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Original Paper

Intraventricular Injection of LKB1 Inhibits the Formation of Diet-Induced Obesity in Rats by Activating the AMPK-POMC Neurons-Sympathetic Nervous System Axis

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Key Words
High-fat diet • Obesity • LKB1/AMPK·AMPK-POMC • neurons-sympathetic nervous system axis • Browning • Epinephrine

Abstract
Background/Aims: Obesity is increasingly becoming a major public health problem worldwide. Peripheral LKB1 inhibits white fat generation, but the effect of central LKB1 on diet-induced obesity (DIO) is unknown. Therefore, we examined whether LKB1 over-expression in the hypothalamus can inhibit the development of obesity. ***Methods:*** Adult male Sprague-Dawley rats were anesthetized and placed in a stereotaxic apparatus. LKB1-AAV-EGFP (2.0×10^8 or 2.0×10^{10} vector genomes) or Control-AAV-EGFP (2.0×10^8 vector genomes) was injected into the third ventricle. After administration, the rats were fed a high-fat diet (HFD) for 9 weeks to induce obesity. Rats fed a chow fat diet were used as normal controls. ***Results:*** LKB1 delivery decreased body weight, energy intake, fat mass, and serum lipid levels. LKB1 also improved HFD-induced hepatic fatty degeneration. Interestingly, LKB1 over-expression in the hypothalamus activated the AMPK-POMC neurons-sympathetic nervous system (SNS) axis, which can release epinephrine to promote white fat browning. Conversely, the elevated expression of MC3R/MC4R inhibited food intake. These two factors worked together to inhibit the development of obesity. ***Conclusions:*** LKB1 in the hypothalamus may have therapeutic potential for DIO through the activation of the AMPK-POMC neurons-SNS axis.

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Introduction

The global obesity issue is associated with an increased risk for metabolic diseases such as type 2 diabetes, stroke, heart disease, hypertension, and cancer [1-3]. Obesity is likely caused by an energy imbalance characterized by high energy intake relative to low energy expenditure [4, 5]. Thermogenesis in adipose tissue is an important factor for overall energy expenditure; therefore, enhancing thermogenesis in adipose tissue is a promising therapeutic strategy to improve obesity conditions [6-9].

LKB1 serine/threonine kinase (also known as stk11) is a tumor suppressor and a key regulator of energy metabolism [10-12]. It is expressed extensively in mammalian cells and plays important roles in various biological processes including cell polarity, cell metabolism, and cancer initiation and progression [13, 14]. These cellular functions of LKB1 are thought to be achieved by the direct phosphorylation of AMP-activated protein kinase (AMPK) at site Thr172 of α subunit [7, 15]. AMPK is a conserved serine/threonine kinase that functions in the regulation of energy metabolism [16]. A previous study showed that LKB1 is necessary for white adipose tissue (WAT) growth and differentiation [17]. Moreover, the LKB1/AMPK signaling pathway negatively regulates WAT [18]. The activation of LKB1 as well as its downstream kinase AMPK inhibits preadipocyte differentiation and adipogenesis and down-regulates the expression of peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer-binding protein α [19]. Adipose tissue-specific LKB1 knockout (KO) mice exhibit reduced amounts of WAT; LKB1 deletion murine embryonic fibroblasts also show impaired adipogenesis [20]. A previous study demonstrated that deletion of LKB1 in adult mice results in body weight reduction and lethality. More recently, Adipoq-Cre KO LKB1 was shown to promote brown adipose tissue (BAT) development and thermogenesis [21]. Collectively, these findings indicate that LKB1 plays an important role in regulating adipocyte growth and differentiation.

The arcuate nucleus (ARC) of the hypothalamus receives and integrates different inputs from peripheral organs and subsequently controls food intake and energy expenditure [22]. ARC neurons are divided into two distinct populations acting together to regulate feeding behavior: those expressing the orexigenic neuropeptides NPY/AgRP (neuropeptide Y/agouti-related peptide) and those expressing the anorexigenic peptides POMC/CART (pro-opiomelanocortin and cocaine- and amphetamine-related transcript) [23]. Different energy sensors have been implicated in the cellular mechanism in the ARC that regulates whole-body metabolism. Down-regulation of AMPKα in the ARC promotes body weight loss and reduces food intake. Thus, AMPK is necessary for glucose sensing in both AgRP and POMC neurons and thereby for the control of energy balance [24]. LKB1-deficient POMC neurons display impaired hepatic glucose metabolism with an underlying reduction in the release of α-melanocyte-stimulating hormone (α-MSH) from the hypothalamus [18].

Adeno-associated virus (AAV) delivery has been demonstrated to be safe and resulted in high and long-term expression in preclinical and clinical studies [25]. Previously, we unexpectedly found that rats with diet-induced obesity (DIO) have a low level of LKB1 in the hypothalamus [26]. In the present study, to precisely analyze the anti-obesity effect of LKB1 in DIO rats, we administered LKB1 through AAV delivery into the third ventricle. We found that LKB1 up-regulation in the hypothalamus activated the AMPK-POMC neurons-sympathetic nervous system (SNS) axis, which can release epinephrine to promote white fat browning. Conversely, the elevated expression of MC3R/MC4R inhibited food intake. These results suggest that LKB1 in the hypothalamus plays a key role in the central regulation of obesity and provide a new approach for the research of centrally regulated energy balance.

Materials and Methods

Animals

Male Sprague-Dawley rats (140-160 g) were purchased from the Beijing Academy of Military Sciences (Beijing, China). The animals were housed in a controlled environment with constant temperature and

maintained in a standard 12:12 h light/dark cycle (lights on at 07:00, lights off at 19:00). To acclimatize to their new environment, all rats were fed with standard laboratory chow and water available *ad libitum* during the first week. After adaptation, the rats were given intraventricular (ICV) injections of AAV. After surgery, the rats were divided randomly into four groups: (1) chow-fat group with Control-AAV-EGFP (CF-AAV; 2.0×10^8 vector genomes), fed with standard laboratory chow (3.8 kcal/g); (2) high-fat group with Control-AAV-EGFP (HF-AAV; 2.0×10^8 vector genomes); (3) high-fat group with a low titer of LKB1-AAV-EGFP (HF-LKB1-L; 2.0×10^8 vector genomes); and (4) high-fat group with a high titer of LKB1-AAV-EGFP (HF-LKB1-H; 2.0×10^{10} vector genomes), fed with a high-fat diet (HFD; 4.76 kcal/g). Body weight was recorded weekly. Food intake was measured daily. After feeding for 9 weeks, adipose tissue, liver, and hypothalamus were collected at the end of the study.

All animal care procedures were performed according to the Guidelines of the Animal Care Committee of Tianjin Medical University.

AAV vector design and preparation

Cloning and mutagenesis were performed by standard techniques. For vector preparation, two types of transgene were used: Control-AAV-EGFP (EGFP gene driven by the CMV promoter) and LKB1-AAV-EGFP (LKB1 gene driven by the CMV promoter). AAV vectors were generated by transfection of AAV-293 cells as described previously [27]. The physical titers (in vector genomes per microliter) were determined by quantitative PCR on a 2720 Thermal Cycler (Applied Biosystems, Camarillo, USA) using an In-Fusion™ PCR Cloning Kit (Clontech, USA) and the following primer set: forward, 5′-TGG AGG TAG TGG AAT GGA TCC CGC CAC CAT GGA CGT GGC TGA CCC CCA GC-3′ and reverse, 5′-CTC ACC ATG GTG GCG GGA TCCTGC TGC TTG CAG GCC GAG AGC-3′.

ICV injections

To administer the over-expression LKB1, the rats were anesthetized by intraperitoneal injection of pentobarbiturate and positioned on a stereotactic frame (KOPF, Germany). The skin over the skull was incised, and a small hole was made in the skull above the target using a microdrill. The stereotactic coordinates were 0.8 mm posterior to the bregma and 6.5 mm ventral of the skull surface [28]. A total volume of 4 μL was injected using a 10-μL syringe (Hamilton Co., Reno, NV). The AAV-mediated LKB1 gene was injected at various titers (low: 2.0×10^8 vector genomes; high: 2.0×10^{10} vector genomes) and at a flow rate of 1 μL/min, after which the needle was left in place for 2 min to prevent backflow before withdrawal.

Dual-energy X-ray absorptiometry scan analysis

At the end of the experiments, measurements were made using peripheral dual-energy X-ray absorptiometry (pDXA), as described previously [29].

Blood lipid analysis

Liver triglyceride (TG), serum total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) concentrations were measured using a chemical reagent kit (Nanjing Jiancheng Biology Engineering Institute, Nanjing, China).

Measurement of epinephrine levels

Serum epinephrine levels were measured using an enzyme-linked immunosorbent assay kit (NOVUS, Ontario, Canada) according to the manufacturer's guidelines.

Histological examinations

At the end of the treatments, WAT and the liver of each rat were removed and stored for histological examinations. Adipose tissue samples were preserved in 4% paraformaldehyde for 48 h, embedded in paraffin, sectioned at 6-μm thickness, and then stained with hematoxylin and eosin using standard procedures. For Oil Red O staining, liver tissues were frozen in liquid nitrogen and cut into 8-μm sections. The sections were stained and analyzed at 200× magnification using a microscope. In addition, the DNA of epididymal adipose tissue was extracted with a DNA kit (Tianjin Baibei Biology Engineering Institute, Tianjin, China). The frozen sections of the hypothalamus were analyzed with immunohistochemical (IHC) staining for NPY and POMC (1:1000 dilution; Abcam, Cambridge, England).

Total RNA extraction and real-time PCR

Total RNA was isolated from adipose tissue by the TRIzol reagent (Invitrogen, Camarillo, USA). The concentration of total RNA was measured by a spectrophotometer, and cDNA was synthesized from RNA (1 μg) using a cDNA Synthesis Kit (Thermo, Camarillo, USA) according to the manufacturer's instructions. Newly synthesized cDNA was amplified with specific primers and SYBR Green reactions using the SYBR Green qPCR Supermix (Invitrogen). β-actin was used as an internal control. PCR for every gene was performed for 40 cycles at 95 °C for 15 s and 60 °C for 1 min. Student's t-test was used to evaluate statistical significance. Sequences of primer sets used in this study are listed in Table 1.

Western blotting

Total cellular protein from adipose tissue and hypothalamus samples was harvested and lysed in RIPA buffer mixed with a complete protease inhibitor cocktail. Protein concentration was determined using a BCA Protein Assay Kit. Then, 30-100 μg of each denatured protein sample was separated on a 10-12% sodium dodecyl sulfate polyacrylamide gel electrophoresis gel and transferred to a polyvinylidene fluoride membrane. The membrane was blocked for 2 h at room temperature in a 5% non-fat milk blocking solution, followed by incubation overnight at 4 °C separately with anti-LKB1, anti-MC3R, anti-MC4R (Abcam); anti-AMPKα, anti-phosphorylated (p)-AMPKα, and anti-UCP1 (Cell Signaling, Massachusetts, USA) antibodies; GAPDH was used as a loading control. Following overnight incubation, the membrane was incubated with an HRP-conjugated secondary antibody (1:8000; Promega, Madison, USA) for 2 h at room temperature. The resultant immune complexes were detected using an enhanced chemiluminescence instrument (CLiNX Science Instruments, Shanghai, China). Protein bands were visualized via autoradiography, and intensities were analyzed using ImageJ.

Statistical analysis

Results are expressed as the mean ± standard error of the mean (SEM). All statistical analysis was performed using SPSS. Data were analyzed for statistical significance using Student's t-test and one-way analysis of variance and Bonferroni's post hoc test. Significance was set at $p < 0.05$.

Results

In vitro validation of AAV vectors for LKB1 over-expression

To over-express LKB1 in the hypothalamus, we cloned LKB1 cDNA into the AAV-brace vector (Fig. 1A). Then, we set out to up-regulate LKB1 expression by co-transfecting HEK293 cells with the p-AAV-CMV β-globin-LKB1 plasmids. After 48 h, the expression levels of LKB1 were quantified by immunoblotting and densitometry band evaluation (Fig. 1B). Control-AAV-EGFP was used as the control. EGFP expression in the hypothalamus was assessed by fluorescence microscopy to validate the ability of the AAV vector to transduce and express the transgene (Fig. 1C). As seen by the EGFP immunoreactivity, these viral vectors could express LKB1 efficiently in the hypothalamus.

Table 1. The primary sequence used for real-time quantitative PCR

Target gene	Forward	Reverse
SIRT1	TTCCAGCCATCTCTGTGTC	GATCCTTTGGATTCCTGCAA
CPT1	ATGACGGCTATGGTGTCTCC	GGCTTGTCTCAAGTGCTTCC
PGC-1α	CCGAGAATTCATGGAGCAAT	GTGTGAGGAGGGTCATCGTT
UCP1	GCCTGCCTAGCAGACATCAT	GGTGGTGATGGTCCCTAAGA
PRDM16	GTGCAGGTACTGTGGCAAGA	GGAGGAGATGCTGAATGACC
SREBP1	CCAGCCTTTGAGGATAACCA	TGCAGGTCAGACACAGGAAG
LPL	CAGCTGGGCCTAACTTTGAG	GGATCCCAATACTTCGACCA
NPY	TCGTGTGTTTGGGCATTCT	TAGTGTCGCAGAGCGGAGTA
POMC	CTATCGGGTGGAGCACTTC	TTCTTGATGATGGCGTTCTT
β-actin	GAGGACCAGGTTGTCTCCTG	TTACTCCTTGGAGGCCATGT

LKB1 changed the body weight and food intake of HFD-fed rats

We evaluated the impact of LKB1 modulation on body weight gain. As shown in Fig. 2A, rats fed the HFD gained more body weight than those fed a chow fat diet (CFD) from week 5 to week 9 ($p < 0.05$). Compared with the rats in the HF-AAV group, the body weight decreased in the rats in the HF-LKB1-H group, beginning at week 4 and continuing until the end of the experiment ($p = 0.037$). A significant difference was seen in body weight between the HF-AAV and HF-LKB1-L groups at week 7 ($p = 0.045$). However, when the LKB1 treatment groups were compared with the CFD group, only the HF-LKB1-H group exhibited a dramatic difference, which occurred at week 8 ($p = 0.034$) (Fig. 2A). Consistently, the rats in the HFD group consumed more calories than those in the CFD group (Fig. 2B). During weeks 8-9, both the HF-LKB1-L and HF-LKB1-H groups reduced their energy and food intake to not being significantly different from intake in the CF-AAV group (Fig. 2B-C). These results indicate that LKB1 administration inhibited body weight gain by suppressing energy intake.

At the end of the experiment, we measured the fat mass and lean mass and calculated body composition. Fig. 2D shows the subregions of rats identified by pDXA. The CF-AAV group had lower %whole body fat than the HF-AAV (-41%), HF-LKB1-L (-31%), and HF-LKB1-H (-29%) groups (Fig. 2E). When compared with the HF-AAV group, this trend was clearly reduced in the HF-LKB1-L and HF-LKB1-H groups,

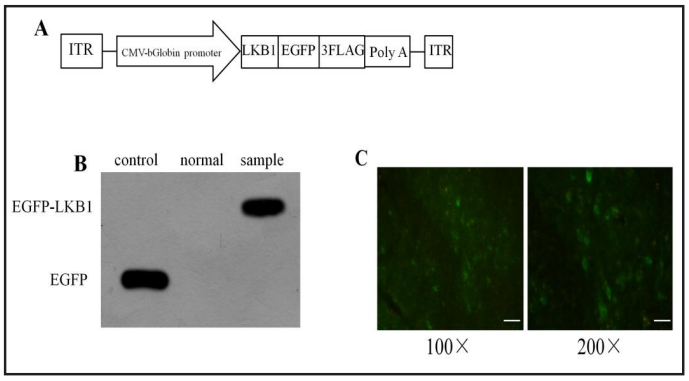

Fig. 1. *In vitro* validation of the LKB1 AAV vector for the up-regulation of LKB1 expression. (A) Schematic representation of the AAV vector. ITR, inverted terminal repeats; PolyA, polyadenosine sequence. (B) Assessment of LKB1 expression by western blot analysis. Control: the GFP gene plasmid was transfected into 293T cells; Normal: 293T cells; Sample: the LKB1 gene plasmid was transfected into 293T cells. (C) Transduction of LKB1-positive expression (EGFP, green) in the hypothalamus (magnification: ×100 and ×200; scale bar: 100 μm).

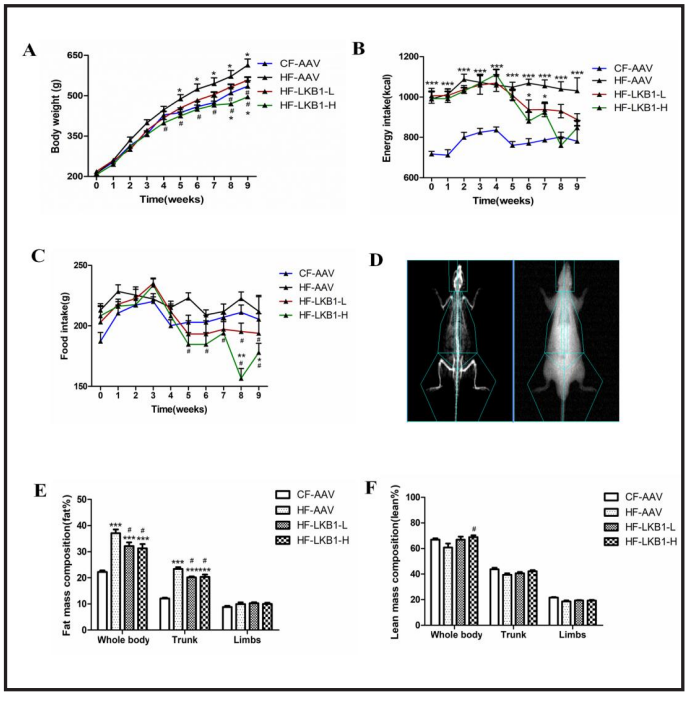

Fig. 2. Effect of LKB1 on HFD-induced obese rats. (A) Body weight changes following LKB1 administration compared with the CF-AAV-control and HF-AAV-control groups. (B-C) Average energy intake and food intake (n = 6 independent experiments in different rats). (D) Subregions of rats identified by pDXA [64]. Fat mass % and lean mass % of the rats in the different groups were measured. Data are the mean ± SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$ vs. CF-AAV group; $^{\#}p < 0.05$ vs. HF-AAV group.

as demonstrated by their 13.5% and 16.2% decrease, respectively. Furthermore, trunk fat mass% showed a similar trend to whole body fat % (Fig. 2E). However, compared with the HF-AAV group, only the HF-LKB1-H group exhibited a marked difference (Fig. 2F). There was no significant difference in limb fat mass or lean mass between the groups ($p > 0.05$). These results suggest LKB1-treated rats gained lean mass and less fat mass.

LKB1 over-expression altered the histopathology of HFD-fed rats

We assessed possible phenotypic alterations of WAT on the modulation of hypothalamic LKB1 expression. The histopathology of WAT showed that the HF-AAV group rats had larger adipocytes than the other groups, whereas total DNA content was the smallest of all groups (Fig. 3A-B). Furthermore, the administration of LKB1 resulted in smaller lipid droplets within WAT, implying high metabolic activity. As for the controls, DNA content was comparable in all groups (Fig. 3B). Oil Red O staining suggested that the HF-AAV rats had larger lipid droplets and developed fatty liver. However, LKB1 treatment resulted in a notable improvement of lipid droplets and hepatic steatosis (Fig. 3C). In addition, we measured liver lipid content. Compared with the CF-AAV group, liver TG levels were significantly increased in the rats of the HF-AAV group ($p = 0.000$). TG levels were significantly decreased in the HF-LKB1-H and HF-LKB1-L groups by 26.7% and 21.6%, respectively, compared with the HF-AAV group (Fig. 3D). As shown in Fig. 3E-F, LKB1-treated rats had significantly more BAT mass than the CF-AAV and HF-AAV groups. These results showed that LKB1 treatment attenuated HFD-induced adipocyte hypertrophy in WAT, prevented hepatic steatosis, and promoted BAT formation.

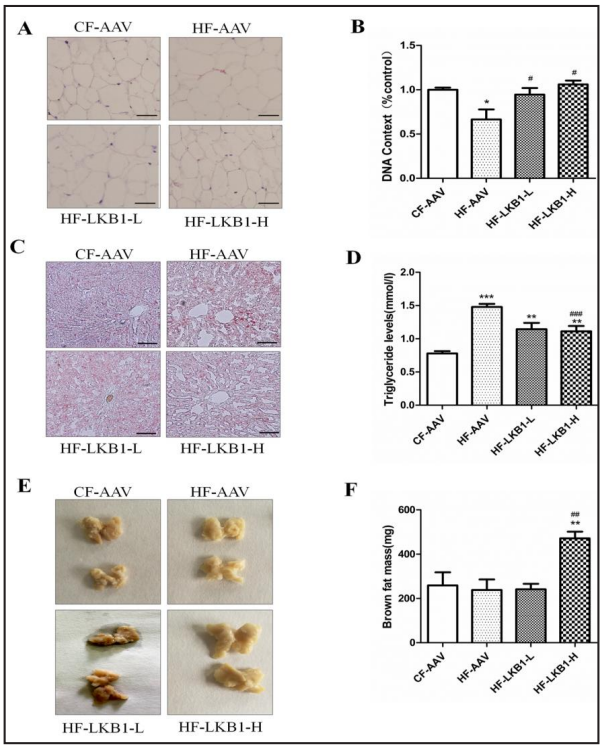

Fig. 3. LKB1 altered the histopathology of rats. (A) Paraffin-embedded sections of WAT were stained with hematoxylin and eosin. (B) DNA content of epididymal WAT was measured (magnification: ×200; scale bar: 100 μm). (C) Frozen sections of liver were stained with Oil Red O. (D) Liver TG levels were measured in all groups. (E) BAT in the interscapular region. (F) Weight of interscapular BAT was measured. Data are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. CF-AAV group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. HF-AAV group.

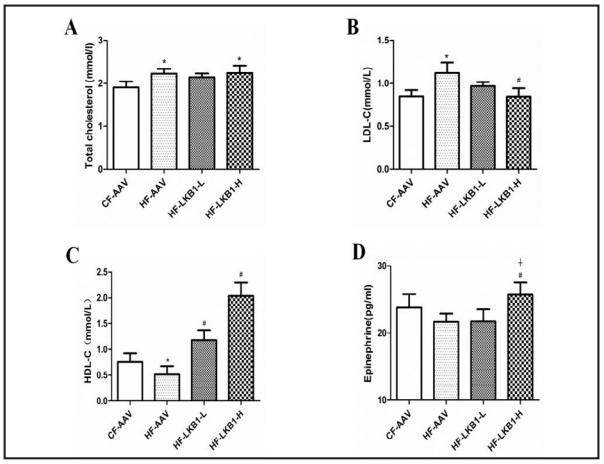

Fig. 4. Effects of LKB1 on the blood lipid profile of HFD-fed rats. (A-D) TC, LDL-C, HDL-C, and epinephrine levels were measured in the serum of each group. Data are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. CF-AAV group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. HF-AAV group.

Effect of LKB1 on the blood lipid profile of HFD-fed rats

Compared with the CF-AAV group, there was a dramatic increase of serum TC and LDL-C levels in the rats of the HF-AAV group (Fig. 4A-B). LDL-C levels were decreased by 30% in the HF-LKB1-H group (Fig. 4B), whereas there was no significant change in TC levels (Fig. 4A). LDL-C levels were decreased by 16.7% in the HF-LKB1-L group (Fig. 4B). The serum HDL-C levels of the HF-LKB1-H group were increased by 1.8-fold compared with the HF-AAV group (Fig. 4C). In addition, serum epinephrine concentrations were significantly increased in the HF-LKB1-H group compared with the HF-AAV and HF-LKB1-L groups ($p = 0.038$). These results suggest that LKB1 treatment beneficially modulated the blood lipid profile.

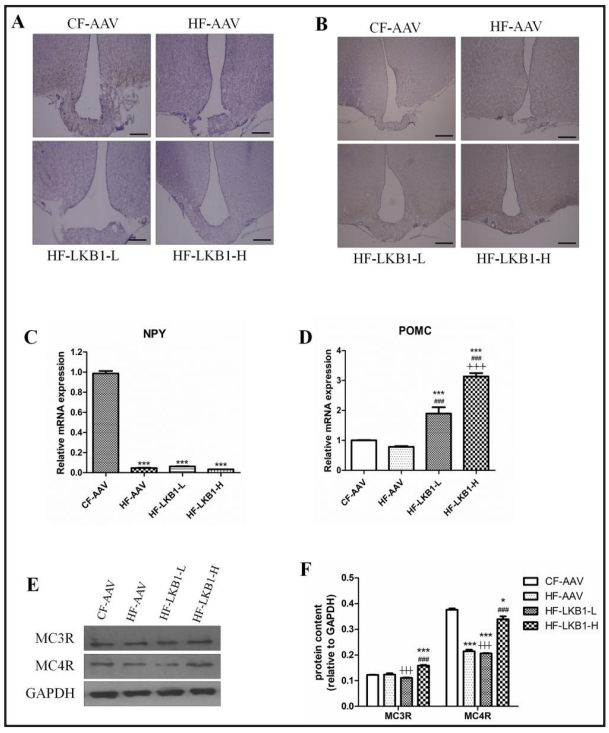

Fig. 5. Effects of LKB1 on the expression of genes in the hypothalamus. [19] NPY and POMC expression in the hypothalamus of rats was detected by IHC (magnification: ×100; scale bar: 100 μm). (C-D) The mRNA expression levels of NPY and POMC in the hypothalamus. The levels of MC3R and MC4R protein were determined [64]. Data are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. CF-AAV group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. HF-AAV-control; $^{\dagger\dagger\dagger}p < 0.001$ HF-LKB1-L group vs. HF-LKB1-H group.

LKB1 regulated the expression levels of factors in the hypothalamus

The protein levels of LKB1 were down-regulated in the hypothalamus of HF-AAV group rats compared with the CF-AAV group (Fig. 6D). The expression of LKB1 protein was increased in the LKB1-treated groups compared with the HF-AAV group. The levels of p-AMPKα in the hypothalamus of LKB1-treated rats were increased compared with the HF-AAV group (Fig. 6D-E). Further, in IHC analysis of the hypothalamus sections of all groups, NPY was substantially decreased in the rats fed an HFD, whereas POMC was increased (Fig. 5A-B). Then, we measured the mRNA levels of NPY and POMC. We found a remarkable increase in hypothalamic POMC mRNA levels following LKB1 administration, whereas NPY mRNA levels were decreased (Fig. 5C-D). As shown in Fig. 5E-F, MC3R and MC4R levels in the hypothalamus of the LKB1-treated groups were increased compared with the HF-AAV-control group. These results suggest that LKB1 treatment inhibited energy intake via the up-regulation of MC3R/4R expression and down-regulation of NPY expression.

LKB1 promoted the browning of WAT

We detected the expression of browning markers such as uncoupling protein 1 (UCP1), PR domain-containing 16 (PRDM16), and PPARγ co-activator 1α (PGC-1α). After administration of LKB1, the mRNA expression levels of browning markers were significantly up-regulated (Fig. 6A). As shown in Fig. 6F-G, we also examined the effect of LKB1 infusion on UCP1 protein expression. LKB1 administration promoted a significant increase in UCP1 protein levels in WAT. Thus, LKB1 treatment led to a significant elevation in carnitine palmitoyl-coA transferase-1 (CPT1) mRNA levels, although no differences were observed in silent information regulator 1 (SIRT1) mRNA expression levels in the LKB1-treated rats (Fig. 6B). In contrast, the expression levels of sterol regulatory element binding protein 1 (SREBP1) and

lipoprotein lipase (LPL) were down-regulated following LKB1 treatment (Fig. 6C), suggesting that lipogenesis was inhibited. Collectively, these results indicate that LKB1 promoted the browning of WAT and inhibited lipogenesis. Collectively, these results, summarized in Fig. 7, indicate that LKB1 promoted the browning of WAT and inhibited lipogenesis.

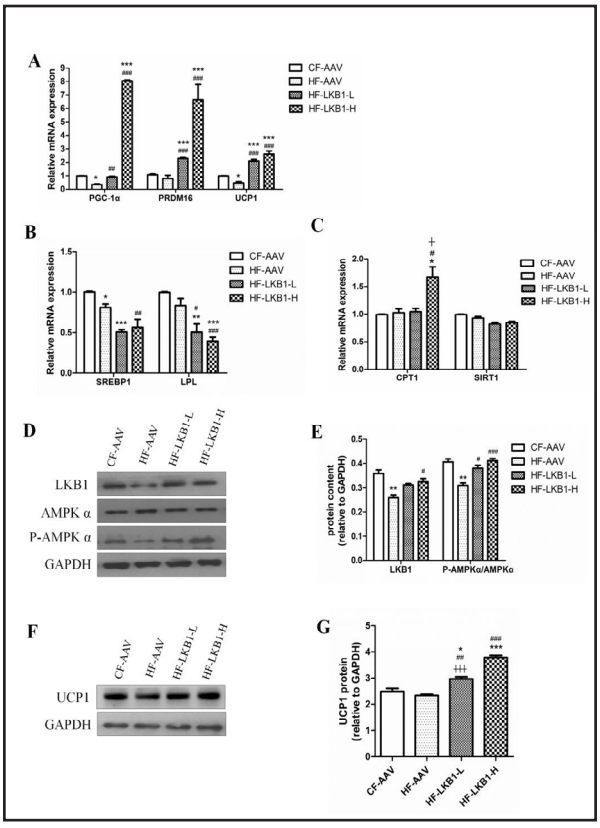

Fig. 6. Effects of LKB1 on the expression of genes. mRNA expression levels of mitochondrial biogenic genes (A), thermogenic genes (B), and adipogenesis genes (C) in WAT. (D-E) Protein expression levels of LKB1, AMPKα, and p-AMPKα in the hypothalamus. (F) Protein expression levels of UCP1 in WAT. Densitometry analyses are presented as a relative ratio to GAPDH. Data are the mean ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ vs. CF-AAV group; $^{\#}p < 0.05$ and $^{\#\#\#}p < 0.001$ vs. HF-AAV group; $^{\dagger\dagger\dagger}p < 0.001$ HF-LKB1-L group vs. HF-LKB1-H group.

Discussion

In this study, ICV injections of an LKB1 over-expression vector into the hypothalamus of HFD-fed rats affected body weight gain and food intake. LKB1 up-regulation resulted in decreased body weight and fat mass, consistent with previous reports [13]. This provides evidence that LKB1 administration triggered a negative energy balance by suppressing energy intake. Additionally, LKB1 over-expression induced browning of WAT by the activation of the AMPK-POMC neurons-SNS axis.

We previously observed dysregulation of the LKB1/AMPK signaling pathway in the hypothalamus of DIO rats [26]. Several other studies showed that the hypothalamus plays an important role in regulating feeding and maintaining energy balance via several neuropeptide signaling systems [30]. Recent studies demonstrated that the LKB1/AMPK signaling pathway negatively regulates adipogenesis in the periphery. Zhang et al. generated a FABP4-Cre-mediated LKB1 KO mouse model and demonstrated that LKB1 is required for WAT growth and differentiation. They showed that a lack of LKB1 leads to a reduction of WAT and early death [18]. KO of LKB1 in skeletal muscle prevents AMPK activation and glucose uptake in response to contraction and phenformin, indicating its important function in energy metabolism [31]. To determine whether ICV LKB1 can inhibit the development of obesity, we delivered LKB1-AAV into the third ventricle before the rats became obese. HFD-fed rats characteristically have significantly increased body weight and fat mass, as well high levels of plasma lipids and fatty liver. However, ICV injections of LKB1 significantly suppressed body weight gain, energy intake, and fat accumulation in a dose-dependent manner. Besides, ICV LKB1 improved blood lipid profile levels. It has been reported that adipose tissue-specific LKB1 KO mice have improved glucose metabolism and insulin sensitivity and are resistant to DIO [32]. DIO is therefore strongly associated with WAT hypertrophy. Our HFD group had a larger volume of adipocytes in epididymal adipose tissue than the CFD group and LKB1 treatment group. However, the LKB1 treatment group exhibited smaller adipocytes. These results suggest that LKB1 inhibited WAT formation. The development of fatty liver is strongly

associated with obesity [13]. HFD feeding induced an accumulation of numerous fatty droplets, which is typical of fatty liver. Treatment with LKB1 reduced this accumulation, thereby ameliorating fatty liver, implying that LKB1 inhibited body weight gain by suppressing energy intake.

The hypothalamus plays a major role in the regulation of BAT thermogenesis through the SNS [33, 34]. The hypothalamus encompasses several anatomically well-defined nuclei, including the ARC, ventromedial hypothalamus (VMH), dorsomedial hypothalamus, lateral hypothalamus, and paraventricular hypothalamus [35]. Some peripheral signals arrive at the VMH to activate thermogenesis in BAT and browning of WAT, such as GLP-1, estradiol, and thyroid hormone [36, 37]. A large number of experiments have shown that AMPK in the VMH is a key negative regulator of sympathetically activated BAT thermogenesis [38]. In addition to controlling feeding behavior, the ARC also regulates energy expenditure. For example, POMC/CART neurons decrease food intake and increase energy expenditure through the activation of MC3R or MC4R via the release of α-MSH [39, 40]. Conversely, AgRP/NPY neurons promote food intake and decrease energy expenditure [41]. In our study, POMC expression was up-regulated and NPY expression was down-regulated in the ARC, which might also contribute to the activation of thermogenesis in the obese rats. A previous study reported that POMC-derived neuropeptides bind to and stimulate MC3R/MC4R to inhibit food intake and increase energy expenditure [42]. In line with our observations, the expression of both MC3R and MC4R protein was up-regulated in the ICV LKB1 rats. Our data showed that a high-titer LKB1 injection increased epinephrine levels. Collins et al. demonstrated that MC4R activation may increase the sympathetic drive to BAT to trigger thermogenesis via norepinephrine stimulation of the β3-adrenergic receptor, which is thought to be principally involved in this response [43]. Such observations suggest that the activation of browning WAT may be via the up-regulation of MC3R and MC4R expression.

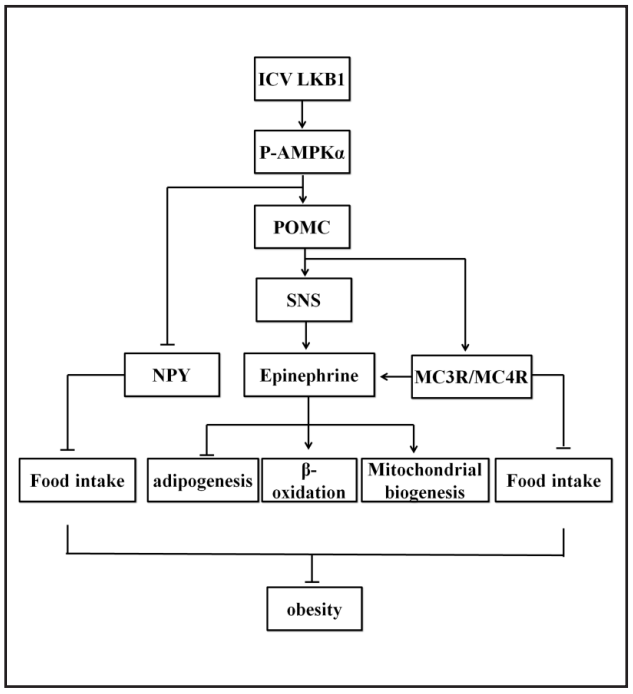

Fig. 7. Mechanism by which LKB1 inhibits DIO through the stimulation of the AMPK-POMC neurons-SNS axis.

Adipose tissue is one of the largest organs in the body and plays an important role in central energy balance and lipid homeostasis [44]. WAT and BAT are the two types of adipose tissue found in mammals [45]. WAT stores energy and BAT specializes in energy expenditure [46]. In addition to classical BAT, a novel type of inducible "brown-like" adipose tissue (named brite adipose) was discovered some years ago [47]. Brite fat cells show multilocular lipid droplets that are comparable to the levels of UCP1 and similar to the physiological functions of classic brown fat cells [48]. Brite adipose tissue has been shown to develop within WAT deposits upon cold or β-adrenergic stimulation [49]. BAT is activated by increased firing of sympathetic neurons, leading to the release of noradrenaline and activation of the β3-adrenergic receptor [50]. Activation of the AMPK pathway is known to inhibit lipid synthesis but promote mitochondrial synthesis [7, 51]. Additionally, the thermogenesis of mature brown adipocytes is activated by agonists of the β-adrenergic receptor, such as norepinephrine and epinephrine, which are released from sympathetic neurons [52]. A high-titer LKB1 injection

increased epinephrine levels. These findings raise the possibility that the administration of LKB1 activated the AMPK-POMC neurons-SNS axis to induce browning. In the present study, LKB1 attenuated HFD-induced adipocyte hypertrophy and increased the number of brite adipose cells in WAT and increased brown adipose mass.

We further measured the expression of related proteins to study the molecular mechanisms associated with the morphological changes in adipose tissue. UCP1 is a hallmark of BAT, and recent studies have revealed that this protein is induced along with an increase in mitochondrial content in WAT during browning [53]. Moreover, accumulating studies have identified several other dominant transcriptional regulators of brown fat development and function, including PPARγ, PGC-1α, and PRDM16 [54]. PGC-1α is α major cofactor known to play a key role in mitochondrial biogenesis [50]. PGC-1α interacts with multiple transcription factors to stimulate mitochondrial metabolic capacity [55]. In muscle-specific PGC-1α transgenic mice, there is significant up-regulation of UCP1 and Cidea mRNA in subcutaneous adipose tissue [56]. PRDM16 is a determining factor of brown-fat development and sufficient to promote brown adipogenesis [57]. PRDM16 transgenic mice display increased energy expenditure and improved glucose tolerance when fed an HFD [58]. The expression of PRDM16 in white fat cells can induce the molecular procedure underlying the development of brown fat cells [59]. Moreover, transgenic mice that over-express PRDM16 in all fat cells exhibit clusters of multilocular brown-like fat cells within their white fat tissue as well as have increased energy expenditure [60]. Deletion of PRDM16 in fat cells, using adiponectin-Cre mice, causes complete ablation of beige fat function, severe insulin resistance, and hepatic steatosis, despite only mild obesity [61]. These results show that LKB1 administration up-regulates the mRNA expression of BAT markers, indicating the increased browning of WAT. CPT1 and SIRT1 play an important role in fatty acid β-oxidation [62, 63]. The levels of CPT protein were increased, suggesting enhanced fatty acid oxidation and mitochondrial biogenesis. Besides, the expression levels of SREBP1 and LPL were decreased, demonstrating an inhibition of adipogenesis. These results reveal that LKB1 administration inhibits the development of DIO by up-regulating the expression of BAT markers and inhibiting adipogenesis.

In conclusion, we provide evidence that the over-expression of LKB1 in the hypothalamus exerts an anti-obesity effect in rats with HFD-induced obesity. It is probable that these actions of central LKB1 were partly because of the increased expression of multiple genes involved in WAT browning via the activation of the AMPK-POMC neurons-SNS axis. These findings offer a better understanding of the central regulation of energy balance and a potential new approach for the treatment of obesity.

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Disclosure Statement

The authors declare no conflicts of interest.

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