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Genetic depletion of *Soat2* diminishes hepatic steatosis *via* genes regulating *de novo* lipogenesis and by GLUT2 protein in female mice

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ABSTRACT

Depletion of the cholesterol esterifying enzyme acyl-Coenzyme A: cholesterol acyltransferase 2 (ACAT2, encoded by *Soat2)* protects mice from atherosclerosis, diet-induced hypercholesterolemia, and hepatic steatosis when fed high-cholesterol diet. The glucose transporter 2 (GLUT2) represents the main gate of glucose uptake by the liver. Lipid synthesis from glucose (*de novo* lipogenesis; DNL) plays a pivotal role in the development of hepatic steatosis. Inhibition of DNL is a successful approach to reverse hepatic steatosis, as shown by different studies in mice and humans. Here we aimed to investigate whether depletion of *Soat2 per se* can reduce hepatic steatosis, also in the presence of very low levels of cholesterol in the diet, and the underlying mechanisms. Female *Soat2*−/− and wild type mice were either fed high-fat or high-carbohydrate diet and both contained <0.05% (w/w) cholesterol. Analysis in serum, liver, muscles and adipose tissues were performed. We found *Soat2*−/− mice fed high-fat, low-cholesterol diet to have less hepatic steatosis, decreased expression of genes involved in DNL and lower hepatic GLUT2. Similar findings were found in *Soat2*-/- mice fed high-carbohydrate, low-cholesterol diet.

Conclusion: Depletion of *Soat2* reduces hepatic steatosis independently of the presence of high levels of cholesterol in the diet. Our study provides a link between hepatic cholesterol esterification, DNL, and GLUT2.

1. Introduction

Non-alcoholic fatty liver disease (NAFLD) is one of the most common liver conditions worldwide and its global prevalence is estimated to be 24% [1]. NAFLD is strongly associated with dyslipidemia, obesity, insulin resistance and type 2 diabetes [2]. Hepatic steatosis represents the initial stage of NAFLD and is characterized by the accumulation of triglycerides (TG) and cholesteryl esters (CE) as cytoplasmic lipid droplets [2]. The diagnosis of NAFLD requires the presence of hepatic steatosis, and exclusion of other causes of liver disease such as excessive alcohol consumption or other chronic liver diseases. No specific drug has yet been approved for the treatment of NAFLD and the management is based mainly on exercise and diet control [3].

Lipid synthesis from glucose (*i.e. de novo* lipogenesis; DNL) plays an important role in the pathogenesis of NAFLD, accounting for about one-fourth of hepatic TG accumulation [4]. DNL is regulated primarily at the transcriptional level. Both insulin and glucose regulate DNL via specific transcription factors: sterol regulatory element-binding protein 1c (SREBP1c, encoded by *Srebf1*) activated by insulin, and carbohydrate-responsive element-binding protein (ChREBP) activated by glucose [5]. Studies have shown regression of hepatic steatosis by targeting DNL through inhibition of acetyl-CoA carboxylase (ACC) [6–8] or stearoyl-CoA desaturase 1 (SCD1) [9].

The DNL is regulated by either the TG-synthesizing enzymes, or by precursor availability, or both. Glucose transporter 2 (GLUT2), encoded by *SLC2A2*, is a bidirectional glucose transporter expressed in the liver, intestine, kidney, and the islets of Langerhans [10]. GLUT2 contributes to the regulation of intracellular glucose, which is the main substrate utilized in DNL. Hence, it has been shown that

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lowering hepatic GLUT2 protein expression results in a reduction in DNL and in hepatic steatosis, in a rat model fed a high-fructose diet [11].

Acyl-Coenzyme A:cholesterol acyltransferase (ACAT) 1 and 2 are encoded by sterol *O*-acyltransferase (*SOAT*) 1 and 2, respectively [12]. ACAT2 is exclusively expressed in enterocytes and hepatocytes [13] and responsible for the synthesis of the CE that constitutes the lipid core of chylomicrons and very low density lipoproteins (VLDL) [14]. In mice, genetic or pharmacological inhibition of *Soat2* protects against atherosclerosis, diet-induced hypercholesterolemia, and cholesterol gallstone disease [15–19]. Depletion of *Soat2* also prevents dietary cholesterol-induced hepatic steatosis in mice [20]. However, no study has yet addressed the association between *Soat2* and DNL in the liver.

In this study we aimed to investigate whether depletion of *Soat2 per se* can reduce hepatic steatosis, also in the presence of very low levels of cholesterol in the diet, and the underlying mechanisms.

2. Material and methods

2.1. Animals

Heterozygote breeding of mice generated the *Soat2−/−* and wild type (WT) mice on a mixed genetic background (C57BL6/SV129). Female mice (eight per group), 9–15 weeks of age, were fed a high-fat diet (HFD) [21% (w/w) fatty acids (65% saturated, 32% monounsaturated, and 3% polyunsaturated fatty acids) and <0.05% (w/w) cholesterol] for eight weeks or a high-carbohydrate diet (HCD) [70% w/w carbohydrates and <0.05% (w/w) cholesterol] for two and a half weeks. The mice were fasted for four hours before collection of blood, liver, muscles, and gonadal adipose tissues. The project was approved by the local ethical committee, Stockholm South ethical committee, Huddinge, Sweden.

2.2. Tissue lipids

Frozen liver sections were stained with Oil red O to visualize lipids. Liver and muscle lipids were extracted using chloroform: methanol (Folch) (2:1 V/V) [21]. Samples were analyzed using TG, total- and free cholesterol (FC) reagents according to the manufacturer's protocols. The amount of esterified cholesterol in the liver was calculated by subtracting free from total cholesterol and multiplied by 1.67.

2.3. Serum analyses

Serum lipoproteins were separated by size-exclusion chromatography followed by determination of total- and FC, and TG [22]. Serum non-esterified fatty acids (NEFA), insulin, c-peptide, and glucose were measured according to the manufacturer's protocols.

2.4. Quantification of lipolytic activity in adipose tissue

The assay was performed by modification of the method described by Dube et al. [23]. Briefly, adipose tissues were homogenized in a buffer solution (150 mM NaCl + 10 mM Tris + 2 mM EDTA + protease inhibitor cocktail, pH7.4) and left on ice for 30 min before centrifugation at 10 000 × *g* for 10 min at +4 °C. The supernatant protein concentrations were determined using the Lowry method. Total lipolytic activities were measured by the enzyme fluorescence method using a Lipoprotein Lipase (LPL) Activity assay kit (Roar Biomedical-SIGMA) which is not specific for LPL.

2.5. RNA extraction, cDNA synthesis and real-time RT-PCR

Total RNA was extracted using Trizol (Thermo Fisher Scientific, Göteborg, Sweden) and transcribed into cDNA using Omniscript (Qiagen, Sollentuna, Sweden). The mRNA expression levels were then quantified and normalized to glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) or cyclophilin (*Ppia*) using specific primers (Supplementary Table 1).

2.6. Western blot

Cytosolic and membrane proteins were prepared from each mouse and an equal amount of protein from all samples in each group was then pooled. Three different protein concentrations were loaded on the gel and the proteins were separated by electrophoresis, transferred to nitrocellulose membranes, blocked, and incubated with antibodies against GLUT2 (Santa Cruz Biotechnology). Bands were visualized using Odyssey CLx (LI-COR, Lincoln, NE) and quantified by the imageJ software. The amount of GLUT2 was quantified by calculating the first derivative of the linear regression curve representing the three titration points for each sample pool. The slopes for the WT mice of respective diet were set equal to 1.

2.7. Statistical analysis

All data are presented as mean ± SEM unless otherwise stated. Data were log transformed before the parametric analysis when homoscedasticity was not present (unequal variance of the different groups). Significances were tested by factorial ANOVA with genotype and diet as factors, to assess the effect of genetic depletion of *Soat2* independently of dietary regimens. Post-hoc comparisons were performed according to the Fisher's least significant difference (LSD) test. The Mann–Whitney U test was used to test differences in body weight between the WT and *Soat2−/−* mice. All statistical analyses were performed using Statistica version 12.0 (StatSoft, Tulsa, OK).

3. Results

Depletion of *Soat2* reduces hepatic steatosis in mice

Female WT and *Soat2−/−* mice were fed HFD for eight weeks or HCD for two and half weeks to investigate whether depletion of *Soat2* protects against hepatic steatosis also in the presence of very low levels of cholesterol in the diet. No significant differences in weight gain over the course of the study were observed between *Soat2−/−* and WT mice (Fig. S1A–B). Liver sections stained for lipid droplets showed less intrahepatic lipid accumulation in *Soat2−/−* compared to WT mice (Fig. 1A). Genetic depletion of *Soat2* reduced hepatic TG, CE, and FC levels independently of the diets (Fig. 1B–D). *Soat2−/−* mice fed HFD or HCD had significant lower hepatic TG (≈ −40% and ≈ −50%, respectively) and hepatic CE (≈ −95% and ≈ −85%, respectively) levels compared to WT mice. Only *Soat2−/−* mice fed HFD had significantly lower hepatic FC (≈ −15%) levels (Fig. 1B–D).

3.1. Genotype and dietary regimens affect serum parameters

Next, we measured serum glucose, insulin, c-peptide, and NEFA levels to investigate whether these parameters were affected by the diminished hepatic lipid accumulation observed in the *Soat2 −/−* mice. *Soat2* depletion significantly lowered serum levels of insulin and c-peptide and increased levels of NEFA independently of the dietary regimen. *Soat2−/−* mice fed HCD had significantly lower serum levels of c-peptide (≈ −50%) and slightly higher levels of NEFA (≈ 10%) compared to WT mice (Fig. 2C–D).

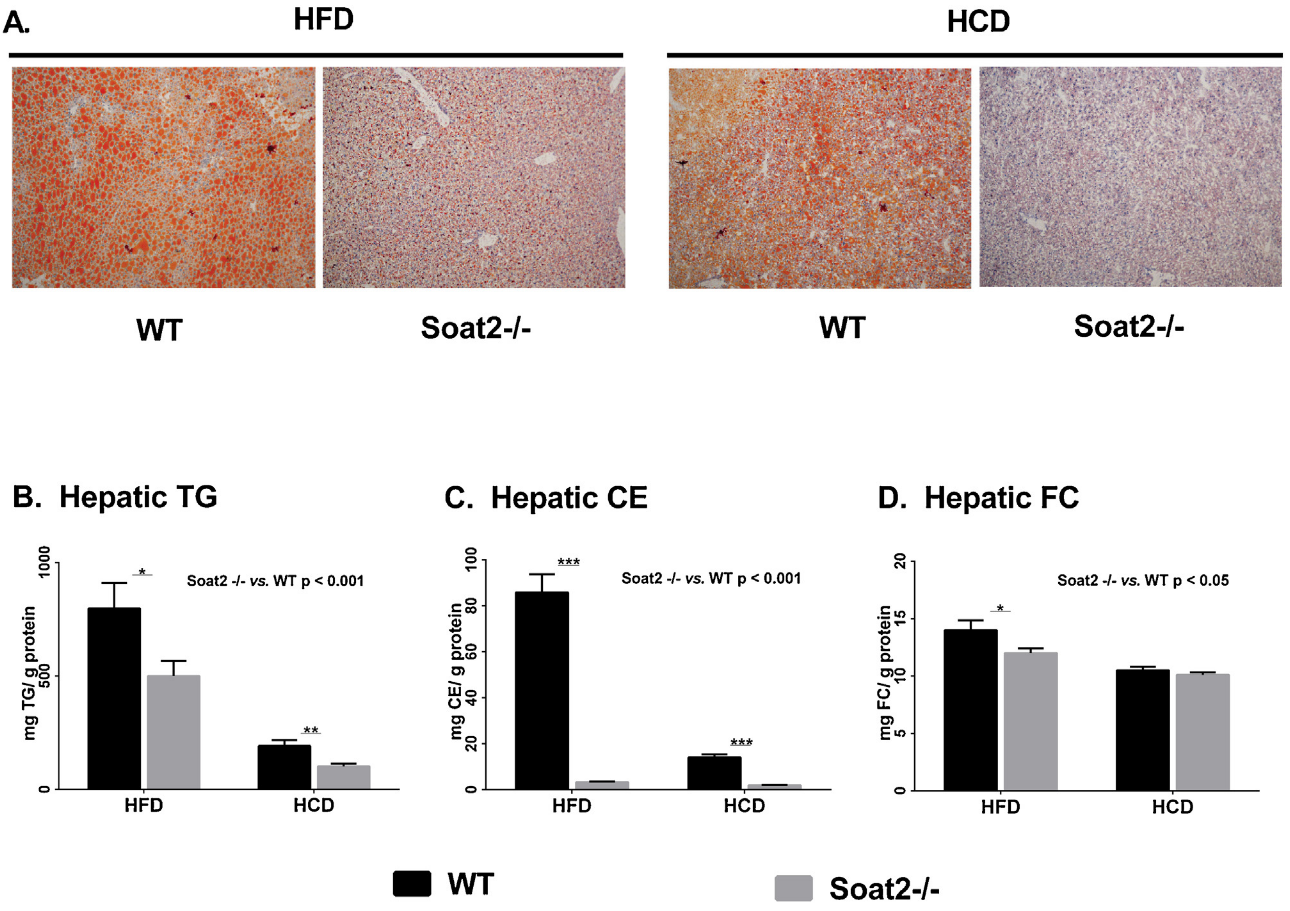

Fig. 1. Depletion of *Soat2* reduces hepatic steatosis in mice.

Soat2−/− and WT mice were fed HFD for eight weeks (n = 8/group) or HCD for two and half weeks (n = 8/group). (A) Frozen liver sections stained with oil red O and (B–D) quantification of hepatic TG, CE, and FC levels. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and diet as factors, followed by post-hoc comparison according to the LSD test; *p < 0.05, **p < 0.01 and ***p < 0.001

Effects of Soat2 *deficiency on genes involved in hepatic DNL, β-oxidation, and lipolysis in mice*

To investigate the underlying mechanisms responsible for the diminished hepatic steatosis following genetic depletion of *Soat2*, some key genes involved in DNL, β-oxidation, and lipolysis were assessed. Genetic depletion of *Soat2* significantly lowered mRNA levels of hepatic fatty acid synthase (*Fasn*) and stearoyl-CoA desaturase 1 (*Scd1*) independently of the diet. *Soat2*−/− mice fed HFD had significantly lower levels of hepatic ATP citrate lyase (*Acly*) (≈ −60%) and *Scd1* (−35%), and lower level of *Fasn* (≈ −60%) when fed HCD compared to WT mice (Fig. 3A). When fed HFD, *Soat2*−/− mice not only had significantly higher hepatic acyl-CoA dehydrogenase medium chain *(Acadm)* (≈ 30%), acyl-CoA dehydrogenase long Chain *(Acadl)* (≈ 25%), and acyl-CoA dehydrogenase very long chain *(Acadvl)* (20%) mRNA levels (Fig. 3B), but also higher hepatic carboxylesterase 2 (*Ces2*) (≈ 30%) level compared to WT mice (Fig. 3C). Genetic depletion of *Soat2* significantly increased the hepatic mRNA levels of *Acadm, Acadvl, Ces2*, and *Acadl* independently of the dietary regimen (Fig. 3B–C). No significant differences were found on hepatic *Srebf1* and *Slc2a2* mRNA levels (Fig. S4).

Depletion of Soat2 *decreases hepatic GLUT2 membrane protein levels*

Since decreased expression of GLUT2 has been linked with reduced DNL and hepatic steatosis in rats [11], we analyzed the GLUT2 protein levels to assess this as a potential mechanism. Indeed, *Soat2*−/− mice fed HFD or HCD had lower hepatic GLUT2 membrane protein levels compared to WT mice (≈ −40%) (Fig. 4A–C). No major differences were observed on the cytosolic hepatic GLUT2 protein (Fig. S2) or mRNA levels (Fig. S4).

Effects of Soat2 *deficiency on serum lipoprotein levels*

Serum lipoproteins were separated by size exclusion chromatography and their major lipid components (total cholesterol, FC and TG) determined online. In *Soat2*−/− mice, significantly lower serum levels of total cholesterol and higher TG levels, independently of the dietary regimen were found (Table 1). Serum levels of VLDL-TG, VLDL-FC, and LDL-TG were increased whereas VLDL-CE was decreased in the *Soat2*−/− compared to WT mice (Table 1).

Effects of Soat2 *deficiency on adipose tissue*

To get more insight about the adipose tissue functionality, we assessed total lipolytic activity in *Soat2*−/− and WT mice fed HFD and HCD. Genetic depletion of *Soat2* significantly increased total lipolytic activity independently of the diet (Fig. S5).We also analyzed some key genes involved in DNL, β-oxidation, and lipolysis in the adipose tissue from *Soat2*−/− and WT mice but did not observe any significant differences (Fig. S3 and S5).

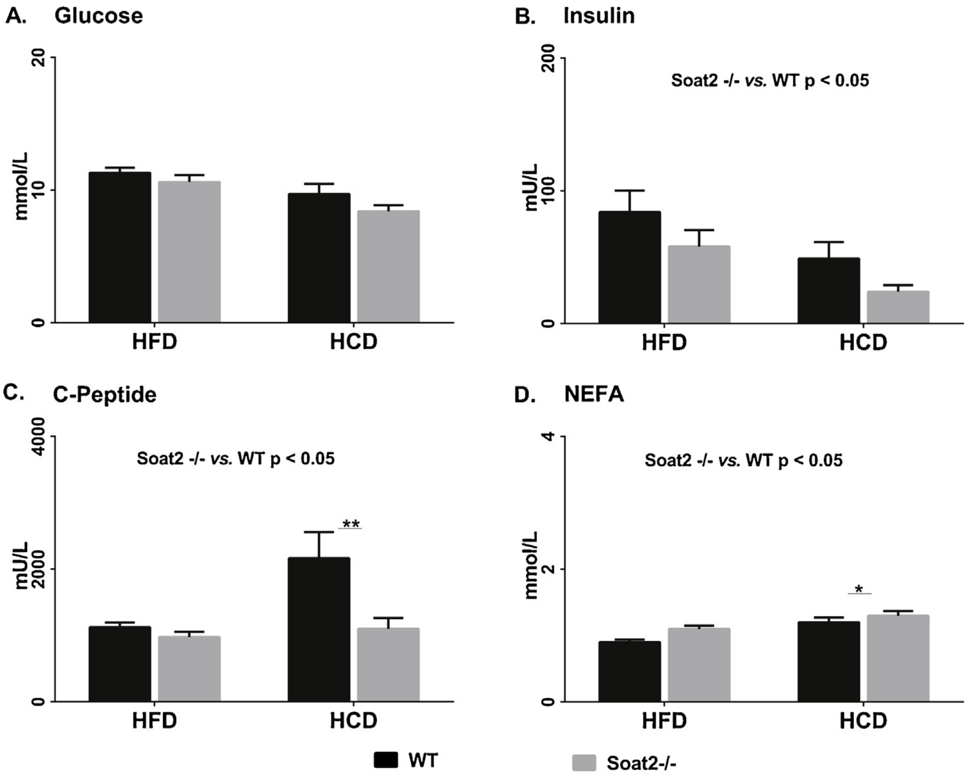

Fig. 2. Genotype and dietary regimens affect serum parameters.
(A–D) Serum glucose, insulin, c-peptide, and NEFA concentrations. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and diet as factors, followed by post-hoc comparison according to the LSD test; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$

Table 1
Serum lipoprotein levels.

	High-fat diet		High-carbohydrate diet		Genotype
	WT	*Soat2−/−*	WT	*Soat2−/−*	
Total triglycerides (mmol/L)	0.36 ± 0.05	0.83 ± 0.1***	0.53 ± 0.08	0.93 ± 0.12**	p < 0.001
VLDL-TG (mmol/L)	0.21 ± 0.04	0.47 ± 0.08*	0.36 ± 0.08	0.61 ± 0.09*	p < 0.01
LDL-TG (mmol/L)	0.05 ± 0.01	0.28 ± 0.0***	0.08± 0.01	0.23 ± 0.0***	p < 0.001
HDL-TG (mmol/L)	0.07 ± 0.01	0.09 ± 0.01	0.11 ± 0.01	0.09 ± 0.01	NS
Total cholesterol (mmol/L)	6.6 ± 0.69	5.38 ± 0.36	4.35 ± 0.4	2.89 ± 0.22**	p < 0.01
VLDL-FC (mmol/L)	0.04 ± 0.005	0.1 ± 0.02**	0.03 ± 0.004	0.03 ± 0.003	p < 0.05
LDL-FC (mmol/L)	0.72 ± 0.17	0.47 ± 0.04	1.4 ± 0.13	0.95 ± 0.06	
HDL-FC (mmol/L)	0.63 ± 0.04	0.62 ± 0.03			
VLDL-CE (mmol/L)	0.04 ± 0.01	0.01 ± 0.01*	0.06 ± 0.01	0.06 ± 0.00	p < 0.05
LDL-CE (mmol/L)	0.65 ± 0.08	0.37 ± 0.03	2.86 ± 0.6	1.85 ± 0.2	
HDL-CE (mmol/L)	4.5 ± 0.46	3.75 ± 0.25			

Data are expressed as mean ± SEM. Abbreviations: TG, triglycerides; VLDL, very low density lipoprotein; LDL, low density lipoprotein; HDL, high density lipoprotein; FC, free cholesterol; CE, cholesteryl ester. Factorial ANOVA with significances indicated in the Table. For differences between *Soat2−/−* and WT mice we used ANOVA followed by the LSD-test.

* $p < 0.05$.
** $p < 0.01$.
*** $p < 0.001$.

Table 2
Intramuscular lipids.

	High-fat diet		High-carbohydrate diet		Genotype
	WT	*Soat2−/−*	WT	*Soat2−/−*	
Triglycerides in T.anterior (mg TG/g protein)	60.7 ± 7.2	56.8 ± 13.5	62.6 ± 5.8	34.1 ± 5.5*	p < 0.05
Cholesterol in T.anterior (mg TC/g protein)	2.47 ± 0.07	2.33 ± 0.08	4.34 ± 0.16	4.01 ± 0.19	NS
Triglycerides in Soleus (mg TG/g protein)	313 ± 68.9	255 ± 59.1	225± 26.1	135 ± 15.9	NS
Cholesterol in Soleus (mg TC/g protein)	6.12 ± 0.21	5.57 ± 0.29	12.29 ± 0.41	12.15 ± 1.3	NS

Data are expressed as mean ± SEM. Abbreviations: TG, triglycerides; TC, total cholesterol. Factorial ANOVA with significances indicated in the Table. For differences between *Soat2−/−* and WT mice we used ANOVA followed by the LSD-test.

* $p < 0.05$.

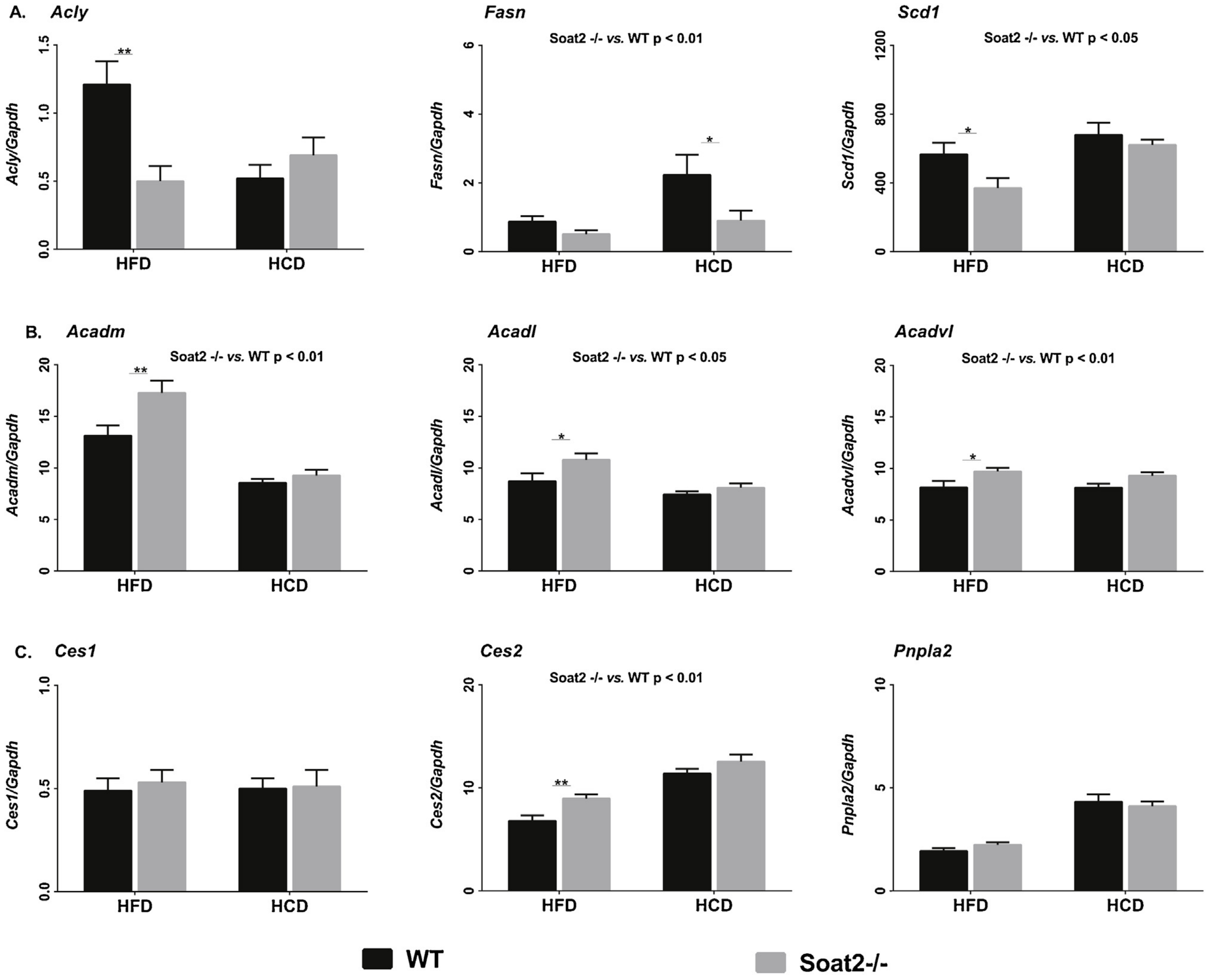

Fig. 3. Effects of *Soat2* deficiency on genes involved in hepatic DNL, β-oxidation, and lipolysis in mice.
Genes involved in (A) DNL; (B) β-oxidation; and (C) lipolysis were assessed in livers from *Soat2*−/− and WT mice. Data are expressed as mean ± SEM. Factorial ANOVA with genotype and diet as factors, followed by post-hoc comparison according to the LSD test; $^{*}p < 0.05$, $^{**}p < 0.01$ and $^{***}p < 0.001$

Effects of Soat2 *deficiency on lipid accumulation in skeletal muscles*

To assess whether *Soat2* depletion affects lipid accumulation in skeletal muscles, we analyzed levels of cholesterol and TG in the Soleus and Tibialis anterior. We found *Soat2*−/− mice fed HCD to have lower TG level in Tibialis anterior (−45%); however, no other significant differences were observed (Table 2).

4. Discussion

Genetic depletion of *Soat2* has previously been reported to prevent hepatic steatosis induced by high levels of cholesterol in the diet [20]. However, whether depletion of *Soat2 per se* — independently by the presence of high dietary cholesterol level – reduces hepatic steatosis, and by which mechanisms, has not been investigated. Hence, we have chosen two different diets, both low in cholesterol content: HCD to stimulate hepatic lipogenesis [24], and HFD to increase hepatic fat uptake.

Here we found that genetic depletion of *Soat2* reduces hepatic steatosis also when the levels of cholesterol in the diets are low. We also found *Soat2* deficiency to lower levels of genes regulating DNL, known to be a driver of NAFLD [25], and levels of hepatic GLUT2 membrane protein.

Depletion of *Soat2* reduces hepatic TG levels by approximately 50% and as DNL is considered to account for up to 25% of hepatic TG accumulation in steatotic livers [4], the reduced DNL seen after *Soat2* depletion may partly explain the diminished hepatic steatosis. Hence, the increased hepatic TG mobilization previously suggested by Alger et al. [20] might complete the picture. The increased TG mobilization from the liver to the circulation is the reason for the hypertriglyceridemia associated with *Soat2*−/− mice [20]. Since it has been reported in rats that inhibition of liver-specific DNL increases VLDL-TG secretion [6], it is likely that this mechanism greatly contribute to the hypertriglyceridemia seen in mice after *Soat2* genetic depletion. Nevertheless, the involvement of VLDL assembly and secretion machinery still remains to be further investigated.

Deficiency of *Soat2* significantly decreases the hepatic GLUT2 membrane protein levels, which should lead to lower influx of glucose into the hepatocyte. As DNL utilizes non-lipid precursors such as glucose as a substrate for the production of fatty acids, the reduced hepatic GLUT2 membrane protein levels would in turn reduce DNL. Thus, genetic depletion of *Soat2* seems to have dual effects: lowering the levels of genes regulating DNL and also the DNL precursor availability.

Downregulation of DNL is also coupled with increased mitochondrial β-oxidation. ACC catalyzes the synthesis of malonyl-CoA,

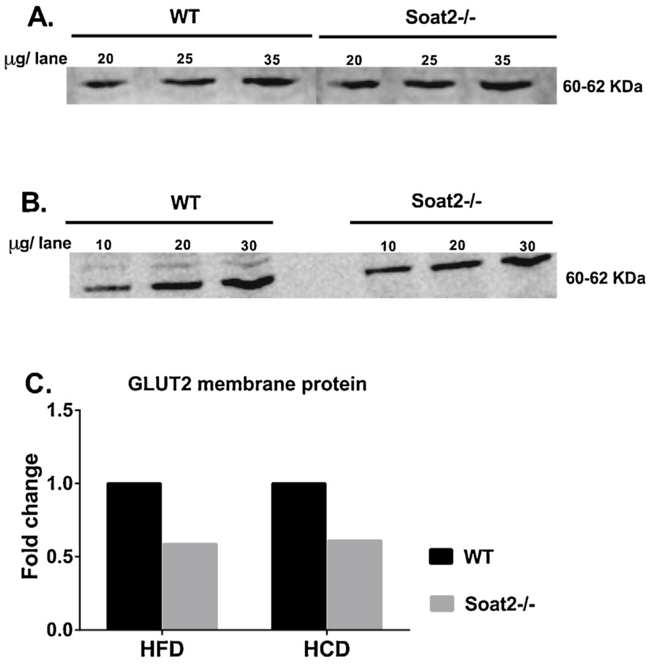

Fig. 4. Depletion of *Soat2* decreases hepatic GLUT2 membrane protein levels in mice. Membrane fractions were prepared from each mouse and an equal amount of protein from samples in each group was pooled. The amount of GLUT2 was quantified by calculating the first derivative of the linear regression curve following (A) HFD and (B) HCD; the slopes for WT mice of respective diet were set equal to 1 (C).

which is both an intermediate in DNL and an allosteric inhibitor of carnitine palmitoyl transferase 1 (CPT1) [26,27]. CPT1 regulates the transfer of fatty acids from the cytosol into the mitochondria for oxidation [28]. Our data shows increased hepatic mRNA levels of some key genes involved in β-oxidation (*Acadm*, *Acadl*, and *Acadvl*) in *Soat2*−/− mice. Genetic depletion of *Soat2* may therefor also affect β-oxidation by increasing fatty acid uptake into mitochondria.

To investigate whether the effects on DNL and β-oxidation also affects other metabolically active tissues, some key genes involved in DNL, β-oxidation, and lipolysis were analyzed in adipose tissue but no major differences between *Soat2* −/− and WT mice were observed. Nevertheless, depletion of *Soat2* significantly increases total lipolytic activity in adipose tissue. Furthermore, skeletal muscles lipid content were quantified, but no major effect of *Soat2* depletion on intramuscular TG levels were detected.

Altogether, the observations in the liver, adipose tissue, and muscles are in line with the hypothesis of an increase in β-oxidation and explain the minor increase in NEFA levels in the circulation, despite the increase of serum TG and total lipolytic activity in adipose tissue.

The amount of hepatic lipids (TG and CE) in WT mice on the HCD was only one fifth of the levels found in WT mice fed the HFD, and an increased hepatic lipid accumulation is known to be associated with insulin resistance. Nevertheless, the reduction of fasting insulin of mice depleted for *Soat2,* while having similar fasting glucose levels as the WT, indicate an improved glucose tolerance that is independent of the level of lipid accumulation in the liver. In conclusion, the diminished hepatic TG accumulation observed in *Soat2* −/− mice, independently of the presence of high dietary cholesterol, may be caused by the decreased availability of glucose secondary to the reduced GLUT2 which in turn lowers DNL. Depletion of *Soat2* seems to protect from hepatic steatosis without negatively affecting adipose and skeletal muscle tissues. This study thus provides a link among hepatic cholesterol esterification, DNL and GLUT2.

Conflict of interest

None declared.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at https://doi.org/10.1016/j.dld.2018.12.007.

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