Hepatocyte-specific Sirt6 deficiency impairs ketogenesis

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Sirt6 is an NADH (NAD+)-dependent deacetylase with a critical role in hepatic lipid metabolism. Ketogenesis is controlled by a signaling network of hepatic lipid metabolism. However, how Sirt6 functions in ketogenesis remains unclear. Here, we demonstrated that Sirt6 functions as a mediator of ketogenesis in response to a fasting and ketogenic diet (KD). The KD-fed hepatocyte-specific Sirt6 deficiency (HKO) mice exhibited impaired ketogenesis, which was due to enhanced Fsp27 (fat-specific induction of protein 27), a protein known to regulate lipid metabolism. In contrast, overexpression of Sirt6 in mouse primary hepatocytes promoted ketogenesis. Mechanistically, Sirt6 repressed Fsp27β expression by interacting with Crebh (cAMP response element–binding protein H) and preventing its recruitment to the Fsp27 (fat-specific induction of protein 27), a protein known to regulate lipid metabolism. In contrast, overexpression of Sirt6 in mouse primary hepatocytes promoted ketogenesis. Mechanistically, Sirt6 repressed Fsp27β expression by interacting with Crebh (cAMP response element–binding protein H) and preventing its recruitment to the Fsp27β gene promoter. The KD-fed HKO mice also showed exacerbated hepatic steatosis and inflammation. Finally, Fsp27 silencing rescued hypoketonemia and other metabolic phenotypes in KD-fed HKO mice. Our data suggest that the Sirt6–Crebh–Fsp27 axis is pivotal for hepatic lipid metabolism and inflammation. Sirt6 may be a pharmacological target to remedy metabolic diseases.

Ketone body biosynthesis primarily occurs in liver (1). When glucose is not available, fatty acids are mobilized for conversion to ketone bodies that function as a vital alternative energy source for extrahepatic tissue (2). Hepatic ketogenesis is governed by an orchestrated series of metabolic processes. First, fatty acids are metabolized to acetyl-CoA via mitochondrial β-oxidation. Acetyl-CoA is then condensed with acetoacetyl-CoA to generate hydroxymethylglutaral (HMG)-CoA by mitochondrial HMG-CoA synthase (Hmgcs2). Subsequently, HMG-CoA is cleaved to acetoacetate by hydroxymethylglutaral-CoA lyase (Hmgcl), the common precursor of the two ketone bodies, β-hydroxybutyrate and acetone (3). Most acetoacetate is further metabolized to β-hydroxybutyrate by β-hydroxybutyrate dehydrogenase.

In addition to hepatic fatty acid β-oxidation, sufficient fatty acid substrates for mitochondrial utilization is another important regulatory step for ketogenesis (4). Lipid droplet (LĐ)-coating proteins delicately balance triglyceride synthesis and breakdown (termed lipolysis) (5), presumably determining the rate of fatty acid utilization. Several critical proteins contribute to hepatic lipolysis, affecting hepatic fatty acid oxidation and ketogenesis (6–8). Perilipin 2, which coats the surface of LDs, prevents hepatic lipolysis and inhibits ketogenesis (7). Atgl (adipose triglyceride lipase) is a key enzyme in lipolysis. Overexpression of Atgl increases the triglyceride breakdown and thereby increases plasma β-hydroxybutyrate level (8). During the chronic fasting, the elevated Atgl level allows triglycerides to be mobilized to support metabolic processes such as fatty acid oxidation and ketogenesis (6). G0s2 (Gα/3; switch gene 2) is an inhibitor of Atgl. Liver-specific knockdown of G0s2 greatly reduces hepatic triglycerides level and enhances ketogenesis (6). Conversely, hepatic G0s2-overexpressing mice show impaired ketogenesis (9).

Fat-specific protein 27 (Fsp27; Cidec is the human homolog) is an LD-coating protein. Abundant expression of Fsp27 in white adipocytes is responsible for the storage of fatty acids as triglycerides in LDs (10). Fsp27 depletion enhances lipolysis and mitochondrial fatty acid oxidation in the white adipose tissues (11). Fsp27 is also expressed in the liver. The hepatic expression of Fsp27 contributes to hepatic lipid accumulation and reduced lipolysis (4, 12). The expression of Fsp27 is positively regulated by Crebh (cAMP response-element binding
Sirt6 regulates ketogenesis

protein H) as a direct target gene. CrebH is a liver-enriched transcription factor that is a key regulator of hepatic lipid metabolism (13). Creb can transcriptionally activate Fsp27 expression and also stimulate LD growth and triglyceride accumulation in the liver (12).

Sirt6 is a NADH (NAD\(^+\))-dependent deacetylase (14). Sirt6 was initially characterized as an NAD\(^+\)-dependent deacetylase of histone (15). Accordingly, Sirt6 acts primarily as a transcriptional repressor of several transcription factors, especially those implicated in lipid metabolism and inflammation. Sirt6 deacetylates histone H3 lysine 9 (H3K9) and lysine 56 (H3K56) on the promoters of Srebp1/2 (sterol regulatory-element binding protein 1/2) and proprotein convertase subtilisin/kexin type 9 to control cholesterol metabolism (16–18). Sirt6 regulates the expression of inflammatory genes by deacetylating H3K9 on the promoters of NF-κB and c-Jun (19, 20). Liver-specific ablation of Sirt6 in mice caused hepatic steatosis (21, 22), but the mechanism remains to be clearly defined. Recent reports suggested that impaired ketogenesis can lead to excess hepatic lipid accumulation (23–25). Thus, it is possible that dysregulation of ketogenesis may contribute to the progression to nonalcoholic fatty liver disease (24) and disequilibrium of hepatic energy metabolism. However, whether Sirt6 has a direct role in regulating ketogenesis remains unknown.

In this report, we show that hepatocyte-specific Sirt6 deficiency impairs fasting- and ketogenic diet (KD)-induced ketogenesis in mice. Conversely, overexpression of Sirt6 in mouse primary hepatocytes promoted ketogenesis. Further investigation revealed that Fsp27 mediated the ketogenesis by Sirt6. Knockdown of Fsp27 alleviated the impaired ketogenesis observed in Sirt6-deficient mice. Mechanistically, Sirt6 repressed Fsp27β expression by interacting with CrebH and preventing its recruitment to the Fsp27β gene promoter. Our data reveal an essential role for Sirt6 in ketogenesis and elucidate the underlying mechanism of Sirt6-regulated ketogenesis.

Results

Sirt6 is regulated by ketogenesis

Sirt6 controls metabolic homeostasis and is regulated by nutrient availability (26, 27). To investigate whether Sirt6 plays a role in ketogenesis, we measured the hepatic Sirt6 expression in mouse models of ketogenesis. Fasting or KD feeding are known to promote ketone body production (3, 28). A 24-h fasting induced the hepatic expression of Sirt6 in C57BL/6J mice (Fig. 1A), which was consistent with a previous report (21). Similarly, KD feeding significantly increased the mRNA and protein levels of Sirt6 in the liver (Fig. 1B and Fig. S1A). In primary hepatocytes, Sirt6 expression was increased after incubation in ketogenic medium (Fig. 1C and Fig. S1B), which is known to induce ketogenesis (29). These data suggest that Sirt6 may function as a ketogenic regulator in response to fasting and ketogenic conditions in mice.

Hepatocyte-specific Sirt6 deficiency impairs ketogenesis

Ketone bodies are primarily produced in the liver (1). To further investigate the function of Sirt6 in ketogenesis, hepatocyte-specific Sirt6 deficiency (HKO) mice were generated by cross-breeding Sirt6\(^{Loxp/Loxp}\) mice (LoXP) with the hepatocyte-specific albumin-Cre mice. The expression of Sirt6 was efficiently knocked down in liver and isolated hepatocytes but not in other tissues (Fig. 2A and Fig. S2, A and B).

\(\beta\)-Hydroxybutyrate is the most abundant circulating ketone body (78%) (30) and is usually used to directly reflect blood levels of ketone bodies (31). We next tested the serum levels of \(\beta\)-hydroxybutyrate under \textit{ad libitum} feeding, fasting, and KD-fed HKO mice and the Loxp control mice. In the \textit{ad libitum} state, the serum level of \(\beta\)-hydroxybutyrate was low and not different between the two genotypes (Fig. 2B). After 24-h fasting, the serum level of \(\beta\)-hydroxybutyrate was greatly increased in Loxp mice. However, the fasting-induced \(\beta\)-hydroxybutyrate level was significantly impaired in HKO mice (Fig. 2C). KD feeding induced ketogenesis in the Loxp mice as expected, but the serum \(\beta\)-hydroxybutyrate level was significantly lower in HKO mice (Fig. 2D). Under CD and KD, the body weight, fat mass, and lean mass was comparable between Loxp and HKO mice (Fig. 2E). The serum levels of triglycerides, cholesterol, and nonesterified free fatty acids (NEFA) were significantly higher in HKO than Loxp mice under \textit{ad libitum} feeding, but not in the fasting or KD feeding state (Fig. 2, B–D). These results suggested that Sirt6 plays a role in ketogenesis.

Sirt6 regulates Fsp27 expression

To understand the mechanism of Sirt6-regulated ketogenesis, we first measured the expression of genes involved in fatty
acid oxidation and ketogenesis in the mouse livers (32). The mRNA levels of fatty acid oxidation genes (Cpt-1α (carnitine palmitoyltransferase 1α), Cpt-2α, and medium-chain acyl-CoA dehydrogenase (Mcad)) and ketone body synthetic genes (Hmgcs2 and Hmgcl) were similar between the HKO and Loxp mice under both KD and CD (Fig. S3, A and B). Consistent with mRNA expression, functional analysis of isolated mitochondria from liver of HKO mice did not differ between two genotypes in the presence of respiratory chain complex I or II substrates (Fig. S4, A–D). In hepatocytes, the mitochondria respiration was also similar (Fig. S4, E and F). Taken together, these results suggest that impaired ketogenesis in HKO mice was unlikely because of the dysregulation of hepatic fatty acid oxidation.

In addition to fatty acid oxidation, sufficient fatty acid substrates for mitochondrial utilization is another key factor in ketogenesis (4). Fsp27, a lipid-coating protein, acts as a barrier of fatty acid oxidation by restricting lipid utilization (4, 11, 33). We suspected that Fsp27 may be involved in the hypoketonia seen in HKO mice. Under the CD, hepatic Fsp27 expression was comparable between HKO and Loxp mice as shown

Figure 2. Hepatocyte-specific Sirt6 deficiency impaired ketogenesis. A, Western blotting analysis of Sirt6 protein expression in liver (left panel) and mouse primary hepatocytes (right panel) in Sirt6Loxp/Loxp (Loxp) and hepatocyte-specific Sirt6 knockout (HKO) mice (n = 3). β-Tubulin was a control. B–D, serum biochemical indices of β-hydroxybutyrate, triglyceride, and NEFA in Loxp and HKO mice under ad libitum feeding (n = 9–11) (B), fasting (n = 5–10) (C), and KD (n = 5–8) (D). E, body weight, fat mass, and lean mass of Loxp and HKO mice both under CD and KD conditions (n = 5). The data are means ± S.E. *, p < 0.05; **, p < 0.01.
However, under the KD, Fsp27 expression was increased, and the increase was more pronounced in HKO than Loxp livers (Fig. 3A). The expression of Fsp27β, a predominant isoform of Fsp27 in the liver was similarly higher in HKO than Loxp livers on the KD (Fig. 3A). In addition, HKO livers showed an increased expression of G0s2, a hepatic lipolysis inhibitor (Fig. 3A). Perilipin-1, another lipid droplet coating protein, was slightly increased in HKO mice (Fig. S5A, upper panel). The expression of Atgl was not changed in the liver of HKO mice (Fig. S5A, lower panel).

These in vivo results were further confirmed in primary hepatocytes that bear the knockout or overexpression of Sirt6. In Sirt6-knockout hepatocytes, the mRNA expression of Fsp27, Fsp27β, and G0s2 was increased (Fig. 3C). Consistent with the mRNA level, the protein level of Fsp27 was significantly elevated in Sirt6 knockout hepatocytes (Fig. 3D). Conversely, overexpression of Sirt6 suppressed the mRNA expression of Fsp27, Fsp27β, and G0s2 (Fig. 3E and Fig. S5B). Consistent with the results in HKO mice, Sirt6 overexpression promoted ketone body production and decreased cellular triglyceride level in primary hepatocytes (Fig. 3F).
and Fig. S5C). Together, these data indicate that Sirt6 might regulate ketogenesis by modulating Fsp27 expression.

**The impaired ketogenesis in HKO mice is Fsp27-dependent**

To directly confirm the effect of Fsp27 on ketogenesis, we overexpressed Fsp27 in primary hepatocytes and monitored the ketone body production by the $\beta$-hydroxybutyrate level. Fsp27 overexpression significantly attenuated $\beta$-hydroxybutyrate level in primary hepatocytes (Fig. 4, A and B). To further validate whether elevated Fsp27 mediated the impaired ketogenesis seen in HKO mice, we injected Ad-shFsp27 in HKO mice to knock down Fsp27 expression under the KD. Ad-shFsp27 injection effectively reduced both the mRNA and protein levels of Fsp27 (Fig. 4, C and D). Notably, Fsp27 silencing increased the serum level of ketone bodies in KD-fed HKO mice (Fig. 4E), with no changes in serum triglycerides, cholesterol, or NEFA level in Ad-shFsp27–infected mice (Fig. 4F). The result was verified by hematoxylin and eosin (H&E) staining of liver sections with reduced inflammatory cell infiltration in Ad-shFsp27–injected mice (Fig. S6C), and the H&E also showed lower vacuolated hepatocytes in Ad-shFsp27–infected mice. Collectively, these results suggest that Fsp27 knockdown ameliorates the impaired ketogenesis in HKO mice.

**Sirt6 interacts with CrebH to regulate Fsp27 expression**

We went on to determine the mechanism of Sirt6-regulated Fsp27 expression. Fsp27 and G0s2 are direct target genes of CrebH (13). In agreement with Fsp27 and G0s2, other CrebH
target genes such as Fads1, Fads2, Apoc2 (encoding apolipoproteins c2), Apoa4, and Apoa5 were increased by Sirt6 knockout. Conversely, Sirt6 overexpression decreased the expression of those Crebh target genes (Fig. S7, A and B). We hypothesized that Sirt6 might negatively regulate Crebh transcriptional activity. The transcriptional activity of Crebh is reliant on the N-terminal fragment of Crebh (activated form of Crebh), which can be liberated from the precursor form of Crebh. The activated form of Crebh translocates to the nucleus and regulates its target genes expression (35, 36). Sirt6 deficiency greatly enhanced the expression of the activated form of Crebh in the liver (Fig. 5A). Consistently, the expression of nuclear-fraction Crebh was substantially increased in Sirt6 knockout hepatocytes (Fig. 5B). In contrast, Sirt6 overexpression decreased the nuclear-fraction Crebh protein level in hepatocytes (Fig. 5C). We further investigated whether Sirt6 physically interacted with Crebh and was recruited to the Fsp27 gene promoter. Immunoprecipitation showed that Sirt6 can interact with Crebh (Fig. 5D). ChIP assay revealed that the interaction of Sirt6 and Crebh prevented the occupancy of Crebh on the promoter of Fsp27 (Fig. 5E). Luciferase reporter assay showed that Sirt6 decreased Crebh transcriptional activity (Fig. 5F). These results indicate that Sirt6 interacts with Crebh to suppress Fsp27 and G0s2 expression.
Hepatocyte-specific Sirt6 deficiency exacerbates hepatic steatosis and inflammation

Ketogenic impairment often leads to lipid metabolism disorders (24). To explore the role of Sirt6 in hepatic lipid metabolism, we quantified hepatic lipid content. Under the CD and KD, Sirt6 deficiency increased triglycerides level in mouse livers (Fig. 6A). Hepatic cholesterol content was increased but not significantly in HKO livers (Fig. S7C). The triglyceride accumulation was further confirmed by hepatic Oil Red O staining, with large-sized and increased LDs in HKO livers (Fig. 6B). In agreement with these phenotypes, fatty acid transport genes Fatp1 and Fatp4 and fatty acid esterification genes Ppap2a (patatin-like phospholipase domain-containing 2a), Ppap2c, and Dgat2 (diacylglycerol O-acyltransferase 2) were significantly increased in HKO livers (Fig. S8, A and B). Ppap2a/Ppap2c and Dgat2 genes are involved in direct esterification of fatty acids with glycerol to synthesize diacylglycerol and triglycerides, respectively (37, 38). Coordinated with increased fatty uptake in liver, the lipolysis was slightly increased in HKO mice, as indicated by fatty acid release from adipose tissue (Fig. S8, C and D). Levels of lipogenic genes such as Srebp-1c, Fas (fatty acid synthase), Acc (acetyl-CoA carboxylase), and Scd-1 (stearoyl-CoA desaturase 1) were similar in the two genotypes (Fig. S9, A and B). Consistently, the lipogenesis measured by \[^{13}C\]acetate incorporation was similar in Ctrl and Sirt6 knockout hepatocytes (Fig. S9C).

Ketogenic insufficiency also causes hepatic inflammation (24). H&E staining showed increased inflammatory cell infiltration in HKO livers (Fig. S9D). Consistent with this phenotype, the expression of macrophage markers (F4/80 and Cd68) and proinflammation cytokines (Mcp-1 (monocyte chemoattractant protein-1), tumor necrosis factor-\(\alpha\), and IL-1\(\beta\)) were greatly induced in livers of KD-fed HKO mice (Fig. 6, C and D). These data suggest that impaired ketogenesis could trigger hepatic inflammation in Sirt6-deficient mouse livers.

Discussion

In this study, we reveal that Sirt6 plays an important role in ketogenesis. Hepatocyte-specific Sirt6 ablation impaired ketogenesis, whereas overexpression of Sirt6 in hepatocytes promoted ketogenesis. Further investigation indicated that Sirt6-regulated ketogenesis was mediated by Fsp27. Knockdown of Fsp27 ameliorated the impaired ketogenesis seen in Sirt6-deficient mice. Mechanistically, Sirt6 interacts with Creb\(\beta\) and attenuates its positive regulation of Fsp27.
Sirt6 regulates ketogenesis

During energy-restricted metabolic states such as caloric restriction and fasting, fatty acids are first metabolized to acetyl-CoA via mitochondrial β-oxidation. Then the fatty acid oxidation product acetyl-CoA serves as the substrate for ketogenesis. Thus, classical ketogenesis requires efficient fatty acid β-oxidation and ketone body biosynthesis in hepatic mitochondria. The nuclear receptor peroxisome proliferator-activated receptor α (Ppara) has been reported as a master regulator of hepatic fatty acid β oxidation and ketone body biosynthesis (32). Ppara-deficient mice show hypoketonemia during fasting (39). Initially, we thought that Sirt6-regulated ketogenesis might be mediated by Ppara. However, levels of Ppara target genes involved in fatty acid oxidation and ketogenesis were comparable in Loxp and HKO mice under both the CD and KD (Fig. S3, A and B). Consistently, the mitochondrial oxidative respiration rate did not differ between the genotypes (Fig. S4, A–F). These results suggest that Sirt6-mediated ketogenesis is independent of Ppara.

We found hepatic Fsp27 expression up-regulated in Sirt6-deficient mice. We suspected that the increased expression of Fsp27 might have mediated the impaired ketogenesis in Sirt6-deficient mice. Several lines of evidence support our hypothesis. First, Sirt6 deficiency and overexpression increased and decreased Fsp27 expression, respectively. Second, Fsp27 knockdown ameliorated the impaired ketogenesis in HKO mice, whereas Fsp27 overexpression suppressed ketogenesis. Finally, Sirt6 interacted with Crebh to control Fsp27 expression. Fsp27-regulated ketogenesis is likely due to its regulation of hepatic LD mobilization. Recent studies found that hepatic Fsp27 helped to maintain the characteristically large unilocular LD structure and triglyceride accumulation in liver (12, 40). Fsp27 could act as a hepatic lipolytic barrier to reduce the release of fatty acids from LDs and restrict fatty acid access to mitochondria. Therefore, enhanced hepatic Fsp27 could limit the amount of fatty acids available for mitochondrial β-oxidation, thereby reducing fatty acid oxidation and ketogenesis. This finding is consistent with a previous report showing that Fsp27 knockdown further enhanced fasting-induced β-oxidation by promoting the efficient mobilization and catabolism of hepatic triglyceride (4).

Crebh was identified as a regulator of hepatic Fsp27 gene expression (12, 13, 34). Fasting-induced hepatic Fsp27 expression was abolished in Crebh-deficient mice (13). Conversely, overexpression of an activated form of Crebh up-regulated the expression of Fsp27 (13). Recent study further revealed that Crebh directly bound to the Crebh response element in the Fsp27 promoter and increased the expression of Fsp27β (12). The Crebh–Fsp27β axis is responsible for hepatic LD growth and triglyceride accumulation (12). Additionally, Crebh was found to up-regulate Fsp27β expression in alcoholic steatohepatitis (34).

Previous studies demonstrated that Sirt6 functions as a transcriptional repressor by deacetylating the histones H3K9 and H3K56 on the promoters of multiple genes (41). Sirt6 also deacetylated nonhistone proteins such as general control non-repressed protein 5 and forkhead box O1 and O3 (14). Therefore, Sirt6 may regulate Crebh transcriptional activity in two ways. First, Sirt6 could directly regulate the acetylation of Crebh. The acetylation level of Crebh has been reported to play a critical role in its transcriptional activity (42). Sirt6 may directly deacetylate Crebh and suppress its transcriptional activity. Alternatively, Sirt6 could attenuate Crebh transcriptional activity via H3K9 or H3K56 deacetylation. Sirt6 is recruited to genomic loci by interacting with Crebh, deacetylating histones H3K9 or H3K56 on the promoters of Crebh target genes, and down-regulating Fsp27 expression. Future study is needed to further define the mechanism by which Sirt6 regulates the transcriptional activity of Crebh.

Hepatocyte-specific disruption of Sirt6 was reported to cause fatty liver formation caused by increased levels of lipogenesis (21). Although we observed a similar phenotype of hepatic steatosis, the expression of lipogenic genes such as Srebp-1c, Fas, Acc, and Scd-1 was not different between livers of Loxp and HKO mice fed a KD. Instead, levels of fatty acid transporters (Fatp1 and Fatp4) were increased in HKO mice fed a KD. Excess influx of fatty acid into hepatocytes is also known to cause fatty liver. In agreement, the expression of genes responsible for fatty acid esterification (Dgat2, Ppap2a, and Ppap2c) was increased in HKO mice fed a KD. Fsp27 aims to prevent free fatty acid release from LDs. The increased LD storage likely explains the fatty liver phenotype seen in HKO mice fed a KD. Also, we observed aggravated inflammation in HKO livers. The expression of macrophage markers (F4/80 and Cd68) and proinflammation cytokines (Mcp-1, tumor necrosis factor-α, and IL-1β) was greatly induced in KD-fed HKO mice.

The Crebh–Fsp27 axis may contribute to the enhanced hepatic steatosis and inflammation in HKO mice. The prosteatotic and proinflammation function of Fsp27 has been documented in several reports (40). Mice with overexpression of the N terminus of Crebh, a nuclear form of Crebh, are susceptible to hepatic steatosis (43). A recent report further confirmed that the Crebh–Fsp27 axis promotes LD enlargement and triglyceride storage in hepatocytes (12). Crebh has a role in inflammation (36). The Crebh–Fsp27 axis may explain the fatty liver and inflammation observed in HKO mice fed a KD. More importantly, Fsp27 knockdown ameliorated the hepatic steatosis and inflammation in HKO mice.

In summary, this study uncovered an important role for Sirt6 in ketogenesis. Hepatic Sirt6 deficiency in mice impaired ketogenesis in response to fasting and a KD. The impaired ketogenesis in HKO mice was caused by increased expression of Fsp27. Mechanistically, Sirt6 interacted with Crebh and suppressed the transcriptional activity of Crebh to Fsp27β, thereby decreasing Fsp27 expression. Additionally, hepatic Sirt6-deficient mice were prone to developing hepatic steatosis and inflammation. Our results suggested that Sirt6 is a promising therapeutic target for treating hepatic metabolic disease.

**Experimental procedures**

**Animal experiments**

All animal experiments were approved by the Institutional Animal Care and Use Committee of Sichuan University. Albumin-Cre and Sirt6<sup>Loxp/lox<sub>P</sub></sup> mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Albumin-Cre mice were mated with Sirt6<sup>Loxp/lox<sub>P</sub></sup> mice to generate hepatocyte-specific
Sirt6 deficiency mice (HKO); Sirt6<sub>Loxp/Loxp</sub> mice (Loxp) were controls (22). C57BL6/J male mice were obtained from the Animal Experimental Center of Sichuan University (Chengdu, China). The mice received CD or KD. KD contained 75.1% fat, 8.6% protein, and 3.2% carbohydrates (F-3666, Bio-serv) for 2 weeks. To knock down Fsp27 expression in liver, the mice were injected with 2 × 10<sup>9</sup> plaque-forming units of Ad-shFsp27 via the tail vein. The mice were then fed the KD for another 10 days. The mice were sacrificed after 24 h of fasting unless otherwise stated, and the livers were excised.

Biochemical analysis

Metabolic biochemical indicators were measured according to the manufacturers’ instructions. Levels of serum β-hydroxybutyrate (Cayman, Ann Arbor, MI) and NEFAs (Wako Chemicals, Richmond, VA) were determined by colorimetric assay kits. Serum triglycerides and cholesterol levels were measured by using commercial kits (Biosino, Beijing). Hepatic triglycerides and cholesterol were extracted as described (44, 45). Briefly, 150 mg of liver tissues were homogenized with 1 ml of phosphate-buffered saline and extracted with chloroform/methanol (2:1, v/v). After aliquots of the extracts were dried, the lipids were resolved in 1% Triton/ethanol. Hepatic triglycerides and cholesterol levels were measured by using the same kits as for serum triglycerides and cholesterol and were normalized to the mass of liver tissues.

Liver histology

Liver tissue specimens were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections (4 μm) were stained with H&E. For Oil Red O staining, frozen sections (8 μm) were fixed in formalin and incubated with 0.5% Oil Red O solution.

Isolation and treatment of mouse primary hepatocytes

Mouse primary hepatocytes were isolated by using a modified two-step method (46). In brief, 6- to 8-week-old mice were anesthetized, and the abdomen was dissected to expose the hepatic vena cava and portal vein. A catheter was inserted into the inferior vena cava, followed by perfusing with Hanks’ buffer salt solution containing 50 mM EGTA and 50 mM HEPES (pH 7.4). After perfusion, the entire liver was transferred to collagenase digestive solution (1 mg/ml collagenase I, 1% BSA in Krebs–Ringer buffer). The digestive suspension was filtered, and washed three times. Mouse primary hepatocytes were seeded at 2 × 10<sup>5</sup>/well in collagen-coated 6-well plates. To measure ketogenesis in vitro, the medium was changed to phenol red-free Dulbecco’s modified Eagle’s medium. At indicated times, the aliquots of medium were collected to measure ketone levels.

Chromatin immunoprecipitation assay

ChIP assays were performed with the EZ-Magna ChIP G Kit (Millipore, Billerica, MA). Mouse primary hepatocytes were infected with Ad-GFP or Ad-Sirt6 for 24 h. Then the protein–DNA complex was cross-linked and fragmented by sonication, followed by immunoprecipitation with anti-Crebh antibody (Karafast, Boston, MA). Immunoprecipitants were then reversed to release DNA, and purified DNA was subjected to RT-PCR.

Real-time PCR

Total RNA from hepatocytes or liver tissues was extracted with TRIzol (Invitrogen). Real-time PCR involved the SYBR Green-based assay with the Bio-Rad CFX 96 PCR system (44). Primer sequences are shown in Table S1.

Luciferase reporter assay

HEK293 cells were seeded in 48-well plates and transiently co-transfected with Crebh luciferase reporter (47). After 24 h of transfection, HEK293 was harvested to detect luciferase. β-Galactosidase activity was used to normalize transfection efficiency.

Western blotting analysis and co-immunoprecipitation

Nuclear extracts were prepared from mouse primary hepatocytes by using nuclear extraction reagents (Pierce, Thermo Scientific). For co-immunoprecipitation, the cells were lysed with non-denaturating Nonidet P-40 lysis buffer containing protease inhibitor mixture as described (48). An amount of 500 μg of protein was immunoprecipitated with anti-Sirt6 or anti-Crebh antibody. Nuclear extracts and immunoprecipitated protein were resolved by SDS-PAGE. The gels were probed with primary antibody against Sirt6 (D12486, CST), Creb (EWS101, Karafast), Fsp27, and β-tubulin (200608, Zen Bio Science).

Oxygen consumption rate of liver mitochondria and mouse primary hepatocytes

Mitochondria in mouse liver were isolated in accordance with differential centrifugation as described (49). Oxygen consumption rate was evaluated by Seahorse analyzer. Mitochondrial complex I substrate (succinate with rotenone) and complex II substrate (glutamate/malate) were used for mitochondrial respiratory chain substrates. ADP (4 mM at final concentration), oligomycin (2.5 μg/ml at final concentration), FCCP (4 μM at final concentration), and antimycin A (4 μM at final concentration) were injected by corresponding ports of a hydrated sensor cartridge. Oxygen consumption rate in mouse primary hepatocytes were performed with Seahorse XF Cell Mito stress test kit (Agilent Technologies, Santa Clara, CA). Isolated mouse primary hepatocytes were plated in collagen-coated Seahorse cell culture microplate. After attachment for 8 h, mouse primary hepatocytes were infected with Ad-GFP or Ad-Cre for 24 h. Prior to the assay, growth medium was replaced with pH-adjusted Seahorse base medium supplemented with 10 mM glucose, 1 mM pyruvate, and 2 mM glutamine, and then cells were incubated in 37 °C non-CO<sub>2</sub> incubator for 45 min. Oligomycin (2 μM at final concentration), FCCP (1 μM at final concentration), and antimycin A (1 μM at final concentration) were sequentially loaded into the hydrated sensor cartridge. All the data were analyzed with the software Wave (Agilent Technologies).

De novo lipogenesis analysis using stable isotopes tracer

 Newly isolated mouse primary hepatocytes were seeded in 10-cm culture dishes and cultured in low-glucose Dulbecco’s
modified Eagle’s medium. Mouse primary hepatocytes were infected with Ad-GFP or Ad-Cre. Then hepatocytes were incubated with fresh medium with 4 mM \([1,2–13C]\)acetate (50). After 24 h, cellular lipid was extracted using dichloromethane/methanol/H\(_2\)O. In brief, the cells were washed with nonbuffered saline and then lysed by adding 1 ml of H\(_2\)O. Cell debris was rest on ice for 10 min. 2 ml of methanol and 0.9 ml of dichloromethane were added, and the contents were vortexed. 1 ml of H\(_2\)O and 0.9 ml of dichloromethane were added again. After vortexed, the content was centrifuged at 1200 rpm for 10 min. Lower organic phase was collected to flesh glass tube. Next, 2 ml of dichloromethane was added to the remains in an extraction tube and vortexed, followed by centrifugation at 1200 rpm for 10 min. Lower organic phase was collected and added to the first extract. Organic solvent was evaporated under a stream of N\(_2\), and samples were dissolved in methanol. Newly synthesized palmitic acid and oleic acid from \([1,2–13C]\)acetate were analyzed using a Dionex Ultimate 3000 LC system (Thermo Scientific) in combination with Thermo Q Exactive Plus mass spectrometer (Thermo Scientific).

**Lipolysis assay in adipose tissue**

Lipolysis assay in adipose tissue was performed as previously described (51). Lipolysis was determined by the amount of glycerol and free fatty acid released from the adipose tissue cultured in the phenol red-free Dulbecco’s modified Eagle’s medium. Glycerol and free fatty acid were determined by a colorimetric assay.

**Statistical analysis**

Quantitative data are presented as means ± S.E. Statistical analysis was determined by unpaired two-tailed Student t test. p < 0.05 was considered statistically significant.

**Author contributions**—L. C. and J. H. conceptualization; L. C. and J. H. data curation; L. C. and J. H. formal analysis; L. C. and J. H. validation; L. C. and J. H. investigation; L. C. and J. H. visualization; L. C., Q. L., Q. T., J. K., H. L., S. P., T. W., X. Y., R. L., J. Z., Z. Z., Y. H., Y. L., T. L., M. G., L. Z., and J. H. methodology; L. C. and J. H. writing—original draft; L. C. and J. H. writing—review and editing; Y. L. and J. H. funding acquisition; J. H. supervision; J. H. project administration; M. Z., W. J., H. W., A. Q., and W. X. discussion and review.

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