Sidt2 regulates hepatocellular lipid metabolism through autophagy

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Abbreviations:

ADFP adipocyte differentiation-related protein
ASMase acid sphingomyelinase
Cpt1 carnitine palmitoyltransferase 1
Cpt2 carnitine palmitoyltransferase 2
Dgat1 diacylglycerol acyltransferase-1
Dgat2 diacylglycerol acyltransferase-2
EBSS Earle's balanced salt solution
EM electron microscopy
Fasn fatty acid synthase
HF high-fat
Hsl hormone sensitive lipase
NAFLD non-alcoholic fatty liver disease

LAMP lysosome-associated membrane protein
LD lipid droplets
LMPs lysosomal membrane proteins
Lipc hepatic lipases
NEFA non-esterified fatty acid
PBS phosphate-buffered saline
Srebf1 sterol regulatory element binding transcription factor 1
TBS tris-buffered saline
TG triglyceride
WT wild-type
Abstract

SID1 transmembrane family, member 2 (Sidt2) is an integral lysosomal membrane protein. To investigate its explicit function, we generated a global Sidt2 knockout mouse model (Sidt2<sup>−/−</sup>). Compared with the littermate controls, Sidt2<sup>−/−</sup> mice exhibited a remarkable accumulation of lipid droplets in liver. Firstly, it was observed that food consumption, hepatocyte fatty acid uptake and de novo lipogenesis, hepatocyte lipolysis and TG secretion in the form of very low-density lipoprotein were comparable between Sidt2<sup>−/−</sup> and WT mice. However, the hepatic β-oxidation of fatty acids decreased significantly as revealed by a low level of serum β-hydroxybutyrate in Sidt2<sup>−/−</sup> mice along with normal mRNA expression of genes involved in fatty acid oxidation. In addition, the classical autophagy pathway marker proteins, p62 and LC3-II increased in liver, along with compromised autophagic flux in primary hepatocytes, indicating a block of autophagosome maturation due to Sidt2 deficiency, which was also supported by electron microscopy image analysis both in livers and in primary hepatocytes from Sidt2<sup>−/−</sup> mice. It was concluded that Sidt2 plays an important role in mouse hepatic lipid homeostasis by regulating autophagy at the terminal stage.

**Key words:** SID1 transmembrane family, member 2; lipid droplets; triglycerides; liver metabolism; autophagy

INTRODUCTION

Lysosomes and lysosome-related organelles form a highly efficient and coordinated metabolic regulatory network. The functions of this system include various physiological processes, such as cholesterol homeostasis(1), pathogen defense(2), plasma membrane repair(3), and bone and tissue
remodeling(4). However, its major task is the degradation of extracellular material and intracellular components taken up by endocytosis and autophagy(1). The degraded products, which may include amino acids, sugars, simple glycolipids, cholesterol, and nucleotides, are salvaged and delivered to other cellular organelles and membranes for reutilization. The proper function of lysosomes is essential for cellular metabolic homeostasis; dysfunction can result in around 50 different types of lysosomal storage disorders(5). In addition, increasing evidence reveals that lysosomes are involved in more widely spread diseases, such as cancer, Alzheimer’s disease, cardiovascular diseases, and non-alcoholic fatty liver disease (NAFLD)(6-9).

Excess fat accumulation in the liver can occur as a result of enhanced fatty acid delivery, increased de novo lipogenesis, reduced lipolysis and fat export in the form of very low-density lipoproteins, and decreased fatty acid oxidation (10, 11). Recent evidence has shown that the lysosomal-autophagic pathway plays an important part in hepatocyte lipid metabolism, and that defective autophagy may contribute to the pathogenesis of NAFLD by decreasing fatty acid oxidation (12). However, the factors that connect autophagy with lipid metabolism urgently await to be discovered.

Lysosomal membrane proteins (LMPs) are vital for lysosomes executive function. They play diverse roles, including acidification of the lysosomal lumen, fusion of the cellular membrane system, and transportation of degradation products (13, 14). Approximately 58 LMPs have been identified (15). Of these, lysosome-associated membrane protein (LAMP)-1 and -2, lysosomal integral membrane protein (LIMP)-2, and CD63 are the most abundant (16). However, more potential LMPs continue to be revealed by proteomics analysis with their exact physical function to be explored (17, 18).

Sidt2 is an integral LMP that is widely expressed in all tissues (19). Previously, to understand the
function of Sidt2 in vivo, we generated a global Sidt2 gene knockout mouse model and observed impaired glucose tolerance likely due to compromised NAADP-involved insulin secretion (20). Here, we demonstrated that Sidt2 also plays an important role in hepatocyte lipid metabolism by regulating hepatic lipid autophagy.

MATERIALS AND METHODS

Animals

Sidt2−/− mice were generated as previously described (21). All mice were housed with standard temperature and humidity and were maintained on a 12 h light/dark cycle. Mice were fed a standard chow diet or a high-fat (HF) diet (60% kcal in fat; Research Diets, D12492) and had free access to water. The HF diet began at 6 weeks of age and continued for a total of 12 weeks. In all studies, gender and age-matched homozygous knockout animals were compared with wild-type (WT) controls which were littermates of the knockout mice. Male mice at 14 weeks of age were used in our study. In selected experiments, 6-week-old male mice were used. All experiments involving mice were approved by Institutional Review Ethics Board of Xinhua Hospital.

Primary hepatocyte isolation and culture

Mouse primary hepatocytes were isolated from 10-week-old male Sidt2−/− and WT mice by 2-step perfusion as previously described (22). The liver was perfused with Hanks’ Balanced Salt Solution (Gibco) containing 1 mM EDTA, then digested with Hanks’ Balanced Salt Solution (Gibco) containing 0.05% collagenase IV (Sigma). The dissociated cells were then filtered through a 100 μm cell strainer and centrifuged at 500 rpm for 5 min at 4°C. Hepatocytes were washed twice with PBS, followed by
centrifugation at 500rpm for 5 min at 4°C, then resuspended and seeded in a 6-well plate at a concentration of $3 \times 10^5$ cells/well in Hepatocyte Medium (ScienCell 5201, 5% fetal bovine serum, 1% penicillin/streptomycin solution, 1% hepatocyte growth supplement). After 24 hours, hepatocytes were maintained in DMEM (Gibco) containing 10% fetal bovine serum, 1% penicillin/streptomycin solution and 1% L-Glutamine.

**Oil Red O Staining**

Oleic acid (OA, Sigma-Aldrich) was conjugated to fatty acid-free bovine serum albumin (BSA), as described Previously (23). In brief, 20 mM OA in 0.1 mol/L NaOH was incubated at 70°C for 30 min, and fatty acid soaps were then complexed with 20% BSA at a 6:1 molar ratio of fatty acid to BSA. Primary hepatocytes were treated with 0.2 mM OA for 24 h in DMEM with 1% fetal bovine serum, which were subsequently fixed in 3.7% formaldehyde for 15 minutes and stained with Oil Red O (Sigma-Aldrich) in 60% isopropanol for 20 minutes. After washing with 60% isopropanol for 30 seconds, the cells were redyed with hematoxylin for 4 minutes. Lipid droplets (LDs) stained in red were assessed by bright-field microscopy and quantified using the Image J software.

**Western blotting**

Cells were washed twice with cold phosphate-buffered saline (PBS) before being lysed on ice in RIPA buffer with PMSF (Sigma-Aldrich). Liver tissue samples (30 mg) were submerged in RIPA buffer supplemented with PMSF, leupeptin, and pepstain inhibitors, then minced with scissors and homogenized with a MagNA Lyser (Roche Applied Science) at 6500 r.p.m for 1 min. Homogenates were centrifuged at 12,000 r.p.m. for 20 min at 4 °C. Supernatants were collected and quantified for protein concentration using a BCA Kit (Bio-Rad).

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Proteins were resolved on SDS-PAGE, and then transferred onto polyvinylidene difluoride membranes (Millipore). Membranes were blocked in Tris-buffered saline (TBS) with 0.1% Tween 20 (TBST) containing 5% non-fat dry milk and incubated with primary antibodies overnight at 4°C. The primary antibodies used in the present study included rabbit anti-LC3B (1:1000, Sigma-Aldrich), rabbit anti-p62/SQSTM1 (1:1000, Cell Signaling Technology), rabbit anti-ADRP (1:1000, Novus Biologicals), rabbit anti-Atg5 (1:1000, Cell Signaling Technology), rabbit anti-Atg7 (1:1000, Cell Signaling Technology), rabbit anti-beclin-1 (1:1000, Cell Signaling Technology), rabbit anti-TFEB (1:1000, Santa), rabbit anti-Phospho-4E-BP1 (1:1000, Cell Signaling Technology), rabbit anti-4E-BP1 (1:1000, Cell Signaling Technology), rabbit anti-Phospho-p70 S6 Kinase (1:1000, Cell Signaling Technology), rabbit anti-p70S6Kinase (1:1000, Cell Signaling Technology), rat anti-Lamp2 (1:1000, Developmental Studies Hybridoma Bank), mouse anti-Cathepsin B (1:400, Abcam), mouse anti-Cathepsin D (1:1000, Abcam), mouse anti-β-actin (1:5000, Beyotime), mouse anti-tubulin (1:1000, Beyotime), and mouse anti-GAPDH (1:5000, KangChen). Membranes were washed 3 times in TBST and incubated with the corresponding HRP-conjugated secondary antibody (1:5000, Santa Cruz Biotechnology) at room temperature for 1 hour. Finally, blots were washed 3 times with TBST and bands were detected using an enhanced chemiluminescence system (Thermo Scientific).

**Histological studies**

Liver tissue was taken from age- and sex-matched mice. Tissues were fixed in 4% formalin and paraffin-embedded, then sections were stained with hematoxylin/eosin. The segments of liver were embedded in optimal cutting temperature compound (SAKURA), and cryostat-sectioned at a thickness of 5 μm for lipid deposition assessment via oil red O staining. Quantification of hepatocytes size and Oil Red
O-stained area was performed by the Image J software after appropriate thresholding.

**Electron microscopy**

Cells cultured in monolayers and liver blocks (1 mm³ in size) were fixed in 2% glutaraldehyde in PBS (pH 7.2) for 2 h, and post-fixed in 1% osmium tetroxide for 2 h. Samples were then dehydrated with an ascending series of alcohol before embedding in Araldite. Ultrathin sections were cut and stained with lead citrate. Images were acquired with an electron microscope (CM-120, PHILIP).

**Microarray analysis of liver tissue**

Microarray analysis was performed in the liver, as previously described (24). Extracted RNA was labeled with biotin and hybridized to Affymetrix Gene Chip Mice Genome 430 2.0 single arrays. The hybridization was preceded according to manufacturer’s instructions. Bioinformatics analysis was performed by Gene Tech, Shanghai, China. Microarray analyses of liver tissue were conducted in Sidt2⁻/⁻ and WT mice at 1, 3, and 6 months of age, respectively. The raw CEL files were background corrected on the perfect match values and normalized by the Robust Multichip Array (RMA) algorithm. Principal component analysis (PCA) was performed and oneway analysis of variance (ANOVA) was performed to identify differentially expressed genes (Fold-Change > 2) among groups.

**Quantitative real-time PCR**

Total RNA was isolated from liver tissue using Trizol (Invitrogen) and cDNA was synthesized from 500ng RNA by the PrimeScript™ RT reagent Kit (Takara), according to the manufacturer’s instructions. Real-time PCR was performed on 7500/7500 FAST Real-Time PCR System (Applied Biosystems) using a SYBR®Premix Ex Taq™ Kit (Takara). Actin levels were measured for normalization. Primer sequences
are available on request.

**Assay of serum non-esterified fatty acid**

Mice were fasted overnight and blood was isolated by retro-orbital bleeding. Serum non-esterified fatty acid (NEFA) was measured with a commercially available kit (Wako), according to the manufacturer’s instructions.

**Assay of hepatic triglyceride secretion**

Triglyceride (TG) secretion was determined as described previously (25). Mice were fasted for 6 h and then injected intraperitoneally with 1g/kg of poloxamer-407 (Sigma-Aldrich). Plasma samples were drawn prior to and after injection for TG measurements. TG production rate was calculated based on serum TG level changes and expressed as μmol/kg/h.

**Analysis of liver lipids**

Mice were fasted overnight and liver TG was determined, as described previously (26). Briefly, approximately 200 mg of liver (wet weight) was digested by ethanolic KOH and incubated overnight at 55°C. On the second day H₂O:EtOH (1:1 v/v) was added to the tube and then the mixture was centrifuged. The supernatant was treated with H₂O:EtOH again followed by neutralization with MgCl₂. The supernatant obtained by centrifugation was ready for further analyses. Liver triglyceride content was determined using a Triglyceride Determination Kit (Sigma-Aldrich).

**Measurement of serum β–hydroxybutyrate**

Serum β-hydroxybutyrate levels were measured in 6 h-fasted serum samples using the β-hydroxybutyrate Assay Kit from Abcam.

**Determination of acid sphingomyelinase activity**
Acid sphingomyelinase (ASMase) activity from liver lysates was determined using a fluorescent substrate (6-HMU-phosphorylcholine, Moscerdam). Homogenates containing 50 μg of protein from liver tissue were incubated with 10 μL of 1.35 mmol/L 6-HMU-phosphorylcholine in 0.1 mmol/L sodium acetate (pH 5.2) for 17 h at 37 °C. The reaction was stopped by adding 200 μL glycine/NaHCO₃ buffer with 0.25% Triton X-100 (pH 10.5). The fluorescence signal was read with a fluorometric plate reader (Perkin Elmer) using an excitation wavelength of 365 nm and an emission wavelength of 450 nm.

**Statistical analysis**

All data are presented as mean ± SEM. A Student’s t-test was used for statistical comparison, and a value of p ≤ 0.05 was considered significant.

**RESULTS**

**Lipid droplets accumulate in the liver of Sidt2⁻/⁻ mice**

The volume of livers in Sidt2⁻/⁻ mice increased. Consistent with their increased liver volume, ratio of liver weight to total body weight also significantly increased compared to control mice (Fig.1A). Although the Sidt2⁻/⁻ mice weighed less than WT mice at 6 weeks of age (21), there was no significant difference in body weight between the two groups after HF diet for 12 weeks (Fig.1B). Histological analysis of liver specimens revealed that abundant round vesicles resembling lipid droplets (LDs) accumulated in Sidt2⁻/⁻ mice fed chow diets. Moreover, hepatocytes from Sidt2-deficient mice had bigger cell sizes than WT mice and appeared hypertrophic, and consequently sinusoids appeared small (Fig.1C). Neither significant inflammatory cell infiltration nor ballooning degeneration were observed, indicating no steatohepatitis at this stage (Fig.1C). In mice fed a HF diet, the liver of the Sidt2⁻/⁻ mice not only contained many more LDs
than those of WT mice, but also showed ballooning degeneration (Fig. 1C). Furthermore, the livers of Sidt2−/− mice fed chow or HF diet contained much more oil red O-positive vacuoles than those of WT mice, suggesting an accumulation of LDs in the liver of Sidt2−/− mice (Fig. 1D). Transmission electron microscopy (EM) studies further confirmed that both the number and size of the hepatic lipid droplets were markedly increased in livers of Sidt2−/− mice fed a chow diet after fasting (Fig. 1E). The accumulation of LDs in Sidt2−/− mice was also confirmed by an elevated level of the adipocyte differentiation-related protein (ADRP or ADFP), an LD-associated protein (Fig. 1G). Finally, we measured the level of TG in the liver of Sidt2−/− mice. TG levels in livers of Sidt2−/− mice fed a chow diet was ~140% higher than in WT mice; and in mice with an HF diet, the proportion was more than 160% (Fig. 1H).

In addition, increased lipid staining with oil red O was observed in primary hepatocytes from Sidt2−/− mice, indicative of an accumulation of LDs both under regular medium (RM) and following a supply of exogenous OA (Fig. 1F). Taken together, these results indicate that Sidt2 deficiency leads to an accumulation of LDs in the liver. Consistent with these findings, Sidt2 deficiency caused a marked elevation of serum ALT and AST in mice fed both chow and HF diets (Fig. 1I), indicating considerable hepatocellular injury by LD accumulation.

Impact of Sidt2 knockout on major pathways of TG metabolism

To investigate the reason of TG accumulation, we first looked into food consumption in Sidt2−/− and WT mice, which did not significantly differ between the two groups (Fig. 2A).

Next, microarray analysis was conducted to examine the mRNA levels of genes usually perceived as important in non-esterified fatty acid uptake to liver. These genes include solute carrier family 27 member 2 (Slc27a2 or Fatp2), solute carrier family 27 member 5 (Slc27a5 or Fatp5), and CD36. The two fatty acid
transport proteins, Slc27a2 and Slc27a5, did not significantly differ (Table 1), which was further demonstrated by quantitative RT-PCR performed in liver tissues of 14-week-old WT or Sidt2−/− mice (Fig.2B). However, CD36 was consistently increased approximately 4 folds in Sidt2−/− mice at 3 and 6 months of age (Table 1). Since CD36-null mice exhibit reduced muscle TG and liver steatosis (27), CD36 may play a limited role in hepatocyte uptake of fatty acids. Instead, fatty acid transport proteins play a predominant role in this cellular process (10). Thus, fatty acid uptake was not significantly increased in Sidt2−/− mice.

Data of microarray and quantitative RT-PCR also showed that most of the genes involved in de novo lipogenesis, including fatty acid synthase (Fasn), diacylglycerol acyltransferase-1 (Dgat1), and diacylglycerol acyltransferase-2 (Dgat2) were not significantly changed in Sidt2−/− mice (Table 1, Fig.2B). Furthermore, the master gene that controls the expression of lipogenic genes, sterol regulatory element binding transcription factor 1 (Srebf1) was not changed (Table 1, Fig.2B). Since lipogenesis is mainly controlled at the transcriptional level (28), it was concluded that de novo lipogenesis was not affected in Sidt2−/− mice.

Liver secretes TG in the form of very low-density lipoprotein to the peripheral blood. We injected fasted mice with poloxamer-407, an inhibitor of lipoprotein lipase. Though TG production rate was decreased by 20% in Sidt2−/− mice compared with WT mice, there was no significant difference between the two groups (Fig. 2C).

Simultaneously, the level of serum NEFA, the product of lipolysis in adipose tissue, was not significantly different between Sidt2−/− and WT mice (Fig. 2D). Consistent with the unchanged serum NEFA, the weights of adipose tissues including gonadal fat, perinephric fat and brown adipose, were comparable
between Sidt2−/− and WT mice (Fig. 2E), suggesting Sidt2 deficiency does not induce excessive lipolysis in adipose tissue.

**Sidt2 knockout reduces hepatic β-oxidation of fatty acids**

Fatty acid β-oxidation is another method of TG disposal. We measured the level of serum β-hydroxybutyrate, the product of hepatic fatty acid β-oxidation. In Sidt2−/− mice, serum β-hydroxybutyrate concentrations decreased to 68% compared to WT mice (Fig. 3A), suggesting that hepatic β-oxidation is impaired in Sidt2−/− mice.

However, the RNA microarray and the quantitative RT-PCR analyses showed the expression of genes involved in hepatic mitochondrial fatty acid oxidation, such as carnitine palmitoyltransferase I (Cpt1), carnitine palmitoyltransferase 2 (Cpt2), and peroxisome proliferator activated receptor alpha (Ppara), were not significantly different between Sidt2−/− and WT mice (Table 1, Fig. 3B). These data suggest that Sidt2 deficiency reduced hepatic mitochondrial β-oxidation of fatty acids without affecting transcription of related enzymes. With the genes providing free fatty acids for mitochondrial β-oxidation unchanged, such as hepatic lipases (Lipc) and hormone sensitive lipase (Hsl) (Table 1, Fig. 3B), we speculated that lipophagy (12), which provides fatty acids to mitochondria, was affected in Sidt2−/− mice.

**Lipid metabolism impaired in 6-week-old mice**

To determine when Sidt2 began to damage hepatic lipid metabolism in mice, we evaluated lipid metabolism in 6-week-old mice. In 6-week-old Sidt2−/− mice, liver TG content and liver ADFP protein expression were increased, and serum β-hydroxybutyrate levels were decreased compared with age-matched WT mice (Fig. 4A-C). These findings confirmed that Sidt2 deficiency led to lipid accumulation by impairing β-oxidation in the liver. Consistent with 14-week-old mice, no significant difference was
found in serum NEFA between 6-week-old Sidt2+/− and WT mice (Fig. 4D).

**Sidt2 knockout blocks hepatic autophagy in vivo**

To explore whether Sidt2 regulates liver steatosis via autophagy, we tested liver responses in Sidt2+/− mice. LC3-II is present in autophagosomes and is a commonly used marker of autophagy activation (29). We found that LC3-II levels were significantly elevated in livers from Sidt2+/− mice fed a chow diet, compared to aged-matched WT mice, suggesting normal or increased autophagosome formation (Fig. 5A, B). In addition, p62/SQSTM1, which functions in guidance of polyubiquitinated protein to autophagosomes and sequential degradation via the autophagy pathway (27, 30), also accumulated in livers of Sidt2+/− mice (Fig. 5A, C). Increase of both LC3-II and p62 levels were also observed in Sidt2+/− mice with HF diet (Fig. 5D–F), suggesting a defect in the autophagy pathway after autophagosome formation.

EM evaluation showed abundant autophagic vacuoles at different stages of autophagy in Sidt2+/− (Fig. 5H-J) compared to WT mice in vivo (Fig. 5G). Sidt2+/− livers had more double/multi-lamellar membrane structures containing cytosol, lipid droplets, or other organelles (Fig. 5H). We also identified autolysosomes, vacuoles surrounded by one limiting membrane enclosing cytoplasmic material and/or organelles, at various stages of degradation (Fig. 5I, J). Therefore, EM examination supported that Sidt2 deficiency impairs maturation of autophagosomes to autolysosomes.

**Sidt2 knockout blocks autophagy in vitro**

To further assess the role of Sidt2 in autophagy, we analyzed autophagosome formation in primary hepatocytes isolated from Sidt2+/− and WT mice. Consistent with our observations in vivo, LC3-II and p62 expression in Sidt2+/− hepatocytes increased in the normal medium (Fig. 6A-B). However, LC3-II levels in Sidt2+/− hepatocytes did not increase further in nutrient-free medium (Fig. 6B). Since either induction of
autophagy or inhibition of autophagosome clearance could account for the increase in LC3-II levels, we measured autophagic flux by neutralizing lysosomal vacuolar pH with chloroquine. As shown in Fig. 6C, WT hepatocytes displayed a drastic increase in the levels of LC3-II in the presence of chloroquine (3.5-folds). By contrast, the response of Sidt2−/− hepatocytes was much less pronounced (1.3-folds), indicating that the autophagic flux had already been blocked by Sidt2 deficiency.

The transmission EM also showed increased autophagic vacuoles at all stages of maturation in primary hepatocytes isolated from Sidt2−/− mice (Fig. 6E-I). And the number of autophagic vacuoles in Sidt2−/− hepatocytes was 3.3 folds of that in WT hepatocytes (Fig. 6K). These in vitro findings confirmed that Sidt2 deficiency does not impair autophagosome formation, but interrupts the later stage.

**Autophagy-related gene expression does not significantly change in livers of Sidt2−/− mice**

Autophagosome formation is regulated at the molecular level by autophagy-related (Atg) genes. Among the corresponding Atg proteins, some are referred to as the ‘core’ molecular machinery as they are indispensable for autophagosome formation (31). In order to analyze whether Sidt2 regulate the expression of Atg genes involved in core molecular machinery in vivo, we performed microarray analyses on Sidt2-deleted livers at 1, 3, and 6 months of age. In the liver transcriptome of Sidt2−/− mice, ULK1/2, Atg3, Atg5, Atg7, Atg12, and Beclin1 levels changed by less than ± 2-folds, indicating they were not significantly affected (Table 2), and these observations were in agreement with real-time PCR analysis (Figures 7A). Hepatic protein levels of autophagy-related proteins (Atg5, Atg7 and Beclin1) in Sidt2−/− were also comparable to WT (Figures 7B). These data suggest that Sidt2 deficiency inhibits autophagy without changing RNA or protein expression of autophagy-related genes. Up-regulation of LC3-II protein levels and increased autophagic vacuoles without enhanced autophagy initiation further confirmed that Sidt2
deficiency leads to blockage during the terminal stage of autophagy.

In the course of autophagy, suppression of mTOR activity results in upregulation of lysosomal function (32). To address the status of mTOR kinase activity in Sidt2-depleted primary hepatocytes, we assessed levels of phosphorylation of S6K1 and 4E-BP1 by Immunoblotting analysis. As shown in Fig. 7C, no significant difference was detected between endogenous phosphorylated S6K1 and 4E-BP1 in Sidt2-deficient hepatocytes compared with WT hepatocytes. Since mTORC1 is also a key upstream kinase that regulates the activity of transcription factor EB (TFEB) (33), we assessed its protein expression by Western Blot (Fig. 7D) and mRNA expression by genechip analysis (table 2). The results showed that neither protein expression nor mRNA expression was changed in Sidt2−/− mice. Our data revealed that Sidt2 deficiency does not significantly affect mTOR signaling pathway.

As an integral lysosomal membrane protein, Sidt2 depletion could affect the structure and function of lysosomes. Therefore, we measured the protein expression levels of lysosome-associated membrane protein2 (Lamp2) and lysosomal proteases including cathepsin B and D. We detected Lamp2 was increased in liver of Sidt2−/− mice (Fig.7E), which could be explained by accumulation of autolysosomes. As shown in Fig. 7F, the levels of mature cathepsin B in hepatic extracts of Sidt2−/− mice was lower (~1.5 folds) compared with the levels in WT mice. Pro-cathepsinB, as a precursor of Cathepsin B, in hepatic extracts of Sidt2−/− mice was comparable to WT mice. Simultaneously, expression analysis of Ctsb gene showed no significant difference between Sidt2−/− and WT mice. Cathepsin B is regulated at multiple levels including transcription, trafficking, activation, so the results suggested that Sidt2 deficiency could impair pro-cathepsin B transport or maturation in the lysosome, consequently, lead to reduced cathepsin B. We did not find significant differences both in level of cathepsin D protein (mature form and immature form).
In addition, ASMase is a lysosomal enzyme expressed ubiquitously. Previous studies have revealed that changes in ASMase activity lead to autophagy dysregulation and contribute to the pathogenesis of fatty liver disease (28, 34). But we found no significant difference in ASMase activity between the two groups (Fig. 7G).

DISCUSSION

Integral lysosomal membrane proteins are involved in various physiological processes, such as acidification of the lysosomal lumen and membrane fusion; thus, identification of more LMPs and further understanding their function has recently drawn more attention (9). In this study, mice with a global knockout of the Sidt2 gene, an integral lysosomal membrane protein, presented with increased liver volume and weight, accumulation of lipid droplets, and elevated hepatic TG levels. These characteristics resemble non-alcoholic fatty liver disease in humans.

In contrast with the abundant content in adipose tissue, the liver has relatively low levels of cytosolic triglyceride lipase and hormone sensitive lipase (35). Thus, the liver needs an additional pathway to digest fat. Autophagy is a critical pathway contributing to the liver's rapid turnover of TG (12). Portions of lipid droplets, or even whole droplets, become sequestrated inside the double membrane-bound vesicles and are delivered to lysosomes, where they are degraded to fatty acids. The autophagy-related lipid degradation pathway allows hepatocytes to rapidly mobilize large amounts of lipids despite their lower levels of cytosolic lipases compared with adipose tissues (36). When pharmacologic or genetic factors inhibit autophagy in hepatocytes, breakdown of stored lipids to supply fatty acids for β-oxidation is blocked (12).
First, enhanced lipogenesis, reduced TG secretion, diminished liver lipolysis and excess lipolysis in adipose tissue were excluded as a possible cause of TG accumulation in the Sidt2−/− liver. Second, several lines of investigation demonstrated that defective autophagy may contribute to the TG accumulation in Sidt2 deficient mice, including elevated p62 and LC3-II, EM evidence of autophagosomes accumulation in the liver, and decreased serum levels of β-hydroxybutyrate.

The terminal stage of autophagy involves the fusion of autophagosomes with endosomes/lysosomes and sequential substrate degradation (37). Our in vitro analysis showed in primary hepatocytes isolated from Sidt2−/− mice the high levels of LC3-II detected in basal conditions were not further increased by chloroquine treatment. This observation revealed that Sidt2 deficiency impaired autophagy at the terminal stage rather than the initial stage of autophagosome formation. This hypothesis was supported by unchanged mRNA expression of autophagy-related genes involved in autophagy initiation. EM analysis of both in vivo and in vitro experiments showed that Sidt2 deficiency caused accumulation of autophagosomes. Thus, we inferred that maturation of autophagosomes was blocked in Sidt2−/− mice.

Cathepsin B is synthesized in the rough endoplasmic reticulum as a preproenzyme and then it turns into pro-cathepsin B after removing the signal peptide. Pro-cathepsin B is glycosylated in the Golgi apparatus and is transported to lysosomes where it is proteolytically cleaved to form a mature protein. Reduced level of mature cathepsin B and unchanged level of pro-cathepsin B in Sidt2−/− liver, along with normal mRNA for Ctsb implied that Sidt2 could affect pro-cathepsin B transport or maturation in the lysosomes. It has been reported that reduction of cathepsin B and D contribute to dysfunctional autophagy by delayed degradation of autolysosomes and defective lysosome reformation(38). The role of decreased mature cathepsin B in Sidt2−/− liver should be further investigated. We found no significant reduced level of
another major lysosomal protease, cathepsin D, underscoring the high sensitivity of cathepsin B to variation of lysosome homeostasis.

The impact of Sidt2 on autophagy is reminiscent of LAMP-2, one of the most abundant lysosomal membrane proteins. LAMP-2 deficiency causes Danon disease in humans and a similar phenotype in mouse models. Previously, autophagosome accumulation in LAMP-2 deficient mice was shown to be secondary to its slow maturation rather than increased formation (39, 40). Further study is necessary to investigate whether there is interaction between Sidt2 and LAMP-2.

Our previous studies revealed that a global Sidt2 gene knockout mouse exhibited age-dependent elevated plasma glucose levels and impaired glucose tolerance at 8 weeks of age(41). However, Sidt2⁻/⁻ mice at 6 weeks of age already displayed elevated liver TG content and decreased serum β-hydroxybutyrate level, which minimized the effect of hyperglycemia on lipid metabolism to some extent. To address the direct effects of Sidt2 on the liver, future studies in liver-specific Sidt2 knockout models should be performed.

In our study, the mice fed a HF diet exhibited a much more pronounced phenotype in fatty infiltration of the liver compared to mice fed a chow diet. Sidt2⁻/⁻ mice fed a HF diet even showed manifestation of steatohepatitis, such as ballooning degeneration. This finding suggests that Sidt2 may play an important role in accommodating a chronic exogenous lipid challenge.

In summary, our study demonstrates that Sidt2 is critical in mouse triglyceride catabolism by regulating autophagy at its terminal stage. Therefore, Sidt2 may be target of therapeutic intervention of nonalcoholic fatty liver disease.
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### Table 1. The mRNA expression of genes involved in lipid metabolism in \( \textit{Sidt2}^{+/} \) relative to wild-type mice

| Gene Symbol | Gene Title | Fold Change (KO/WT) |
|-------------|------------|---------------------|
|             |            | 1 month  | 3 months | 6 months |
| **Fatty acid uptake and transport** | | |
| Slc27a2 | solute carrier family 27 member 2 | 1.06 | 1.05 | -1.52 |
| Slc27a5 | solute carrier family 27 member 2 | -1.04 | -1.16 | -1.32 |
| Cd36 | CD36 antigen | 2.04 | 4.73 | 3.64 |
| Acsl1 | acyl-CoA synthetase long-chain family member 1 | 1.20 | -1.50 | -2.70 |
| Fabp1 | fatty acid binding protein 1 | -1.02 | 1.07 | -1.68 |
| **Lipogenesis** | | |
| Srebf1 | Sterol regulatory element binding transcription factor 1 | -1.82 | 1.29 | -1.18 |
| Acaca | acetyl-CoenzymeA carboxylase alpha | -1.13 | -1.09 | 1.02 |
| Fasn | fatty acid synthase | -1.04 | -1.14 | -1.25 |
| Elov6 | ELOVL family member 6, elongation of long chain fatty acids | 1.50 | 1.04 | -1.03 |
| Scd1 | stearoyl-Coenzyme A desaturase 1 | -1.46 | -1.11 | 1.37 |
| Agpat1 | 1-acylglycerol-3-phosphate O-acyltransferase 1 | -1.01 | -1.18 | 1.03 |
| Lpin1 | lipin 1 | 2.84 | -1.88 | -1.02 |
| Dgat1 | diacylglycerol O-acyltransferase 1 | 1.04 | -1.06 | -1.12 |
| Dgat2 | diacylglycerol O-acyltransferase 2 | -1.19 | -1.00 | -1.18 |
| **Lipolysis** | | |
| Hsl | lipase, hormone sensitive | -1.10 | -1.31 | 1.01 |
| Atgl | adipose triglyceride lipase | -1.05 | -1.30 | -1.22 |
| Lipc | lipase, hepatic | -1.18 | 1.47 | -2.02 |
| **Fatty acid oxidation** | | |
| Cpt1a | carnitine palmitoyltransferase 1a, liver | -1.18 | -1.11 | -1.57 |
| Cpt2 | carnitine palmitoyltransferase 2 | 1.58 | 1.03 | -1.52 |
| Cact | mitochondrial carnitine/acylcarnitine translocase | 1.37 | -1.07 | -1.24 |
| Ppara | peroxisome proliferator activated receptor alpha | 1.05 | 1.03 | -1.85 |
## Lipoprotein assembly

*Mttp* microsomal triglyceride transfer protein  
1.27  
1.43  
-1.28

### Table 2. The mRNA expression of autophagy-related genes in *Sidt2*−/− relative to wild-type mice

| Gene Symbol | Gene Title                                      | Fold Change (KO/WT) |
|-------------|------------------------------------------------|----------------------|
| *Ulk1*     | Unc-51 like kinase 1                            | -1.25 1.10 -1.44    |
| *Ulk2*     | Unc-51 like kinase 2                            | -1.04 1.24 -1.34    |
| *Atg13*    | ATG13 autophagy related 13 homolog              | -1.04 1.02 -1.01    |
| *Map1lc3b* | Microtubule-associated protein 1 light chain 3 beta | -1.05 -1.09 1.22 |
| *Atg5*     | Autophagy related 5                             | 1.41 1.19 0.85      |
| *Atg12*    | Autophagy related 12                            | 1.20 1.06 0.92      |
| *Atg3*     | Autophagy related 3                             | 1.15 1.24 -1.47     |
| *Atg7*     | Autophagy related 7                             | -1.13 -1.13 -1.35   |
| *Beclin 1* | Beclin 1, autophagy related                     | 1.19 1.31 -1.08     |
| *Atg14*    | Autophagy related 14                            | -1.12 -1.39 -1.10   |
| *Uvrag*    | UV radiation resistance associated gene         | -1.05 -1.06 -1.22   |
| *Atg9a*    | Autophagy-related 9A                            | -1.13 -1.08 1.01    |
| *Atg9b*    | ATG9 autophagy related 9 homolog B              | 1.23 1.02 1.09      |
| *Atg10*    | Autophagy related 10                            | 1.08 1.49 -1.05     |
| *Tcfeb*    | Transcription factor EB                         | -1.09 1.03 1.31     |
Figure 1.
Figure 1. *Sidt2* knockout causes lipid accumulation in the liver. (A) Gross view and weights of livers in chow and HF diet groups (n=5). (B) Body weights of WT and *Sidt2*−/− mice maintained on a HF diet for 12 weeks. (C) H&E staining of liver from mice fed with chow diet (scale bar 50 µm). Graphs show the quantification of hepatocytes size in more than 100 cells from three different mice in each group. (D) Oil red O staining (scale bar 100 µm). (E) Electron micrographs of liver from chow diet-fed mice without intake of food for 20 h. Scale bars, 5 µm (left) and 2 µm (right). Graphs show quantifications of LD number and size (n=3). (F) Oil Red O staining of primary hepatocytes from *Sidt2*−/− and WT mice treated with 0.2 mM OA for 24 h. Microscopic images (left, 400X) and quantification of Oil Red O-stained area (right). n=46-54 cells from three independent experiments. (G) Immunoblot analysis of ADFP levels in livers from mice fed with chow diet (n=4). (H) Hepatic TG concentration of mice fed chow or HF diets (n=6). (I) Serum ALT and AST levels (n=6). * represents $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Abbreviation: TBW, total body weight.
Figure 2

Figure 2. Sidt2 knockout does not influence lipogenesis and TG secretion. (A) Total food consumption of WT or Sidt2−/− mice under a chow diet measured for 4 weeks beginning at 8 weeks of age (n=8). (B) QPCR analysis of genes involved in lipid transport and lipogenesis in the livers of 14-week-old WT or Sidt2−/− mice fed a chow diet (n=3). (C) Hepatic TG production rate (n=6). (D) Serum NEFA level (n=6). (E) The weight of adipose tissues from WT or Sidt2−/− mice under a chow diet (n=6). Abbreviation: TBW, total body weight.
Figure 3

Figure 3. Sidt2 knockout reduces hepatic β-oxidation of fatty acids. (A) Serum β-hydroxybutyrate levels of mice fed a chow diet (n=6). (B) QPCR analysis of genes involved in lipolysis and fatty acid oxidation in livers of 14-week-old WT or Sidt2−/− mice fed a chow diet (n=3). ** represents p < 0.01.
Figure 4. Lipid metabolism in 6-week-old mice fed with chow diet. (A) Liver TG levels (n=5). (B) Immunoblot and densitometry analysis of ADFP levels in murine livers (n=4). (C) Serum β-hydroxybutyrate levels (n=5). (D) Serum NEFA levels of 6-week-old mice. (n=5). * represents p < 0.05.
Figure 5. Sidt2 knockout blocks hepatic autophagy in vivo. (A-C) Immunoblot analysis of LC3-II and p62 levels in livers from 14-week-old mice fed with chow diet (n=4). (D-F) Immunoblot analysis of LC3-II and p62 levels in livers from mice fed with HF diet (n=4). EM of liver tissues from WT (G) and Sidt2−/− (H).
(H-J) mice. Abundant vacuolar structures were observed in Sidt2" livers. White arrows indicate autophagosomes and multi-lamellar membrane structures. Black arrows indicate autolysosomes.

* indicates lipid droplets. Scale bars: G, 2 μm; H, I, and J, 1 μm. (K) Quantification of autophagic vacuoles (n=3). * represents $p < 0.05$. **$p < 0.01$. 
Figure 6

Figure 6. Sidt2 knockout blocks autophagy in vitro. (A) Immunoblot analysis of p62 levels of primary hepatocytes from Sidt2−/− and WT mice in condition of normal medium. (B) Immunoblot analysis of LC3-II levels in primary hepatocytes from Sidt2−/− and WT mice. Cells were either kept in normal medium or starved in EBSS medium for 2 h. (C) Primary hepatocytes were treated for 2 h with or without 30 μM
chloroquine, then the protein levels of LC3-II were detected by Western blot (n=4). (D-I) Electron micrograph of primary hepatocytes cultured in normal medium from WT (D) and Sidt2−/− mice (E-I). (F-I) Higher magnification images of the boxed area in E showing autophagosomes marked by white arrows and autolysosomes marked by black arrows. Scale bars for D and E, 1 μm; F, 0.5 μm; G-I, 0.2 μm. (J) Graph shows quantification of number of autophagic vacuoles (AVs) in hepatocytes (n=14 cells). * represents $p < 0.05$, **$p < 0.01$, and ***$p < 0.001$. Abbreviation: CQ, chloroquine.
Figure 7. Autophagy-related gene expression does not significantly change in livers of Sidt2−/− mice

(A) QPCR analysis of genes involved in autophagy and lysosomal proteases in livers of 14-week-old WT or Sidt2−/− mice fed a chow diet (n=3). (B) Immunoblot analysis of Beclin1, Atg5 and Atg7 levels in livers from 14-week-old mice fed with chow diet (n=4). (C) Phospho-S6K1, S6K1, Phospho-4E-BP1 and 4E-BP1 of primary hepatocytes in regular medium from Sidt2−/− and WT mice (n=4). (D-F) Immunoblot analysis of TFEB (D), Lamp2 (E), cathepsin B and cathepsin D (in their immature and mature forms) (F) in mouse livers. (G) ASMase activity in liver lysates from Sidt2−/− and WT mice fed a chow diet (n=3-4). * represents p < 0.05. Abbreviation: Ctsb: Cathepsin B, Ctsd: Cathepsin D.