did not differ between genotypes (Figure 2E). These results indicate that CREBH overexpression reduces dietary cholesterol absorption and cholesterol efflux from small intestine, resulting in lower liver and plasma cholesterol levels and higher fecal excretion.

3.3. CREBH Tg mice fed a LD have reduced intestinal cholesterol transporter expression

Based on the finding that CREBH overexpression prevents LD-induced hypercholesterolemia and cholelithiasis, we performed real time-PCR to identify possible changes in the intestinal and hepatic expression levels of lipid metabolism-related genes. Expression levels *Npc1l1* and scavenger receptor class B member 1 (*Srb1*), genes encoding two cholesterol transporters that remove cholesterol from the lumen and into enterocytes, were lower in the small intestine of CREBH Tg mice than WT mice. In addition, expression levels of *Abcg5* and *Abcg8*, encoding sterol transporters that excrete cholesterol from enterocytes into the lumen, were lower in the small intestine of CREBH Tg mice. *Abca1*, which plays a crucial role in HDL biosynthesis [29,30], was also lower in the small intestine of CREBH Tg mice. The expression of liver X receptor β (*Lxrb*), a transcriptional regulator of *Abcg5* and *Abcg8*, was lower in the small intestine of CREBH Tg mice (Figure 3A). Western blotting analysis confirmed that NPC1L1 protein levels were significantly reduced in the small intestine of CREBH Tg mice compared to WTs following a LD (Figure 3B). These results may partially explain how intestinal CREBH overexpression increases fecal cholesterol and decreases plasma cholesterol.

We then compared expression levels of hepatic genes related to cholesterol synthesis and conversion between WT and CREBH Tg mice fed a LD. Expression levels of sterol regulatory element-binding factor (*Srebf*) family members and their target genes that regulate hepatic lipid synthesis did not differ between genotypes. Similarly, hepatic expression levels of the lipid oxidation genes *Ppara* and carnitine palmitoyltransferase 1a (*Cpt1a*) did not differ between genotypes. In contrast, expression levels of farnesoid X receptor (*Fxr*) and small heterodimer partner (*Shp*), genes regulating bile acid synthesis, were significantly higher in CREBH Tg mice, resulting in reduced expression of cytochrome P450 family 7 subfamily a polypeptide 1 (*Cyp7a1*). These expression changes suggest reduced hepatic conversion of cholesterol to bile acids in CREBH Tg mice fed a LD compared to WTs. Therefore, bile acid synthesis was downregulated in liver of CREBH Tg mice presumably due to decreased cholesterol influx from the small intestine.

3.4. CREBH reduced *Npc1l1* promoter activity

To explain the reduced *Npc1l1* expression in CREBH Tg mice, we examined the effects of CREBH on *Npc1l1* promoter activity using a series of mouse *Npc1l1* promoter-luciferase constructs. The mouse *Npc1l1* promoter luc vector and the active form of the CREBH expression vector were co-transfected into the Caco-2 colon cancer cell line. CREBH decreased the *Npc1l11* promoter activity in a dose-

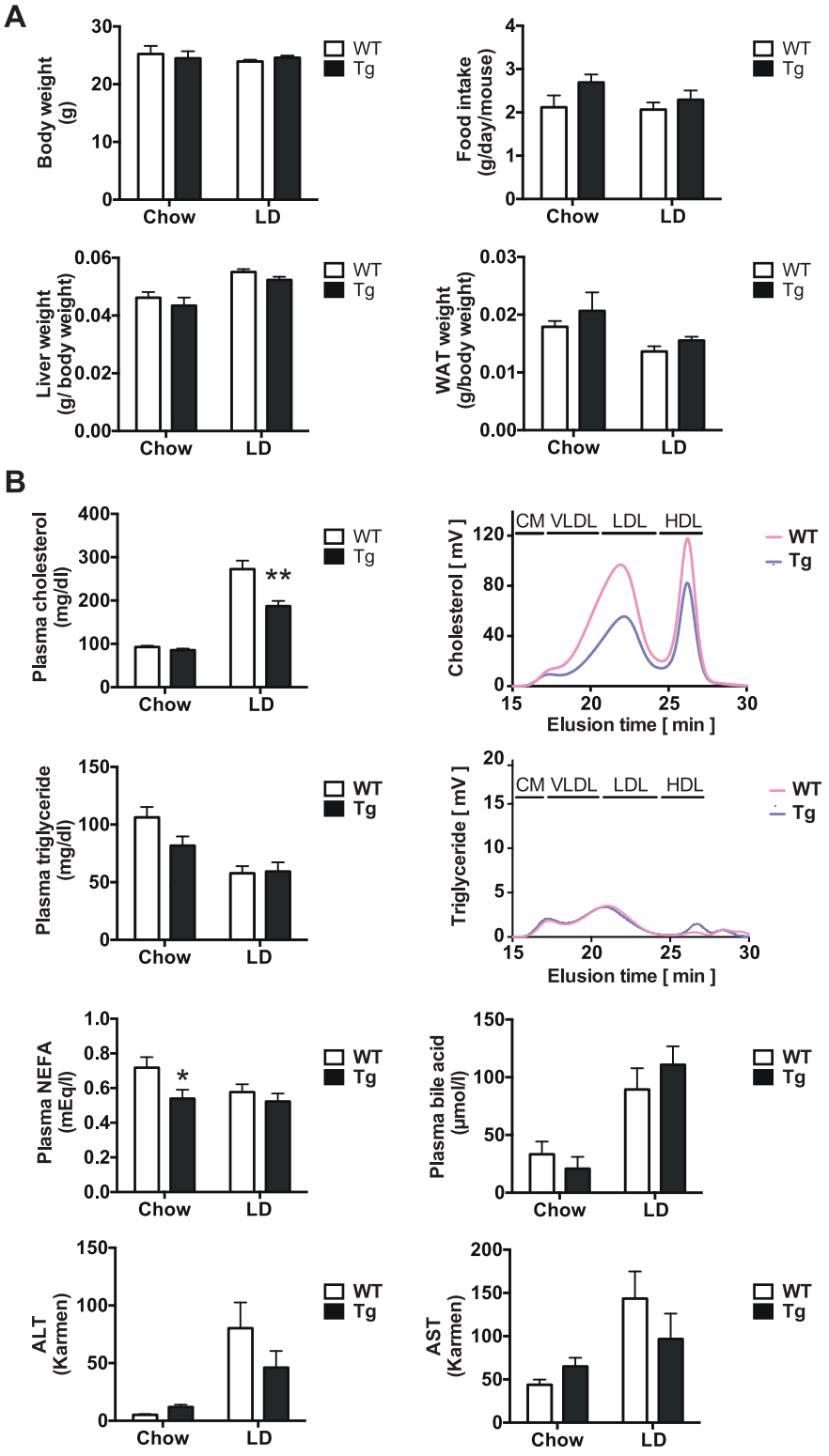

Figure 1: Overexpression of CREBH in the small intestine reduces plasma cholesterol levels in mice fed a lithogenic diet (LD). Eight-week-old male WT and CREBH Tg mice were fed a standard chow diet (Chow) or lithogenic diet (LD) for 2 weeks. (A) Body weight, food intake, liver weight, and white adipose tissue (WAT) weight. (B) Plasma cholesterol (n = 19–31/mice group), triglyceride (n = 10–26/group), NEFA (n = 14–23/group), bile acids (n = 24–38/group), ALT (n = 15–27/group), and AST (n = 15–27/group). Lipoprotein particle distributions were determined by HPLC analysis. Results presented as mean ± SEM. Differences between the genotypes on either nutrient state, $^{**}p < 0.01$, $^{*}p < 0.05$.

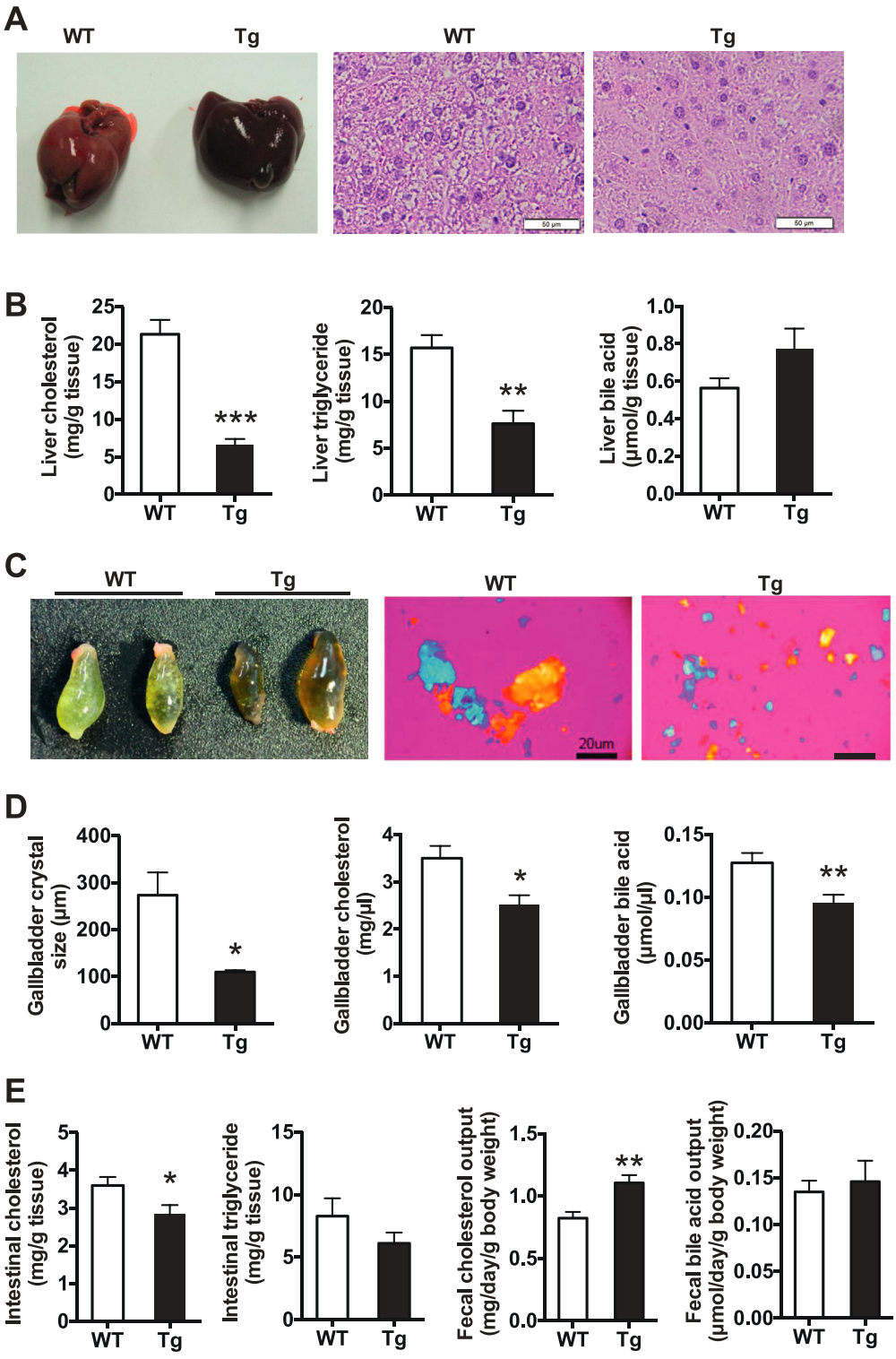

Figure 2: Intestinal CREBH overexpression reduces entero-hepatic system tissue cholesterol in mice fed a LD. (A) The representative pictures of whole livers and liver sections stained with hematoxylin and eosin (H&E) from LD-fed WT and CREB3 Tg mice. (B) Liver cholesterol, triglyceride, and bile acid levels (n = 8–15 mice/group). (C) Representative pictures of the gallbladder and images of gallbladder bile analyzed by light polarizing microscopy for the presence of cholesterol monohydrate crystals and sandy stones. (D) Average gallbladder crystal size in the bile (n = 5/group), and cholesterol and bile acid levels (n = 14–18/group) in gallbladder. (E) Intestinal cholesterol levels (n = 6/group), intestinal triglyceride levels (n = 6/group), fecal cholesterol output (n = 7–9/group), and fecal bile acid output (n = 4–5/group). Results presented as mean ± SEM. $^{**}p < 0.01$, $^{*}p < 0.05$.

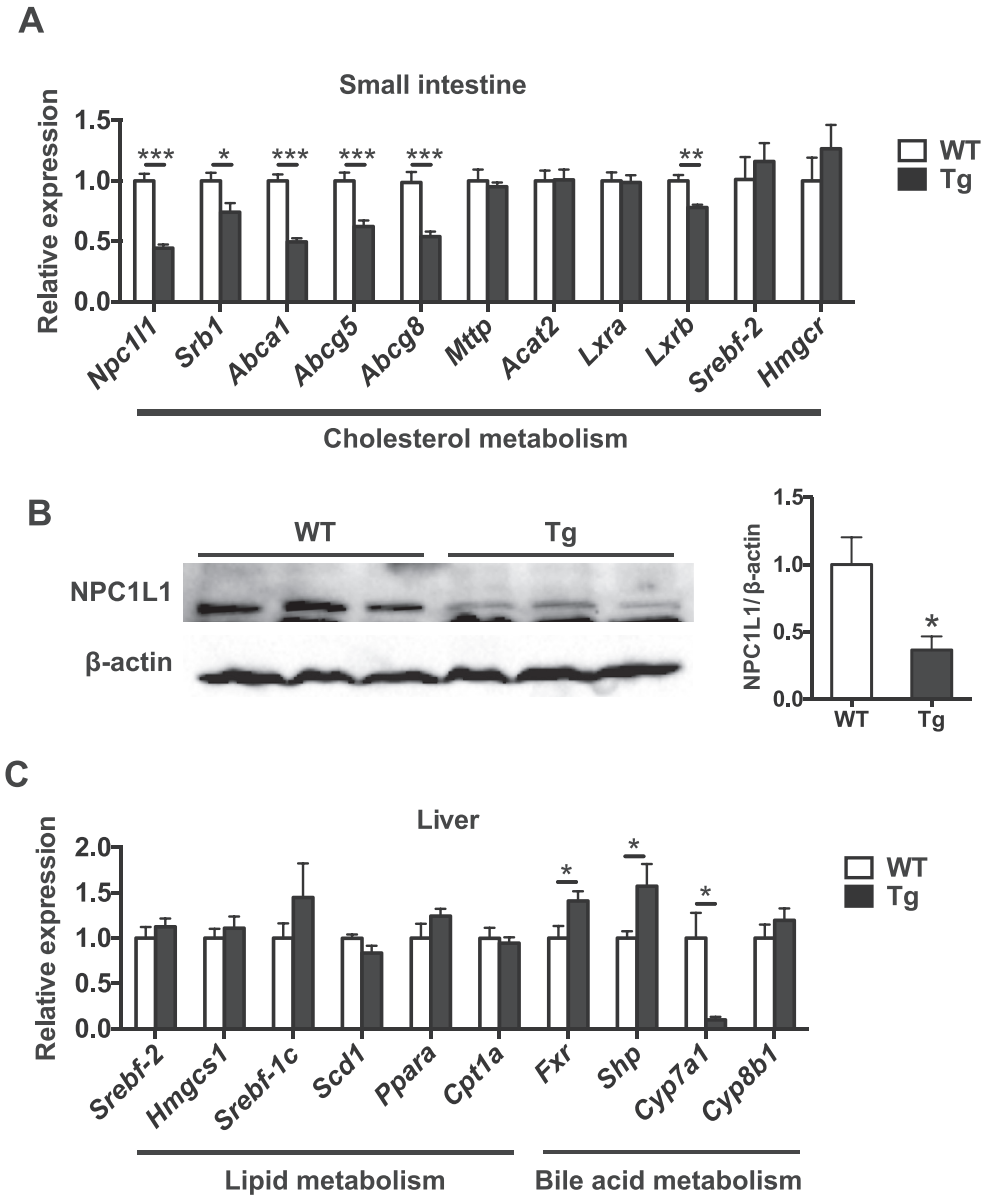

Figure 3: Changes in cholesterol-related gene expression patterns in intestine and liver of WT and CREBH Tg mice fed a LD. Intestinal and hepatic mRNA was extracted from WT and CREB Tg mice fed a LD for 2 weeks. (A) Expression of cholesterol-related genes in small intestine (n = 6–14 mice/group). (B) Intestinal protein was extracted from WT and CREBH Tg mice. Western blotting was performed with anti-NPC1L1 antibody. (C) Expression of genes related to lipid and bile acid metabolism in liver (n = 6–7/group). Results presented as mean ± SEM. ***p < 0.001, **p < 0.01, *p < 0.05.

dependent manner using the −0.3 kbp mouse *Npc1l1* promoter luc vector (Figure 4A). A previous study reported that SREBP-2 increased *Npc1l1* promoter activity [31], and CREBH significantly suppressed SREBP-2-induced *Npc1l1* promoter activity (Figure 4B). SREBP-2 did not induce −92 bp luc vector, suggesting that the binding site of SREBP-2 might lie in the region from −300 to −92 bp of the *Npc1l1* promoter. CREBH expression decreased the luciferase activity of the −93 bp mouse *Npc1l1* promoter but did not change the activity of the control luc vector, suggesting that the promoter region responsible for CREBH regulation lies −93 bp to the transcription start site (Figure 4B). To confirm the binding site of CREBH in the mouse *Npc1l1* promoter, we performed the EMSA assay. In vitro translated CREBH proteins bound to −32 and +5 bp of *Npc1l1* promoter, but not to other regions (Figure 4C). Taken together, these results indicate that CREBH directly regulates *Npc1l1* expression.

3.5. CREBH null mice showed severe hypercholesterolemia

If the phenotypes observed in Tg mice are indeed due to intestinal CREBH overexpression, CREBH deficiency would be expected to have opposite effects on cholesterol absorption, accumulation, excretion, and transporter expression. Indeed, CREBH null mice showed significantly higher plasma cholesterol levels than WT mice (Figure 5A). Plasma TG levels were also higher in CREBH null mice, but the difference was not significant. There were no differences in plasma NEFA levels between WT and null mice. In contrast to CREBH Tg mice, the livers of CREBH null mice showed

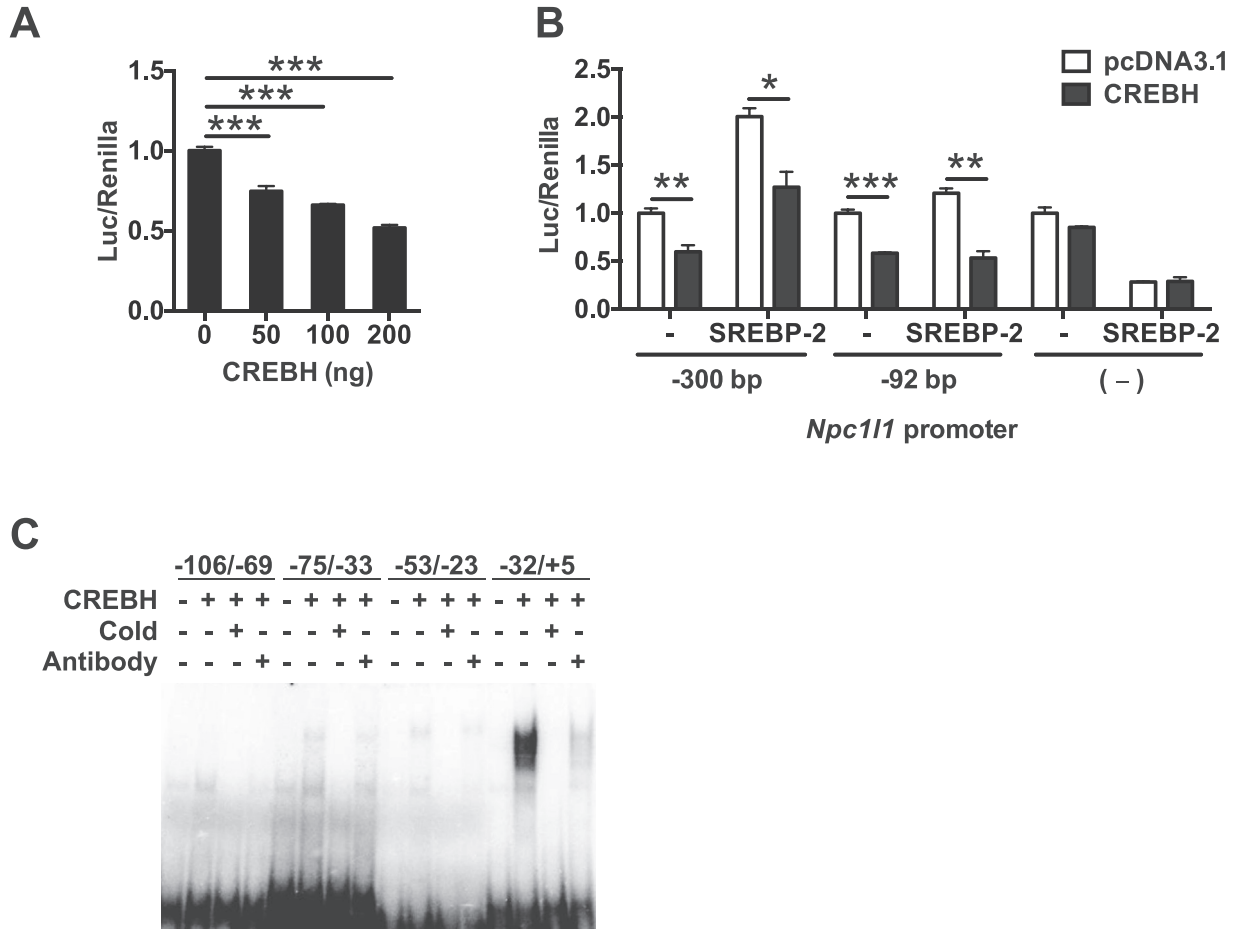

Figure 4: CREB3L3 reduces *Npc1l1* promoter activity. Caco-2 cells were co-transfected with a luciferase reporter plasmid containing segments of the mouse *Npc1l1* promoter and a CREBH or empty expression plasmid. Forty-eight hours after transfection, the cells were harvested and luciferase assay was performed. (A) CREBH inactivated the −0.3 kbp *Npc1l1* promoter luc in a dose-dependent manner. (B) The effects of CREBH and SREBP-2 on a series of *Npc1l1* promoter luc vectors. (C) EMSA assay indicated that CREBH directly bound to the region from −32 to +5 bp of the *Npc1l1* promoter. n = 3–4/group. ***$p < 0.001$, **$p < 0.01$, and *$p < 0.05$.

massive lipid accumulation (Figure 5B) as revealed by H&E staining (Figure 5B). Also contrary to the phenotype of LD-fed CREBH Tg mice, CREBH null mice exhibited higher liver and intestinal cholesterol levels compared to WT mice (Figure 5C and D). CREBH null exhibited lower fecal cholesterol output than WTs (Figure 5E). Intestinal *Npc1l1* expression by CREBH null mice was significantly higher than that by WT mice under the normal chow diet. Even if feeding a LD suppressed *Npc1l1* expression, CREBH null mice had a tendency to increase its expression, but not significantly (Figure 5F). These findings indicate that CREBH deficiency increases cholesterol absorption in small intestine, possibly by allowing *Npc1l1* upregulation, thereby elevating plasma, liver, and intestinal cholesterol.

4. DISCUSSION

CREBH is highly expressed in liver and small intestine, suggesting that CREBH regulates both nutrient absorption and metabolism. CREBH null mice exhibited increased plasma triglyceride and NEFA levels but decreased plasma cholesterol when fasting [32] and on an atherogenic high-fat diet [33]. CREBH enhanced LPL activity by upregulating the expression of hepatic apolipoprotein genes *Apoa4*, *Apoc2*, and *Apoa5*, thereby increasing triglyceride clearance [32]. In addition, CREBH upregulated hepatic fatty acid oxidation by increasing *Ppara* and its target genes [21]. These effects explain the plasma TG-lowering effects of liver CREBH. Recently, we generated liver-specific CREBH knockout (CREBH L-KO) mice using the CRISPR/Cas9 system [34] and found higher plasma cholesterol, triglyceride, and NEFA levels. Expression of hepatic cholesterol synthesis genes such as *Hmgcs1* and *Hmgcr* were also increased in the liver of CREBH L-KO mice, consistent with the higher plasma cholesterol levels [34]. Conversely, mice infected with an adenovirus encoding the active form of CREBH showed reduced plasma cholesterol associated with downregulated hepatic expression of *Hmgcs1* and *Hmgcr* [21]. Taken together, these findings indicate that CREBH has a crucial role in hepatic cholesterol synthesis. However, the functions of CREBH in small intestine and enterohepatic circulation remained unclear.

In the current study, we show that CREBH Tg mice have lower plasma cholesterol levels than WT mice when fed a LD, suggesting that intestinal CREBH contributes to cholesterol metabolism in small intestine under cholesterol and cholic acid loading. LD-fed CREBH Tg mice also exhibited reduced liver cholesterol, liver injury, intestinal cholesterol, and bile acid levels in gallbladder, and increased fecal cholesterol. These changes suggest that CREBH Tg mice have lower intestinal cholesterol absorption. Indeed, gene expression analysis

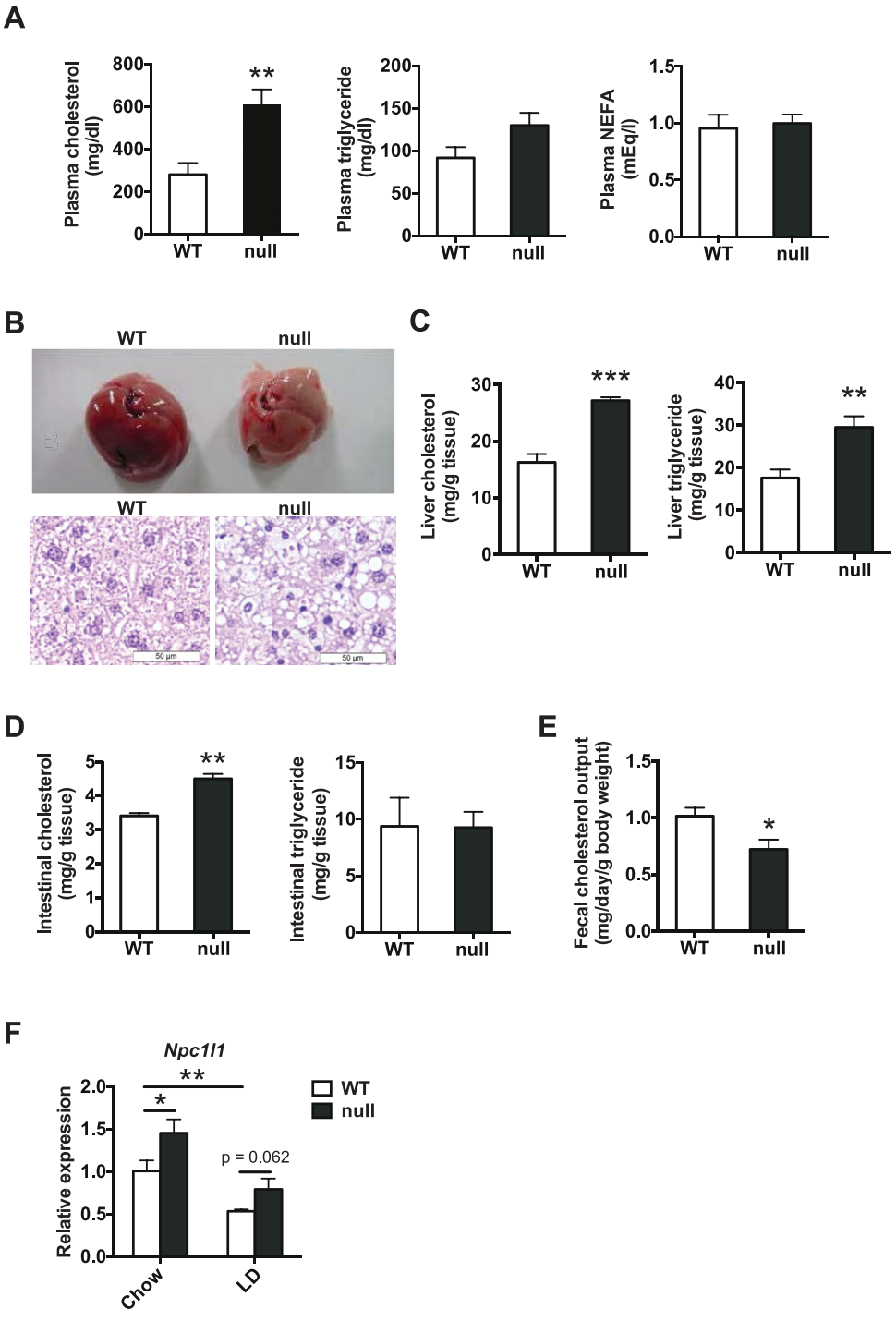

Figure 5: CREBH knockout induces severe hypercholesterolemia in mice fed a LD. (A) Plasma cholesterol (n = 6–11/group), triglyceride (n = 10/group), and NEFA levels (n = 10/group) in WT and CREBH null mice fed a LD for 2 weeks. (B) Representative pictures of whole livers and. H&E stained liver sections from LD-fed WT and CREBH null mice. (C) Liver cholesterol and triglyceride levels (n = 6–10/group). (D) Intestinal cholesterol and triglyceride levels (n = 3–5/group). (E) Fecal cholesterol output (n = 4–5/group). (F) Expression of *Npc1l1* in small intestine following normal chow diet or LD. Results presented as mean ± SEM. *p < 0.05.

revealed downregulation of cholesterol transporters *Npc1l1*, *Srb1*, *Abca1*, and *Abcg5/8* in the small intestine of CREBH Tg mice. NPC1L1 is a rate-limiting transporter of cholesterol absorption in small intestine and a key modulator of systemic cholesterol homeostasis [8,16]. Ezetimibe, an anti-hypercholesterolemia drug, is an inhibitor of NPC1L1 [35]. *Npc1l1* is expressed in the liver and small intestine in human but is expressed only in the small intestine in mice [8]. Thus, the effect of Ezetimibe administration mainly depends on the small intestine. Ezetimibe induces a complete resistance to cholesterol gallstone formation [36] and nonalcoholic fatty liver disease in addition to its effect on hypercholesterolemia in mice on a Western diet [37]. Ezetimibe inhibits the development of fatty liver in mice fed

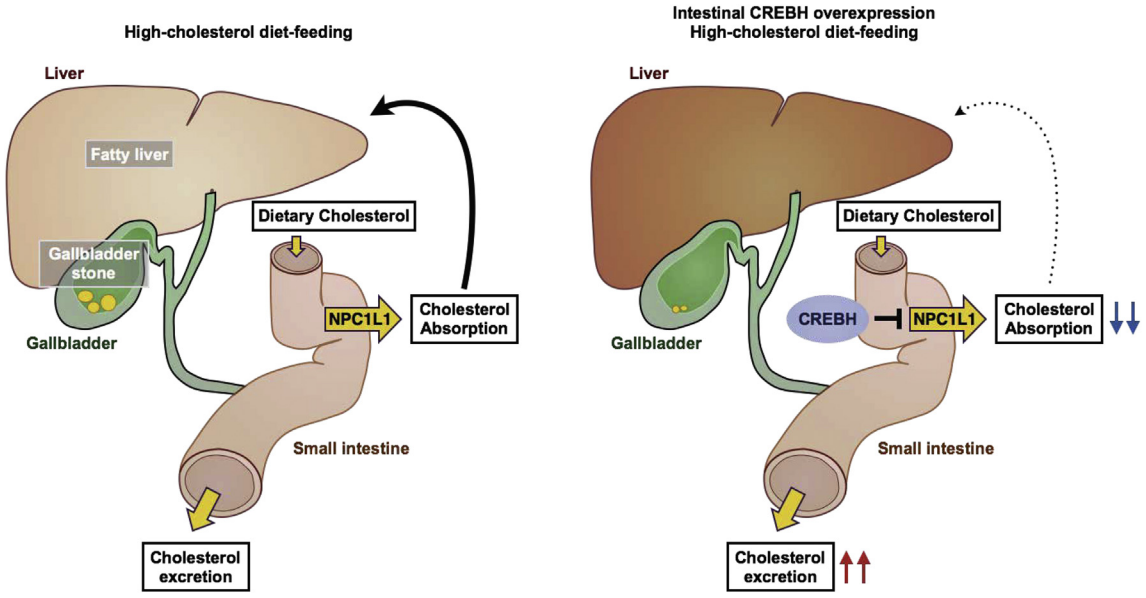

Figure 6: The scheme of cholesterol flow in CREBH Tg mice.

a high-fat diet by inhibiting SREBP-1 activation [38]. As LD-fed NPC1L1 KO mice showed lower plasma and liver cholesterol levels [16,39], the reduction of NPC1L1 in the small intestine of CREBH Tg mice likely contributes to the low plasma cholesterol phenotype and the suppression of fatty liver development. An *Npc1l1* deficiency in small intestine led to reduced intestinal expression of *Abca1* and *Abcg5/8* [7,37], genes encoding sterol transporters. Net cholesterol uptake into enterocytes of the small intestine depends on the balance between NPC1L1-mediated uptake [8] and ABCG5/8-mediated excretion [40]. CREBH Tg mice showed a reduction in intestinal cholesterol levels and an increase in fecal cholesterol, suggesting that the predominant effect of CREBH overexpression is reduced NPC1L1-mediated uptake (Figure 6). Intestinal ABCA1 deficiency leads to deficient HDL biogenesis, thereby reducing cholesterol influx into the circulation [29,30]. SRB1 is involved in the production of intestinal chylomicrons [41], which mediate the uptake of cholesterol from the lumen of the proximal small intestine [42,43]. Reduced expression of these genes in small intestine of CREBH Tg mice likely also contributes to the reduced absorption and elevated excretion of cholesterol. As *Abca1*, *Abcg5/8*, and *Srb1* are regulated by LXRβ in the small intestine [44–47], the decrease of *Lxrb* expression may contribute to the reduced these expression in LD-fed CREBH Tg mice. Excessive amounts of cholesterol are lipogenic through activation of LXR by its metabolites [48]. The decrease of intestinal cholesterol levels in CREBH Tg mice also reduces endogenous ligands of LXR and subsequent LXR activation. Intestinal *Npc1l1* expression is upregulated by SREBP-2 and downregulated by PPARα [31,49]. Promoter analysis revealed that CREBH dose-dependently suppressed *Npc1l1* promoter activity and SREBP-2-induced *Npc1l1* expression. We identified the direct binding sites for CREBH in −32 to +5 bp of the *Npc1l1* promoter. Both CREBH and SREBP-2 are membrane bound transcription factors that respond to changes in cellular energy charge. In liver, CREBH functions in opposition to SREBP family members in the regulation of triglyceride and cholesterol homeostasis. As CREBH directly binds to SREBP-1 and suppresses SREBP-1 transcriptional activity (Nakagawa, unpublished data), there is a possibility that CREBH also binds to SREBP-2. SREBP-2 upregulates *Npc1l1* expression in response to cholesterol depletion [31]. Similarly, intestinal CREBH and SREBP-2 may act reciprocally to ensure balanced systemic cholesterol levels through regulation of absorption.

Systemic cholesterol levels depend on the balance among intestinal absorption, hepatic synthesis, and hepatic conversion into bile acids. In light of the low cholesterol phenotype conferred by intestinal CREBH overexpression, we suggest that CREBH contributes to systemic cholesterol homeostasis by regulating cholesterol absorption in small intestine as well as by modulating hepatic synthesis and conversion. In the liver of CREBH Tg mice, the conversion of cholesterol into bile acids was likely downregulated as a secondary consequence of reduced liver cholesterol input. As we are generating small intestine-specific CREBH KO mice now, we will reveal the function of CREBH in the small intestine in detail.

This study and our unpublished data indicate that intestinal CREBH regulates TG and cholesterol absorption. Hepatic CREBH regulates TG and cholesterol catabolism [10,33,34]. Taken together, we propose that CREBH governs systemic lipid metabolism via entero-hepatic circulation.

In summary, this study suggests that intestinal CREBH functions as a metabolic regulator to attenuate diet-induced hypercholesterolemia and cholelithiasis by decreasing expression of the transporter gene *Npc1l1*. It is known that hepatic CREBH has the potential to ameliorate hypertriglyceridemia. This study identifies intestinal CREBH as another possible therapeutic target for the treatment of hypercholesterolemia and related metabolic diseases. Thus, drugs controlling CREBH activity throughout the intestinal–hepatic system could be highly efficacious treatments for metabolic diseases.

AUTHOR CONTRIBUTIONS

Y.N. and H. Shimano designed the experiments and wrote the manuscript. T.K., K. Orihara., F.O., S-I.H., M.K., K. Okuda., A.S., Y.O., Y.T., and Y.A. performed the experiments. T.M., H.I., S.Y., M.S., N. Yahagi, H. Suzuki, and H. Sone were involved in project planning. N. Yamada supervised this study and contributed crucial ideas to the project.

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DISCLOSURE STATEMENT

The authors have nothing to disclose.

APPENDIX A. SUPPLEMENTARY DATA

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.molmet.2016.09.004.

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