Hepatic steatosis is a hallmark of nonalcoholic fatty liver disease (NAFLD) and is promoted by dysregulated de novo lipogenesis. ATP-citrate lyase (ACLY) is a crucial lipogenic enzyme that is up-regulated in individuals with NAFLD. A previous study has shown that acetylation of ACLY at Lys-540, Lys-546, and Lys-554 (ACLY-3K) increases ACLY protein stability by antagonizing its ubiquitylation, thereby promoting lipid synthesis and cell proliferation in lung cancer cells. But the functional importance of this regulatory mechanism in other cellular or tissue contexts or under other pathophysiological conditions awaits further investigation. Here, we show that ACLY-3K acetylation also promotes ACLY protein stability in AML12 cells, a mouse hepatocyte cell line, and found that the deacetylase sirtuin 2 (SIRT2) deacetylates ACLY-3K and destabilizes ACLY in these cells. Of note, the livers of mice and humans with NAFLD had increased ACLY protein and ACLY-3K acetylation levels and decreased SIRT2 protein levels. Mimicking ACLY-3K acetylation by replacing the three lysines with three glutamines (ACLY-3KQ variant) promoted lipid accumulation both in high glucose–treated AML12 cells and in the livers of high-fat/high-sucrose (HF/HS) diet–fed mice. Moreover, overexpressing SIRT2 in AML12 cells inhibited lipid accumulation, which was more efficiently reversed by overexpressing the ACLY-3KQ variant than by overexpressing WT ACLY. Additionally, hepatic SIRT2 overexpression decreased ACLY-3K acetylation and its protein level and alleviated hepatic steatosis in HF/HS diet–fed mice. Our findings reveal a posttranscriptional mechanism underlying the up-regulation of hepatic ACLY in NAFLD and suggest that the SIRT2/ACLY axis is involved in NAFLD progression.

Nonalcoholic fatty liver disease (NAFLD) represents a spectrum of liver disorders ranging from simple steatosis to nonalcoholic steatohepatitis, cirrhosis, and even hepatocellular carcinoma (1–3). NAFLD is strongly associated with obesity, insulin resistance, hypertension, and dyslipidemia, making it the liver manifestation of metabolic syndrome (4, 5). It has been estimated that over a third of the adult population and ~5–10% of children in the United States develop fatty liver (2, 6–8). Although the “two-hit” hypothesis has been widely accepted to depict the pathogenesis of NAFLD, the molecular mechanism underlying the initiation and progression of NAFLD remains imperfectly understood. Meanwhile, effective therapeutic strategies to prevent the disease progression of NAFLD patients are lacking (9, 10).

Hepatic steatosis, the excessive accumulation of lipid in the liver, is an early hallmark of NAFLD, which results from abnormally enhanced de novo lipid synthesis and fat delivery from excessive adipose tissue as well as decreased fatty acid oxidation and lipid export (11). Previous studies have established a close association between obesity and aberrant stimulation of hepatic lipogenesis. ATP-citrate lyase (ACLY) is a crucial lipogenic enzyme that catalyzes an ATP-consuming reaction to generate acetyl-CoA from citrate, and acetyl-CoA is the key building block for de novo lipidogenesis (12). Acetyl-CoA is further converted to malonyl-CoA, from which fatty acids are subsequently synthesized. Thus, ACLY links cellular glucose catabolism and de novo lipid synthesis. ACLY expression levels are up-regulated in the livers of db/db mice that have morbid obesity, fatty liver, and type 2 diabetes. Hepatic ACLY abrogation by short hairpin RNA (shRNA) reduces hepatic lipogenesis to protect against fatty liver and ameliorate insulin resistance in db/db mice (13). Therefore, aberrant up-regulation of hepatic ACLY could be an important factor promoting the progression of NAFLD. However, how ACLY is up-regulated during the development of NAFLD, especially at posttranscriptional levels, is still not completely understood.

Lysine acetylation is a conserved protein posttranslational modification in the regulation of a wide range of cellular processes, including cellular metabolism (14). Numerous studies have shown that many metabolic enzymes can be acetylated, and the functional importance of their acetylation has been elucidated (15–17). For example, the M2 isoform of pyruvate kinase is acetylated at lysine 433, which promotes its nuclear localization; TSA, trichostatin A; ALT, alanine aminotransferase; TNFα, tumor necrosis factor α; IL1β, interleukin 1β; H&E, hematoxylin and eosin; pfu, plaque-forming units; ACLY-3K-Ac, ACLY-3K acetylation.
accumulation and protein kinase activity to facilitate cell proliferation and tumorogenesis (18). Acetylation of aldehyde dehydrogenase inhibits its enzyme activity, thereby suppressing breast cancer stem cells (19). In a previous study, ACLY was shown to be acetylated at lysine residues 540, 546, and 554 (ACLY-3K) with sirtuin 2 (SIRT2) as its deacetylase. Acetylation on ACLY-3K increases ACLY protein stability by antagonizing its ubiquitylation and proteasome-mediated degradation to promote lipid synthesis and cell proliferation in lung cancer cells (20). However, whether the above regulatory mechanism for ACLY also functions in other cellular or tissue contexts or under other pathophysiological conditions is not clear. In this study, we demonstrate that ACLY-3K acetylation enhances ACLY protein stability and promotes lipid accumulation in the mouse hepatocyte cell line AML12 and aggravates hepatic steatosis in high-fat/high-sucrose (HF/HS) diet–fed mice. Overexpression of SIRT2 leads to impaired acetylation of ACLY on 3K sites, decreased ACLY protein stability with attenuated lipid accumulation in AML12 cells, and alleviated hepatic steatosis in HF/HS diet–fed mice. We also show an up-regulation of ACLY protein and ACLY-3K acetylation levels, with a decline of SIRT2 protein level, in the livers of mice and humans with NAFLD. Our findings reveal a SIRT2/ACLY axis that could be involved in the pathogenic development of NAFLD.

Results

SIRT2-mediated ACLY deacetylation on lysine residues 540, 546, and 554 (3K sites) decreases ACLY protein stability in the mouse hepatocyte cell line AML12

Although ACLY-3K acetylation has been shown to promote protein stability of ACLY by antagonizing its ubiquitylation and proteasome-mediated degradation and facilitate cell proliferation in lung cancer cells (20), the functional importance of this regulatory mechanism in other cellular or tissue contexts awaits further investigation. To study the functional role of ACLY-3K acetylation in hepatocytes, mouse hepatocyte cell line AML12 cells were transfected with the plasmid encoding wildtype (WT) ACLY (Fig. 1A) or ACLY-3KQ variant (Fig. 1B) that mimics ACLY-3K acetylation. Then the cells were treated with cycloheximide (CHX) for the indicated time points and subjected to Western blotting to test protein stability. As shown in Fig. 1C, ACLY-3KQ variant shows higher protein stability than WT ACLY, suggesting that ACLY-3K acetylation enhances the protein stability of ACLY in AML12 cells. To study the involvement of the ubiquitin/proteasome degradation pathway in regulating ACLY protein stability in AML12 cells, the proteasome inhibitor MG132 was used. AML12 cells were transfected with the plasmid encoding FLAG-tagged WT ACLY (FLAG-ACLY-WT) or 3KQ variant of ACLY (FLAG-ACLY-3KQ) and treated with or without MG132. Inhibiting the ubiquitin/proteasome degradation pathway by MG132 led to increased protein level of FLAG-ACLY-WT, whereas it had no effect on the protein level of FLAG-ACLY-3KQ (Fig. 1D). In addition, MG132 did not affect the mRNA level of ACLY (Fig. 1E). Therefore, these results support the notion that ACLY-3K acetylation enhances ACLY protein stability by preventing the degradation of ACLY through the ubiquitin/proteasome pathway.

To identify the deacetylase for ACLY in AML12 cells, NAM, an inhibitor of the SIRT family deacetylases, and trichostatin A (TSA), an inhibitor of histone deacetylases, were used to study their roles in ACLY-3K acetylation. AML12 cells were transfected with the plasmid encoding FLAG-ACLY-WT. After 24 h, cells were treated with TSA or NAM before being harvested and subjected to immunoprecipitation with anti-FLAG antibody. The immunoprecipitates were then subjected to Western blotting with the antibody against ACLY or that against ACLY-3K acetylation (ACLY-3K-Ac). Treatment of the cells with NAM, but not with TSA, obviously increased the ACLY-3K acetylation level (Fig. 1F), which suggests that SIRTs are responsible for deacetylating ACLY. Seven SIRTs (SIRT1–SIRT7) have been identified in mammals. SIRT1, SIRT6, and SIRT7 are principally found in the nucleus. SIRT3–5 are located in the mitochondria, whereas SIRT2 is mainly located in the cytoplasm (21). ACLY is recognized as a cytoplasmic protein. Therefore, the localization of ACLY in the cells seems similar to that of SIRT2. Besides, SIRT2 has been shown to be a deacetylase of ACLY in lung cancer cells. As a result, the role of SIRT2 in the deacetylation of ACLY-3K in AML12 cells was examined. As shown in Fig. 1G, overexpression of SIRT2 obviously decreased the ACLY-3K acetylation level, which indicates that SIRT2 functions as a deacetylase of ACLY to remove ACLY-3K acetylation in AML12 cells. Consistently, overexpression of SIRT2 did not affect the mRNA level of ACLY (Fig. 1H) but caused an obvious decrease of ACLY protein level (Fig. 1I). Taken together, these data demonstrate that SIRT2-mediated deacetylation of ACLY on 3K sites leads to decreased protein stability of ACLY in the mouse hepatocyte cell line AML12.

Increased ACLY protein level and ACLY-3K acetylation level, with decreased SIRT2 protein level, are detected in the livers of HF/HS diet–fed mice and db/db mice

Because ACLY is an important enzyme for lipogenesis and dysregulated expression of ACLY contributes to the development of NAFLD, the levels of total ACLY protein, ACLY-3K acetylation, and its candidate deacetylase SIRT2 were examined in the mice with hepatic steatosis. Mice fed with HF/HS diet for 12 weeks are known to suffer from fatty liver (22, 23). HF/HS diet–fed mice had significantly higher hepatic triglyceride levels than chow diet–fed mice (Fig. 2A), which confirms the hepatic steatosis induced by HF/HS diet feeding. As shown in Fig. 2B, compared with the chow diet–fed mice, the protein level of ACLY is higher, whereas the protein level of SIRT2 is lower, in the livers of HF/HS diet–fed mice. To investigate the ACLY-3K acetylation level, we examined ACLY-3K acetylation by normalizing it to ACLY protein level. As Fig. 2, C and D, show, higher ACLY-3K acetylation level is detected in the livers of HF/HS diet–fed mice. The db/db mice also have obvious hepatic steatosis (Fig. 2E). Similar to HF/HS diet–fed mice, the levels of ACLY protein and ACLY-3K acetylation are also higher, whereas the level of SIRT2 protein is lower, in the livers of db/db mice than in the livers of control lean mice (Fig. 2, F–H). These data suggest that enhanced hepatic ACLY-3K acetylation could play some role in the development of hepatic steatosis in mice.
Increased ACLY protein level and ACLY-3K acetylation level, with decreased SIRT2 protein level, are detected in the livers of NAFLD patients

To further study the correlation of ACLY, ACLY-3K acetylation, and SIRT2 level in the progression of NAFLD, paraffin-embedded human liver tissues from control patients and NAFLD patients were used to perform immunohistochemistry (IHC) staining. As shown in Fig. 3, A–D, the levels of ACLY protein and ACLY-3K acetylation are increased, whereas the level of SIRT2 protein is decreased, in the livers of NAFLD patients compared with the livers of control patients. Because ACLY-3K acetylation could increase ACLY protein level, to better analyze the ACLY-3K acetylation level, we normalized the relative intensity of ACLY-3K acetylation to the relative...
intensity of ACLY and found that normalized ACLY-3K acetylation level is still higher in the livers of NAFLD patients than in the livers of control patients (Fig. 3E). Linear progression analyses were also performed. ACLY protein level negatively correlates with SIRT2 protein level (Fig. 3F). Similarly, the higher ACLY-3K acetylation level correlates with the lower SIRT2 protein level (Fig. 3G). Moreover, the ACLY-3K acetylation level positively correlates with the ACLY protein level (Fig. 3H). Taken together, these results further suggest that ACLY-3K acetylation and SIRT2 could be implicated in the pathogenic development of NAFLD.

**ACLY-3K acetylation enhances lipid accumulation in high glucose–treated AML12 cells**

The good correlation between ACLY-3K acetylation and NAFLD as shown in Figs. 2 and 3 prompted us to investigate the functional role of ACLY-3K acetylation in hepatic steatosis. To this end, the endogenous ACLY was knocked down by small interfering RNA (siRNA) against ACLY (siACLY), and in the meantime, the RNAi-resistant WT ACLY (ACLY-WT) or ACLY-3KQ variant was reexpressed in AML12 cells. At the mRNA level, the endogenous ACLY was efficiently knocked down, and the reexpressed ACLY-WT or ACLY-3KQ reached a level similar to that of the endogenous ACLY (Fig. 4A). At the protein level, a similar result was obtained, except that the level of the reexpressed ACLY-3KQ was about 1.9-fold higher than the level of endogenous ACLY and the reexpressed ACLY-WT (Fig. 4, B and C). The results above are consistent with the results in Fig. 1 that ACLY-3K acetylation promotes ACLY protein stability. To determine the effect of ACLY-3K acetylation on lipid accumulation in hepatocytes, AML12 cells were cultured in complete medium with high glucose (25 mM), which
can lead to slight lipid accumulation in the cells (Fig. 4D). Oil Red O staining demonstrated that knocking down ACLY caused an obvious decrease in lipid accumulation, which indicates an important role of ACLY in the lipid homeostasis in hepatocytes (Fig. 4, D and E). In addition, reexpressing ACLY-3KQ restored the lipid accumulation more efficiently than reexpressing ACLY-WT (Fig. 4, D and E). Therefore, these in vitro data suggest a functional role of ACLY-3K acetylation in promoting lipid accumulation in AML12 cells.

ACLY-3K acetylation aggravates hepatic steatosis in mice challenged by HF/HS diet

On the basis of in vitro results, we next examined whether ACLY-3K acetylation also plays a role in HF/HS diet–induced hepatic steatosis in mice. We used a recombinant adenovirus to deliver the shRNA and the shRNA-resistant expression constructs to mouse livers through tail vein injection. Mice fed an HF/HS diet for 12 weeks were infected once a week with adenoviruses harboring the indicated shRNA and expression constructs via tail vein injection and sacrificed 15 days after the first virus injection. The knockdown of ACLY and the reexpression of ACLY-WT or ACLY-3KQ in the mouse livers was confirmed at both the mRNA level and the protein level. The shRNA against ACLY led to significant down-regulation of ACLY mRNA level, and the reexpression of ACLY-WT or ACLY-3KQ yielded mRNA levels similar to the endogenous ACLY mRNA level (Fig. 5A). At the protein level, however, ACLY-3KQ was about 1.6-fold higher than that of ACLY-WT (Fig. 5, B and C), supporting the notion that ACLY-3K acetylation promotes ACLY protein stability. Knockdown of ACLY ameliorated hepatic steatosis in the livers of HF/HS diet–fed mice and its accompanying pathology as shown by decreased fat accumula-
Enhanced ACLY acetylation promotes NAFLD

Figure 4. ACLY-3K acetylation enhances lipid accumulation in high glucose–treated AML12 cells. A, AML12 cells cultured in high-glucose (25 mM) medium were transfected with control siRNA (siNC) or siRNA against ACLY (siACLY) together with the plasmids encoding GFP, RNAi-resistant WT ACLY (ACLY-WT), or ACLY-3KQ variant. At 48 h after transfection, cells were harvested, and the mRNA level of ACLY was determined by RT-qPCR. For RT-qPCR results in A, data are normalized to the GFP + siNC group. For statistical analysis, all groups were compared with the GFP + siNC group. B, AML12 cells were treated as in A, and at 48 h after transfection, cell lysates were subjected to Western blotting with the indicated antibodies. β-Actin serves as an internal control. C, the band intensity of ACLY in the Western blotting results of B was quantified by ImageJ. The values are normalized to the GFP + siNC group. D, AML12 cells were treated as in A, and at 48 h after transfection, Oil Red O staining of the cells was performed. Scale bar, 50 μm. E, Oil Red O (ORO) was extracted from cells in D and measured by absorbance at 490 nm. For statistical analysis, one-way analysis of variance and Bonferroni’s post hoc tests were carried out in A, C, and E. **, p < 0.01; ***, p < 0.001; #, p > 0.05. All values are represented as means with error bars representing S.D. n = 6 for each group.

Figure 5. SIRT2 deacetylates ACLY on 3K sites, which stabilizes ACLY by antagonizing its ubiquitination, thereby promoting lipid synthesis and cell proliferation (Fig. 5D), decreased hepatic triglyceride (Fig. 5E), attenuated liver inflammation (Fig. 5F), and reduced plasma alanine aminotransferase (ALT) level (Fig. 5G). Consistent with the in vitro study, reexpression of ACLY-3KQ more potently restored the hepatic steatosis phenotypes than the reexpression of ACLY-WT (Fig. 5, D–G). Taken together, these data demonstrate the functional importance of ACLY-3K acetylation in hepatic steatosis and its associated pathology.

Deacetylation of ACLY-3K contributes to SIRT2-mediated inhibition of lipid accumulation in high glucose–treated AML12 cells

Because SIRT2 deacetylates ACLY to decrease its protein stability, we asked whether overexpressing SIRT2 in AML12 cells could ameliorate lipid accumulation. SIRT2 was overexpressed in AML12 cells (Fig. 6A), and SIRT2 overexpression led to decreased ACLY protein level (Fig. 6, B and C) but not ACLY mRNA level (Fig. 6A). Indeed, overexpression of SIRT2 attenuated lipid accumulation in high-glucose–treated AML12 cells (Fig. 6, D and E). To examine the contribution of ACLY-3K deacetylation in the above SIRT2-mediated phenotype, simultaneous overexpression of ACLY-WT or ACLY-3KQ was performed. Overexpression of ACLY-WT and ACLY-3KQ resulted in comparable up-regulation of ACLY mRNA level (Fig. 6A). However, compared with overexpressing ACLY-WT, overexpressing ACLY-3KQ more efficiently restored the ACLY protein level that is otherwise reduced by overexpressing SIRT2 (Fig. 6, B and C). Consistently, overexpressing ACLY-3KQ more potently reversed SIRT2-mediated inhibition of lipid accumulation than overexpressing ACLY-WT (Fig. 6, D and E). Collectively, these results suggest that deacetylation of ACLY on 3K sites could play an important part in the inhibition of hepatocyte lipid accumulation mediated by SIRT2.

Overexpression of hepatic SIRT2 decreases ACLY-3K acetylation and its protein level with alleviated hepatic steatosis in mice challenged by HF/HS diet

We further investigated the role of SIRT2 in hepatic steatosis in vivo. Mice fed an HF/HS diet for 12 weeks were infected once a week with adenoviruses harboring the gene encoding GFP or SIRT2 via tail vein injection and sacrificed 15 days after the first virus injection. Hepatic overexpression of SIRT2 did not affect the mRNA level of ACLY (Fig. 7A) but led to a decreased ACLY protein level (Fig. 7B) and impaired ACLY-3K acetylation (Fig. 7C) in the mouse livers. These results are consistent with our in vitro studies showing that SIRT2 deacetylates ACLY on 3K sites to decrease its protein stability (Fig. 1). Moreover, overexpression of hepatic SIRT2 in mice alleviated HF/HS diet–induced fatty liver and its accompanying pathology as shown by decreased fat accumulation in the livers (Fig. 7D), decreased hepatic triglyceride (Fig. 7E), attenuated liver inflammation (Fig. 7F), and reduced plasma ALT level (Fig. 7G). These results confirm the function of SIRT2 in regulating ACLY-3K acetylation and its protein stability in vivo and also indicate a protective role of SIRT2 against HF/HS diet–induced hepatic steatosis in mice.

Discussion

The aberrant activation of hepatic lipogenesis is known to be implicated in the pathogenesis of obesity–associated metabolic disorders, particularly NAFLD. However, the regulatory network that governs the pathophysiological activation of the lipogenic program is still incompletely understood (4, 11). ACLY is a critical enzyme regulating the initial step in lipid biosynthesis, and its aberrant up-regulation in liver contributes to the derangement of hepatic lipid metabolism during the progression of NAFLD (13). In this study, we demonstrate that ACLY-3K acetylation enhances ACLY protein stability and aggravates hepatic steatosis. Our work also suggests that SIRT2-mediated deacetylation of ACLY on 3K sites could be beneficial in preventing the pathogenic development of NAFLD.

Covalent lysine acetylation has been found to play a broad and critical role in the regulation of multiple metabolic enzymes (17). A previous study shows that ACLY is acetylated at 3K sites, which stabilizes ACLY by antagonizing its ubiquitylation, thereby promoting lipid synthesis and cell prolifera-
tion in lung cancer cells (20). However, it is not clear whether the above regulatory mechanism for ACLY also plays a part in other cellular or tissue contexts or under other pathophysiological conditions. In the present study, we investigated the functional importance of ACLY-3K acetylation in hepatocyte lipid metabolism and in the pathogenic development of NAFLD. Mimicking ACLY-3K acetylation by replacing ACLY-3K with ACLY-3KQ did increase ACLY protein stability in the mouse hepatocyte cell line AML12 (Fig. 1, A–C). Compared with WT ACLY, ectopic expression of ACLY-3KQ more potently enhanced lipid accumulation in high glucose–treated AML12 cells (Fig. 4) and hepatic steatosis in HF/HS diet–fed mice (Fig. 5). Importantly, higher ACLY protein level and higher ACLY-3K acetylation level are detected in the livers of both mice and humans with NAFLD than in the livers of non-NAFLD controls (Figs. 2 and 3). Therefore, our results suggest a

**Enhanced ACLY acetylation promotes NAFLD**

Figure 5. ACLY-3K acetylation aggravates hepatic steatosis in mice challenged by HF/HS diet. Male C57BL/6J mice fed an HF/HS diet for 12 weeks were injected once a week with adenovirus harboring shNC or shACLY together with adenovirus harboring the gene encoding LacZ, RNAi-resistant WT ACLY (ACLY-WT), or ACLY-3KQ variant through the tail vein. Mice were sacrificed, and measurements were performed 15 days after the first virus injection. The mRNA levels and protein levels of ACLY in the mouse livers were determined by RT-qPCR (A) and Western blotting (B), respectively. For RT-qPCR results in A, data are normalized to the LacZ + shNC group. For statistical analysis, all groups were compared with the LacZ + shNC group. β-Actin serves as an internal control in B. C, the band intensity of ACLY in the Western blotting results of B was quantified by ImageJ. The values are normalized to the LacZ + shNC group. D, representative images of H&E staining of mouse liver sections are shown. Scale bar, 50 μm. E, hepatic triglyceride levels were determined and plotted. F, the mRNA levels of the indicated inflammatory genes in mouse livers were determined by RT-qPCR. The data are normalized to the LacZ + shNC group. G, plasma ALT levels of the mice were determined and plotted. For statistical analysis, one-way analysis of variance and Bonferroni’s post hoc tests were carried out in A, C, E, and G, and two-way analysis of variance and Bonferroni’s post hoc tests were carried out in F. *, p < 0.05; **, p < 0.01; ***, p < 0.001; #, p < 0.05. All values are represented as means with error bars representing S.D. n = 5 for each group.
potential, important role of dysregulated ACLY-3K acetylation in the development of NAFLD.

The sirtuin family consists of seven family members (SIRT1–7), each containing a conserved catalytic core domain with NAD\(^+\)/H\(_9\) -dependent deacetylase, deacylase, and/or ADP-ribosyltransferase activities (24, 25). SIRT1–7 have different subcellular localization and function, regulating the function of cellular proteins through various posttranslational modifications (21, 26). SIRT1 and -3 are the most studied sirtuins that regulate hepatic carbohydrate and lipid metabolism, insulin signaling, and inflammation and are recognized as potential therapeutic targets for NAFLD (21, 27). SIRT2 is primarily a cytoplasmic protein, and its expression is elevated in calorie-restricted mice (21). Interestingly, caloric restriction can reverse the modifications to some physiological parameters caused by metabolic syndrome and protect against metabolic disorders (24). SIRT2 deacetylates ACLY on 3K sites to inhibit ACLY protein stability (Fig. 1). Moreover, overexpressing SIRT2 inhibits lipid accumulation in high glucose–treated AML12 cells (Fig. 6). Compared with WT ACLY, simultaneously overexpressing ACLY-3KQ more efficiently impairs the SIRT2-mediated inhibition of lipid accumulation in AML12 cells (Fig. 6). Moreover, hepatic overexpression of SIRT2 led to decreased ACLY protein level, impaired ACLY-3K acetylation, and alleviated fatty liver in HF/HS diet–fed mice (Fig. 7). Therefore, it is proposed that down-regulation of hepatic SIRT2 may contribute to the pathogenic development of NAFLD at least partially through enhancing ACLY-3K acetylation and up-regulating ACLY protein level (Fig. 8).

In summary, our results in this study suggest that enhanced ACLY-3K acetylation may play a role in promoting hepatic steatosis, adding to the recent findings showing that dysregulated acetylation of metabolic enzymes is involved in the derangement of hepatic lipid homeostasis (23, 30). We have also uncovered a potential SIRT2/ACLY axis that may be involved in the pathogenic development of NAFLD. Although we have not

Figure 6. Deacetylation of ACLY-3K contributes to SIRT2-mediated inhibition of lipid accumulation in high glucose–treated AML12 cells. A, AML12 cells cultured in high-glucose (25 mM) medium were transfected with the plasmid encoding GFP or SIRT2 together with the plasmid encoding ACLY-WT or ACLY-3KQ variant. At 48 h after transfection, cells were harvested, and the mRNA levels of SIRT2 and ACLY were determined by RT-qPCR. Data are normalized to the GFP group. B, AML12 cells were treated as in A, and at 48 h after transfection, cell lysates were subjected to Western blotting with the indicated antibodies. β-Actin serves as an internal control. C, the band intensity of ACLY in the Western blotting results of B was quantified by ImageJ. The values are normalized to the GFP group. D, AML12 cells were treated as in A, and at 48 h after transfection, Oil Red O staining of the cells was performed. Scale bar, 50 μm. E, Oil Red O (ORO) was extracted from cells in D and measured by absorbance at 490 nm. For statistical analysis, two-way analysis of variance and Bonferroni’s post hoc tests were carried out in A, and one-way analysis of variance and Bonferroni’s post hoc tests were carried out in C and E. *, \( p < 0.05; **, \ p < 0.01; \ ***, \ p < 0.001; \ #, \ p > 0.05. \) All values are represented as means with error bars representing S.D. \( n = 6 \) for each group.
explored the impact of chemical modulators in this study, the results merit exploration of these as therapeutic agents for treating NAFLD.

**Experimental procedures**

**Cell culture and reagents**

AML12 cells were maintained and propagated in Dulbecco’s modified Eagle’s medium/F-12 with 10% fetal bovine serum, ITS Liquid Media Supplement (Sigma), and 0.1 µM dexamethasone. For fat-overloading induction of cells, AML12 cells were exposed to high glucose (25 mM) for 48 h. The primary antibodies used in this study are as follows: anti-ACLY (Epitomics, 1699-1), anti-SIRT2 (Santa Cruz Biotechnology, sc-20966), anti-β-actin (Santa Cruz Biotechnology, sc-47778), anti-HA (Abclonal, AE036), and anti-FLAG (Abclonal, AE005). Antibodies specifically recognizing ACLY-3K were prepared as described previously (20). Liver triglycerides were assayed using a triglyceride assay kit (GPO-POD, Applygen Technologies Inc., Beijing, China). Plasma ALT levels were measured using kits from Stanbio. Lipofectamine 2000 (Invitrogen) and Lipofectamine RNAiMAX (Invitrogen) were used to transfect

**Figure 7.** Overexpression of hepatic SIRT2 decreases ACLY-3K acetylation and its protein level with alleviated hepatic steatosis in mice challenged by HF/HS diet. Male C57BL/6J mice fed an HF/HS diet for 12 weeks were injected once a week with adenovirus harboring the gene encoding GFP or SIRT2 through the tail vein. Mice were sacrificed, and measurements were performed 15 days after the first virus injection. A, the mRNA levels of SIRT2 and ACLY in the mouse livers were determined by RT-qPCR. Data are normalized to the GFP group. B and C, the mouse livers were lysed and subjected to Western blotting with the indicated antibodies. β-Actin serves as an internal control in B. The ACLY-3K acetylation levels were compared against total ACLY protein levels in C. D, representative images of H&E staining of mouse liver sections are shown. Scale bar, 50 µm. E, hepatic triglyceride levels were determined and plotted. F, the mRNA levels of the indicated inflammatory genes in mouse livers were determined by RT-qPCR. The data are normalized to the GFP group. G, plasma ALT levels of the mice were determined and plotted. For statistical analysis, unpaired two-tailed Student’s t tests were used in E and G, and two-way analysis of variance and Bonferroni’s post hoc tests were carried out in A and F. Data were compared with the GFP group in E, F, and G. **, p < 0.01; ***, p < 0.001; #, p < 0.05. All values are represented as means with error bars representing S.D. n = 6 for each group.

**Figure 8.** A proposed model for the role of dysregulated ACLY-3K acetylation in the pathogenic development of NAFLD. The expression of SIRT2 in the liver is decreased after overnutrition, thereby increasing the acetylation level of ACLY on lysine residues 540, 546, and 554 (3K sites). Enhanced ACLY-3K acetylation increases the protein stability of ACLY, which promotes de novo lipogenesis. The effect above contributes to hepatic steatosis and could be involved in the pathogenic development of NAFLD.
Enhanced ACLY acetylation promotes NAFLD

plasmids and synthetic siRNA oligonucleotides, respectively, into cells according to the manufacturer’s instructions.

Animals

Male C57BL/6J mice, db/db mice, and their control littermates were purchased from the Model Animal Research Center of Nanjing University. These mice were maintained on normal diet. To produce diet-induced fatty liver, 6-week-old C57BL/6J mice were fed HF/HS diet (D10012G, Research Diet) for 12 weeks with normal chow diet–fed mice as control. Then adenovirus injection was performed in HF/HS diet–fed mice. All studies involving animal experimentation were approved by the Animal Care and Use Committee of the Fudan University Shanghai Medical College and followed the National Institutes of Health guidelines on the care and use of animals.

Western blotting

The cells and tissues were harvested and homogenized with lysis buffer containing 1% sodium dodecyl sulfate (SDS), 50 mM Tris-HCl (pH 8.0), 10 mM DTT, 10% glycerol, 0.002% bromphenol blue, protease inhibitor mixture (Roche Applied Science), and 10 mM NAM (Sigma). For protein stability assay, cells were treated with CHX (Sigma) at a final concentration of 10 μM for the indicated time before harvest. To study the involvement of ubiquitin/proteasome degradation pathway in regulating ACLY protein stability, MG132 (Sigma), at a final concentration of 10 μM, was added to the medium 4 h before harvesting the cells. Equal amounts of protein were resolved on an SDS-polyacrylamide gel by electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), immunoblotted with primary antibodies, and visualized with horseradish peroxidase–coupled secondary antibodies.

Immunoprecipitation assay

Immunoprecipitation assay was performed as described before (31–33). Cells were washed with PBS, scraped off, and collected by centrifugation. Then cells were suspended in radioimmunoprecipitation assay buffer (50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Nonidet P-40, 0.5% sodium deoxycholate, and 0.1% SDS) in the presence of protease inhibitors, 10 mM NAM, and 10 μM TSA (Sigma), at a final concentration of 10 μM, was added to the medium 4 h before harvesting the cells. Equal amounts of protein were resolved on an SDS-polyacrylamide gel by electrophoresis, transferred to a polyvinylidene difluoride membrane (Millipore Corp., Bedford, MA), immunoblotted with primary antibodies, and visualized with horseradish peroxidase–coupled secondary antibodies.

RNA extraction and reverse transcription (RT)-qPCR

Total RNA from cells and tissues was extracted using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. Complementary DNA (cDNA) was synthesized from total RNA with PrimeScript reverse transcriptase and random primers (TaKaRa Bio, Otsu, Japan). cDNAs were amplified with Power SYBR Green PCR Master Mix (Applied Biosystems, Carlsbad, CA) and a Prism 7500 instrument (Applied Biosystems) with 36b4 as an endogenous control. The qPCR was done in triplicate and repeated at least three times. The primers for qPCR are as follows: 5’-AACTTTCTTGAACCCCTCG-3’ (ACLY-forward), 5’-TCCACATGCCCACATTC-3’ (ACLY-reverse), 5’-CCGCAAGGTCTCAGTGGT-3’ (SIRT2-forward), 5’-TCTGGTAAAGGAAGTTG-3’T (SIRT2-reverse), 5’-AGGCCACAGTCTGTATC-3’ (TNFa-forward), 5’-CTCCCTTGCTGAAGACTCAG-3’ (TNFa-reverse), 5’-TGGCAACTGTCCTGAACCTCAA-3’ (IL1β-forward), 5’-AGCAGCTCCTCATCTTTTGG-3’ (IL1β-reverse), AGGTCTCTGTCTGCTTG (CCL2-forward), TCTGGACCATCTCTTTTG (CCL2-reverse), 5’-GAAACTGTCGCTACATCCG-3’ (36b4-forward), and 5’-GCTGGCAACAGTGACCTC-3’ (36b4-reverse).

Oil Red O staining and hematoxylin and eosin (H&E) staining

Cells were washed with cold PBS and fixed for 10 min with 3.7% formaldehyde. Oil Red O (Sigma; 0.5% in isopropanol) was diluted with water (3:2), filtered through a 0.45-μm filter, and incubated with the fixed cells for 4 h at room temperature. The cells were washed with water, and stained fat droplets in the cells were examined by light microscopy and photographed. To measure the Oil Red O staining level, Oil Red O was extracted from the cells and measured by absorbance at 490 nm. H&E staining of mouse liver tissues was conducted as described previously (31).

Generation and administration of recombinant adenoviruses

Recombinant adenoviruses for overexpression and knockdown were generated using the ViraPower™ Adenoviral Expression System (Invitrogen) and BLOCK-IT™ Adenoviral RNAi Expression System (Invitrogen), respectively. High-titer stocks of amplified recombinant adenoviruses were purified by Sartorius Adenovirus Purification kits. Viral titers were determined by the tissue culture infectious dose 50 (TCID50) method using 293A cells. For in vivo studies of ACLY-3K acetylation function, 3 × 10⁸ plaque-forming units (pfu)/mouse adenoviruses harboring control shRNA (shNC) or shACLY were mixed with 2 × 10⁳ pfu/mouse adenoviruses harboring shRNA-resistant WT ACLY or ACLY-3KQ variant and then diluted to 100 μl with PBS and administered through tail vein injection (once a week). For overexpression of hepatic SIRT2, 4 × 10⁹ pfu/mouse
adenoviruses harboring the gene encoding GFP or SIRT2 were administered through tail vein injection (once a week). Measurements were performed 15 days after the first virus injection.

**Immunohistochemistry**

Human liver samples were acquired from Zhongshan Hospital of Fudan University. A physician obtained informed consent from the patients. The procedures related to human subjects were approved by the Ethics Committee of Zhongshan Hospital, Fudan University and conform to the principles outlined in the Declaration of Helsinki (34). All of the NAFLD patients were defined as simple steatosis according to histological manifestation. Patients were excluded if alcohol consumption was more than 40 g/day for men and more than 20 g/day for women and if other liver diseases were detected by serological testing and imaging studies. Paraffin-embedded liver tissue was cut, deparaffinized, and hydrated, and immunohistochemistry was performed as described previously (23). Images were captured using a charge-coupled device camera and analyzed using ImageJ software. Relative staining intensity was calculated by dividing the raw staining intensity by cell number in the same area. Data obtained were expressed as mean values ±S.D. Differences were considered significant if the p value was less than 0.05.

**Statistics**

Results are expressed as means with error bars representing S.D. Comparisons between groups were made using unpaired two-tailed Student’s t tests. For comparison of three or more independent groups with only one variable, one-way analysis of variance plus Bonferroni’s post hoc tests was performed. For comparison of two or more independent groups with two variables, two-way analysis of variance plus Bonferroni’s post hoc tests was performed. The statistical analyses are also indicated in the legends of each figure, with p < 0.05 being considered statistically significant. All experiments were repeated a minimum of three times, and representative data are shown.

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