RESEARCH ARTICLE

Cyclin Y Is Involved in the Regulation of Adipogenesis and Lipid Production

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Abstract

A new member of the cyclin family cyclin Y (CCNY) is involved in the regulation of various physiological processes. In this study, the role of CCNY in energy metabolism was characterized. We found that compared with wild-type (WT) mice, Ccny knockout (KO) mice had both lower body weight and lower fat content. The Ccny KO mice also had a higher metabolic rate, resisted the stress of a high-fat diet, and were sensitive to calorie restriction. The expression levels of UCP1 and PGC1α were significantly higher in the brown adipose tissue (BAT) of the Ccny KO mice than that of the WT littermate controls, whereas there was no significant difference in BAT weight between the WT and the Ccny KO mice. In addition, the down-regulation of Ccny resulted in suppression of white adipocyte differentiation both in vivo and in vitro, while the expression of Ccny was up-regulated by C/EBPα. Furthermore, both hepatocytes and HepG2 cells that were depleted of Ccny were insensitive to insulin stimulation, consistent with the significant inhibition of insulin sensitivity in the liver of the Ccny KO mice, but no significant changes in WAT and muscle, indicating that CCNY is involved in regulating the hepatic insulin signaling pathway. The hepatic insulin resistance generated by Ccny depletion resulted in down-regulation of the sterol-regulatory element-binding protein (SREBP1) and fatty acid synthase (FASN). Together, these results provide a new link between CCNY and lipid metabolism in mice, and suggest that inhibition of CCNY may offer a therapeutic approach to obesity and diabetes.

Introduction

Dysregulation of lipid metabolism results in many pathological disorders, such as type 2 diabetes, fatty liver, and cardiovascular disease [1–4]. Adipose tissue and the liver are the major effectors of lipid homeostasis, and they are mainly controlled by the insulin signaling pathway [5]. One of the important downstream targets that is regulated by the insulin pathway is the group of sterol regulatory element binding proteins (SREBPs). SREBPs belong to the basic
helix-loop-helix leucine zipper (bHLH-LZ) family of transcription factors (TFs), which are capable of regulating the expression of many enzymes required for the hepatic biosynthesis of fatty acids, endogenous cholesterol and triglycerides [6,7]. There are three isoforms of SREBP transcription factors, SREBP1a, SREBP1c and SREBP-2. All three isoforms are synthesized as inactive precursors that are tethered to the endoplasmic reticulum membrane, and then cleaved to the mature forms when the sterol levels decrease or when there is insulin stimulation [8,9]. The mature forms of SREBPs translocate to the nucleus to activate the transcription of target genes [8]. Importantly, SREBP1c is expressed at particularly high levels in hepatocytes, and this expression is mainly regulated by insulin at the transcription level through AKT/PKB [10].

Cyclins are a family of cell cycle proteins that share a conserved region of approximately 100 amino acid residues, termed the cyclin box [11]. Cyclins bind specific CDKs through their cyclin box to form functional protein kinase complexes. Cyclin Y (CCNY) is a new member of the cyclin family that was originally cloned from a testis cDNA library [12] and was first characterized by its function in cell cycle regulation [13]. CCNY has been identified as a membrane-binding protein that can activate the kinase activity of cdk14 through direct binding to cdk14 [14]. In addition to its emblematic function of regulating the cell cycle, CCNY is also involved in many other cellular developmental processes. Ccny deletion impairs Drosophila development and mice spermatogenesis [15,16]. In the nervous system, Ccny drives synapse removal to regulate the maturation of neural circuits [17].

In the present study, by analyzing Ccny-deficient mice using a range of assays, we showed that CCNY is involved in adipogenesis and lipid accumulation in mice through regulation of the insulin signaling pathway and other regulatory pathways related to lipid homeostasis.

Materials and Methods

Animal studies

Ccny flox mice were generated by the Shanghai Research Center For Model Organisms (Shanghai, China). Crossing Ccny flox mice with EIIa-Cre mice generated Ccny heterozygous mice. The Ccny heterozygous mice were maintained on a mixed background. We backcrossed the mice for three generations with C57BL/6 mice purchased from SLAC laboratory (Shanghai, China). The Ccny KO mice were generated by crossing Ccny heterozygous mice. Tail biopsies of the mice were analyzed using genomic PCR. WT Ccny was detected using the primers P-F: 5′- AATACAGCTCTTGCTCCACCA-3′ and P-R: 5′- ATACAGCTCTTGCTCCACCA-3′. The PCR product was 400 bp in length. Ccny deletion was detected using primer P-F: 5′- GCT ACCGCTGTATTGCTGAA-3′ and P-R: 5′- ATACAGCTCTTGCTCCACCA-3′. The PCR product was 700 bp in length.

The mice were fed either a normal chow diet or a high-fat diet (HFD) (Research Diets, Inc., NJ, USA) starting at 8 weeks of age. The HFD was maintained for up to 12 weeks. The body fat content was determined by nuclear magnetic resonance using a Minispec LF50 nuclear magnetic resonance analyzer (Bruker Optics). For calorie restriction, the mice were fed 90% of the normal food intake every day for a period of four weeks. At the end of the studies, serum and tissues were collected for further analysis. For in vivo studies of insulin signaling, male mice were fasted overnight, followed by an IP injection of insulin (5 U/kg body weight). The mice were humanely destroyed after 5 minutes, and the liver, white adipose tissue and muscle were excised and snap frozen for immunoblotting.

Plasmids, antibodies, and reagents

The wild-type C/EBPβ, C/EBPα fragments were generated from the mouse cDNA library using the PCR method. The fragments were cloned into pcDNA3-HA vector.
We purchased the following antibodies: antibody against CCNY from Abcam (Cambridge, MA, USA); antibodies against phospho-insulin receptor β (Tyr1150/1151), AKT (11E7), phospho-AKT (Ser473), GSK3β, phospho-GSK3β (Ser9), UCP1 and PGC1α from Cell Signaling Technology (Beverly, MA, USA); antibodies against SREBP1 (K-10), PPARγ, C/EBPα, aP2 and the secondary antibody (horseradish peroxidase-conjugated anti-rabbit and anti-mouse IgG and donkey anti-goat IgG) from Santa Cruz Biotechnology (Santa Cruz, CA, USA); anti-FASN from R&D Systems (Minneapolis, MN, USA).

LabAssay Triglyceride and LabAssay Cholesterol kits were purchased from Wako (Japan). A free fatty acid quantification kit was purchased from Biovision (CA, USA). Mouse insulin ELISA kit was purchased from Mercodia (Uppsala, Sweden). Mouse neuropeptide-Y ELISA kit was purchased from Mlbio (Shanghai, China). Insulin, dexamethasone (DEX), 1-methyl-3-isobutylxanthine and oil red O were purchased from Sigma Aldrich (St. Louis, MO, USA).

Cell culture and transfection

HepG2 cells (American Type Culture Collection, Manassas, VA, USA) and primary hepatocytes were cultured in 5.5 mmol glucose Dulbecco’s modified Eagle’s medium (DMEM) (GIBCO, Life Technologies, NY, USA) supplemented with 10% FBS and 1% penicillin/streptomycin. HEK293T (American Type Culture Collection) and 3T3-L1 cells (American Type Culture Collection) were kept in DMEM supplemented with 10% FBS and 1% penicillin/ streptomycin. All cells were kept at 37°C in 5% CO₂. Transient transfection was performed using lipofectamine reagent (Invitrogen, Life Technologies, NY, USA).

Measurement of the metabolic rate and activity

The metabolic rate and locomotion were measured using a Comprehensive Laboratory Animal Monitoring System (CLAMS) (Columbus Instruments). After the mice acclimated to the system for 24 hours, the oxygen consumption (VO₂), carbon dioxide production (VCO₂), respiratory exchange ratio (RER) and food intake were individually monitored for the subsequent 24 hours. Energy expenditure was normalized to body weight. The activity of the mice was monitored using x-axis beam breaks.

Glucose tolerance test and insulin tolerance test

The GTT was performed as previously described [18]. Briefly, for the GTT, the mice were fasted overnight and injected with glucose at 2 g/kg body weight. For the ITT, the mice were fasted 4 hours and injected with insulin at 0.75 U/kg body weight. The blood glucose concentrations were measured 15, 30, 60, and 120 minutes after the IP injection.

Paraffin sectioning and hematoxylin and eosin (H&E) staining

The white adipose tissue (WAT) was cut from 8-week-old mice and fixed overnight using 4% polyoxymethylene at 4°C. The fixed tissue was washed for 2 hours to remove the polyoxymethylene. Then, the tissue was dehydrated using ethanol and xylene. The tissue was embedded in paraffin and cut into slices. The slides were treated with xylene to remove the paraffin and with ethanol for rehydration. H&E staining was performed according to the protocol of Beyotime Institute of Biotechnology (jiangSu, China).

Isolation of various primary cells from mice

The primary adipocytes and stromal vascular cells were isolated from the WAT tissue of the 8-week-old mice. The WAT tissue was placed into pre-chilled PBS and washed three times to
remove the blood. Then, the floating tissue was transferred to a dish and quickly cut into pieces. The tissue was then washed another two times and transferred to a new tube for digestion with 1 mg/ml collagenase and 4% BSA in PBS. The tube was incubated at 37°C for 30 min and centrifuged at 1,000 rpm for 10 min. The supernatant was discarded, and the pellets were re-suspended using erythrocyte lysis buffer (154 mM NH₄Cl, 10 mM KHCO₃, and 0.1 mM EDTA) for 1 min to eliminate red blood cells. The cell suspension was filtered using a 70-μm filter, and the cells were centrifuged at 1,000 rpm for 10 min. The cells were re-suspended using DMEM with 10% FBS, and the medium was changed 4 h after incubation. Primary hepatocytes were isolated by infusing the mouse liver with collagenase buffer, and cells were filtered through a 100-μm filter. The cells were seeded on collagen-coated 6-well plates at a density of 1x10⁶/well.

**Adipocyte differentiation and detection with oil red O staining**

The 3T3-L1 pre-adipocytes and primary pre-adipocytes of the mice were kept at 37°C in 10% CO₂ and were grown to confluence for two days (called D0). Then, the cells were cultured with 1 mg/ml insulin, 1 mM DEX and 0.5 mM 1-methyl-3-isobutylxanthine in DMEM supplemented with 10% FBS for two days. Next, the cells were treated with fresh medium containing 1 mg/ml insulin, and the medium was changed every two days until day 8. The differentiated adipocytes were stained with oil red O. We extracted the oil red O in triglyceride droplets using 100% isopropanol and quantified them by measuring the OD at 510 nm.

**Stable Ccny knockdown cell lines**

To establish stable Ccny knockdown cell lines, a retrovirus system was introduced based on the manufacturer’s recommendations (Clontech). The targeted sequences of Ccny were cloned into the pSiren-RetroQ vector, and the targeted sequences of Ccny and shuffle are shown in S1 Table. Retroviruses were produced by cotransfecting 293T cells with recombinant pSiren-RetroQ plasmids and pCL10A1 helper plasmid. Then, we infected the proliferating HepG2 and 3T3-L1 cells using the 293T cell culture supernatants, which were supplemented with 8 μg/ml polybrene and passed through a 0.45-mm filter. After 12 hours, the cell lines were selected in medium supplemented with 3 μg/ml puromycin.

**RNA extraction and RT-qPCR**

The total RNA of the samples was isolated using TRIzol Reagent (Life Technologies). cDNA was reverse transcribed using a ReverTra Ace qPCR RT Kit (TOYOBO, Osaka, Japan). Quantitative PCR analysis was performed using an ABI Prism 7500 detection system (Life Technologies). The reaction buffer was SYBR Green (TOYOBO). GAPDH was simultaneously detected as a control.

**Chromatin immunoprecipitation (ChIP) assay**

The ChIP assay was performed as previously described by Pei et al [19]. The primer sequences used for detecting the precipitated DNA are shown in S1 Table.

**Statistical analysis**

All results were analyzed using Microsoft Excel software. Student’s t-test was used to compare the significance between two groups and was judged at *, P< 0.05; **, P< 0.01; ***, P<0.001.
Ethics statement

All animals were maintained in strict accordance with the guidelines of the Institutional Animal Care and Use Committee of the Institute of Biochemistry and Cell Biology. All animals were kept on a 12-hour light/dark cycle and were given ad libitum access to food and water. All of the experimental procedures were approved by the Chinese Academy Science ethics commission (Permit Number: SIBCB-NAF-14-001-s304-007). The mice were anesthetized with sodium pentobarbital after an overnight fast. All efforts were made to minimize suffering.

Results

Ccny-deficient mice showed dysfunctional lipid metabolism

To investigate the potential role of CCNY in energy homeostasis, we first compared the body weight of the Ccny KO mice with the WT littermate controls during growth. The body weight did not significantly differ between the Ccny KO mice and the WT controls during the first 5 weeks; afterward, the Ccny KO mice weighed significantly less than the WT controls (Fig 1A). By 14 weeks, the Ccny KO males weighed a dramatic 20% less than the WT males (Fig 1A).

Nuclear magnetic resonance analysis showed that the Ccny KO mice had a 30% decrease in total body fat mass compared with that of the WT controls; there was also a 6% increase in lean body mass (Fig 1B). In addition, the weights of the retroperitoneal, inguinal, perirenal and arm-pit fat pads in the Ccny KO mice were all lower than those in the WT controls (S1A and S1B Fig). Moreover, histologic analysis of the white adipose tissue (WAT) revealed that the Ccny KO mice had smaller adipocytes than those of the WT littermate controls (Fig 1C). Taken together, these results indicate that suppression of CCNY expression results in lipid metabolic dysfunction in mice.

Because WAT is an important endocrine organ and contributes to the metabolic state, a reduction in WAT might lead to lipodystrophy syndrome [4]. To assess the possibility of the lipodystrophic phenotype, we analyzed the total plasma- cholesterol, triglyceride, free fatty acid and liver-triglyceride levels. The plasma cholesterol, triglyceride, free fatty acid and liver-triglyceride levels decreased in the Ccny KO mice (Fig 1D). In contrast, the GTT assay showed no significant difference in the glucose tolerance between the Ccny KO mice and the WT controls (Fig 1E). And the ITT experiment also showed no significant difference in the insulin tolerance between the Ccny KO mice and the WT controls (Fig 1F). Additionally, there was no difference in the fasting basal glucose and insulin levels between the Ccny KO mice and the WT controls (Fig 1D and 1E).

Using metabolic cages (Comprehensive Lab Animal Monitoring System), we analyzed the metabolic states of the mice. The VO2 (S2A Fig), VCO2 (S2B Fig), RER (S2C Fig), spontaneous locomotor activity (S2D Fig) increased significantly in the Ccny KO mice. These results suggest that the Ccny KO mice have increased energy expenditure compared with that of the WT controls. Therefore, we analyzed the weights of brown adipocyte tissue (BAT). The result showed there was no significant difference in BAT weights between the WT and Ccny KO mice (S1 Fig). On the other hand, both mRNA and protein levels of Ucp1, which is a mitochondria protein for energy dissipation, were significantly higher in the BAT of Ccny KO mice than that of their WT littermate controls (Fig 2). In addition, the expression level of transcriptional coactivator Pgc1α, a critical positive regulator of UCP1 expression, was also significantly upregulated in BAT of the Ccny KO mice (Fig 2). Taken together, these results suggest that the increased energy expenditure in the Ccny KO mice is at least partially due to a higher activity of brown adipocytes.
Ccny-deficient mice are resistant to a HFD and sensitive to calorie restriction

We expected that the Ccny KO mice and the WT controls would respond differently to energetic stresses. The mice were first subjected to a HFD. The body weight of the Ccny KO mice was lower than that of the WT control mice on the HFD (Fig 3A and S3A Fig). The body fat contents in the Ccny KO mice, including the retroperitoneal, inguinal, perirenal and armpit fat pads, were also smaller than that in the WT control mice under such treatments (Fig 3B and S3B Fig). In addition, the Ccny KO mice had greater variations in their triglyceride, total plasma cholesterol and free fatty acid levels compared with those of the WT controls (Fig 3C). These results suggest that the Ccny KO mice were resistant to the HFD treatment.

We also administered these two murine genotypes to calorie restriction. The 10% reduction in food intake could not lower the body weight of the WT mice (Fig 3D, also see ref. [20]), whereas the body weight of the Ccny KO mice decreased significantly under such treatments (Fig 3D). In addition, the Ccny KO mice had greater variations in their triglyceride, total plasma cholesterol and free fatty acid levels compared with those of the WT controls (Fig 3C). These results suggest that the Ccny KO mice were resistant to the HFD treatment.

We also administered these two murine genotypes to calorie restriction. The 10% reduction in food intake could not lower the body weight of the WT mice (Fig 3D, also see ref. [20]), whereas the body weight of the Ccny KO mice decreased significantly under such treatments (Fig 3D). In addition, the calorie restriction also significantly decreased the body fat contents of the Ccny KO mice (Fig 3E). Furthermore, the Ccny KO mice had significantly lower triglyceride, total plasma cholesterol and free fatty acid levels compared with those of the WT controls on the calorie restriction (Fig 3F). Taken together, these results indicate that the Ccny KO mice are more sensitive to calorie restriction than the WT mice.
CCNY is involved in adipocyte differentiation

We found that the Ccny mRNA level of the white adipocytes from the mice with HFD-induced obesity was significantly higher than that of the non-obese mice that consumed a normal diet; and the similar situation was also found for the adipogenesis-specific marker PPARγ (Fig 4A). In addition, comparing to preadipocytes, the CCNY protein level was elevated in the adipocytes that were differentiated from primary stromal vascular cells of the normal mice (Fig 4B). These results indicate that CCNY expression is positively associated with adipogenesis. Furthermore, the stromal vascular cells isolated from the adipose tissue of the Ccny KO mice also displayed impaired adipogenesis (Fig 4C), wherein the protein and mRNA levels of PPARγ, C/EBPα, and aP2 also declined (Fig 4D and 4E). Taken together, these results suggest that CCNY is required for the adipogenesis.

To further define the role of CCNY during adipogenesis, we used 3T3-L1 pre-adipocytes as an in vitro differentiation model [21]. The results showed that the CCNY expression in the
induced adipocytes was much higher than that in pre-adipocytes, and other adipogenesis-specific markers, such as PPARγ, C/EBPα, and aP2 were also elevated in the adipocytes (Fig 4F).

Next, we generated six stable Ccny knockdown 3T3-L1 cell lines through retrovirus-mediated shRNA targeting of six different regions of Ccny (see Materials and Methods). The Ccny knockdown efficiency was determined by the mRNA and protein levels of Ccny, with or without MDI induction (S4A and S4B Fig). These six stable cell lines and shuffle were then subjected to a standard adipogenic differentiation protocol. The oil red O staining assay showed that Ccny knockdown resulted in inhibition of the differentiation of 3T3-L1 pre-adipocytes (Fig 4G), suggesting that CCNY is needed for adipocyte differentiation.

Importantly, both the mRNA and protein levels of the middle and later adipogenesis-specific markers, including PPARγ, C/EBPα, and aP2, were all inhibited in the Ccny knockdown cells (S4C and S4D Fig), whereas the early adipogenesis-specific markers, including C/EBPα, C/EBPβ, Klf5 and Krox20, were not significantly changed in these cells (S4E Fig). These results suggest that CCNY does not function during the early stage of adipogenesis, although it is required for adipogenesis during the middle stage.

We further analyzed TFs that possibly interact with the promoter region of Ccny to regulate Ccny expression using a TF-prediction database (http://www.cbrc.jp/research/db/TFSEARCH.html), and found several potential TFs, including C/EBPα that is activated during the middle stage of adipogenesis [22]. Because Ccny was up-regulated during the middle stage of adipocyte differentiation (Fig 4D and 4E and S4C and S4D Fig), C/EBPα might be responsible for regulating Ccny expression. Therefore, we generated stable Cebpα knockdown cells and found that the CCNY expression level in the Cebpα knockdown cells was lower than that of the wild-type controls after the differentiation process (Fig 5A). In addition, a luciferase reporter assay showed that the Ccny transcription was elevated for the co-expression with C/EBPα but not C/EBPβ (Fig 5B). These results suggest that Ccny is a target of C/EBPα. Then, we performed chromatin immunoprecipitation (ChIP) assays to test whether C/EBPα could be directly bound to the endogenous Ccny promoter in 3T3-L1 cells during adipogenesis. The PPARγ promoter was detected at day 4 and peaked at day 8 after MDI induction; the Ccny promoter had similar binding activity (Fig 5C). However, these samples did not bind to the IgG antibody (Fig 5C). These results suggest that C/EBPα could directly bind to the Ccny promoter and activate the expression of Ccny during the middle stage of adipogenesis.

**CCNY is involved in insulin signaling and the regulation of its downstream targets**

The insulin signaling pathway plays an important role in regulating energy metabolism [23–25]. In the present study, we analyzed the insulin signaling pathway in stable Ccny knockdown HepG2 cells and differentiated 3T3-L1 cells using a retrovirus system and primary hepatocytes from Ccny KO mice. The results showed that the insulin-induced phosphorylation of AKT and GSK3β was partially inhibited in the Ccny knockdown HepG2 cells and primary hepatocytes from Ccny KO mice, but no such changes were detected in the 3T3-L1 adipocytes (Fig 6A and
suggested that CCNY is needed for the full function of the hepatic insulin signaling pathway. Furthermore, the insulin induced phosphorylation of AKT and GSK3β was decreased in the liver of the Ccny KO mice compared with that in the WT controls (Fig 6C and 6D).
Fig 6. Ccny deficiency inhibits the activation of insulin signaling. Stable Ccny knockout HepG2 cells, differentiated stable Ccny knockdown 3T3-L1 cells and primary hepatocytes of Ccny KO mice were stimulated with insulin for 15 min (HepG2, 100 nmol; 3T3-L1, 100 nmol; Hepatocytes, 10 nmol). (A) Western blot analysis of the phosphorylation of insulin receptor β (IRβ), AKT, and GSK3β. (B) The relative ratios of phosphorylated AKT and GSK3β were quantified (HepG2, from three independent experiments; 3T3-L1, from three independent experiments; Hepatocytes, n = 3). (C) Ccny KO mice and littermate controls (n = 2) of age 16 weeks were fasted overnight, IP injection of insulin (5U/kg body weight). The mice were humanely destroyed after 5 minutes and the liver, WAT and muscle were excised and used in western blotting analysis. (D) The relative ratios of phosphorylated AKT and GSK3β. *, P<0.05; **, P<0.01 WT vs Ccny KO, or con vs Ccny-Ri. Error bars, S. E.

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other hand, there were no significant differences in the insulin induced phosphorylation of AKT and GSK3β between the WAT and muscle of the Ccn y KO mice and the WT controls (Fig 6D). Taken together, these results suggest that CCNY involves in the insulin signaling pathway mainly in mouse liver.

Previous studies reported that insulin stimulation could activate the transcription of SREBP1 through the PI3K-AKT pathway [10]. We analyzed the protein level of SREBP1 in the stable Ccn y knockdown HepG2 cells and the primary hepatocytes from the Ccn y KO mice. The results showed that protein levels of the mature SREBP1 forms (nSREBP1) were lower in both Ccn y-deficient cells, whereas there was no significant difference in the levels of the SREBP1 precursor (Pre-SREBP1) between the Ccn y-deficient cells and the WT controls, with or without insulin stimulation (Fig 7A and 7B). Furthermore, we found that the insulin treatment significantly elevated the protein levels of nSREBP1 in the normal CCNY-expressing HepG2 cells or hepatocytes, whereas the insulin treatment did not increase the levels of nSREBP1 in both Ccn y-deficient cells (Fig 7B). Taken together, these results suggest that CCNY promotes the insulin-induced maturation of SREBP1 mainly in the liver cells.

Because fatty acid synthase (FASN) is a downstream target of SREBP1, we also analyzed the FASN expression under the same conditions. The results showed that the protein level of FASN in the HepG2 cells and the WT hepatocytes was significantly higher than that in both Ccn y-knockdown HepG2 cells and the hepatocytes of Ccn y KO mice (Fig 7A and 7B). We further analyzed the expression of FASN and SREBP in the liver of the Ccn y KO mice, and got the similar results (Fig 7A and 7B). In addition, we analyzed the mRNA levels of Fasn, stearoyl-CoA desaturase-1 (Scd-1) and L-pyruvate kinase (Lpk), which are downstream targets of SREBP1. The mRNA levels of these three enzymes in the Ccn y KO hepatocytes and liver tissue were all significantly lower than that in the WT hepatocytes and liver (Fig 7C). Taken together, these results suggest that CCNY is required for the activation of the insulin signaling pathway, functional insulin signaling results in the elevation of mature SREBP1, and SREBP1 up-regulates the expression of downstream genes such as FASN and SCD-1.

Discussion

CCNY involves in regulation of energy expenditure

Based on analyzing the metabolic states of the mice using metabolic cages, we showed that the Ccn y KO mice had a significant increase in food intake compared to that of the WT controls (S2E Fig). So we detected the level of neuropeptide-Y (NPY), which acts as the neurotransmitter involving the regulation of the food intake [26]. The result showed that the level of NPY in the Ccn y KO mice was a little bit higher than that in the WT controls, while this difference did not show statistical significance (S2F Fig).

Previous studies showed that NPY and other hormones involved in the regulation of activity or development of brown adipocytes [27–29], particularly, the knockdown of NPY expression promoted the development of brown adipocytes [27]. So we also analyzed BAT development of the mice, and found there was similar BAT weight between the WT and Ccn y KO mice (S1 Fig), which is consistent with the result there was no significant difference of NPY level between the Ccn y KO mice and the WT controls (S2F Fig). On the other hand, our results showed that the expression levels of UCP1 and its transcriptional coactivator Pgc1α were significantly higher in BAT of the Ccn y KO mice than that of the WT littermate controls (Fig 2). It is proposed that multi-factors from gut and brain participate in the regulation of the BAT activity [30], and the present data suggest that CCNY might also involve in regulating the BAT activity.
Since previous reports showed that the BAT thermogenesis is tightly correlated with the food intake in rodents [31,32], we propose that the up-regulation of BAT activity in the Ccny KO mice elevates energy expenditure and partially results in the increase of the food intake (S2E Fig). Taken together, increase of physical activity (S2D Fig) and BAT activity in the Ccny KO mice could generate the hyperphagic behaviors.
CCNY promotes differentiation of white adipocytes

The present study shows that the mRNA and protein levels of CCNY are up-regulated in adipocytes, both in vivo and in vitro (Fig 4A, 4B and 4F), and this increase might be induced by C/EBPα during the middle stage of 3T3-L1 pre-adipocyte differentiation (Fig 5). Moreover, the Ccny-deficient cells have a reduced capacity to differentiate into adipocytes, both in vivo and in vitro (Fig 4C and 4G). Therefore, these results indicate that CCNY plays a positive role in adipogenesis.

Because a number of cell cycle proteins are devoted to adipogenesis through regulation of clonal expansion during adipogenesis [33,34], we analyzed the CCNY effects on 3T3-L1 cell proliferation. There was no significant difference in the proliferation rate between the Ccny knockdown cells and the control cells at the exponential growth and clonal expansion stages (S5 Fig), indicating a cell-cycle independent role for CCNY during adipogenesis.

Since previous studies showed that CCNY was mainly located in the cell membrane [14–17], we suspect that CCNY regulates adipogenesis through its interaction with other adipogenesis-related signaling pathways. A previous report showed that CCNY in the membrane could activate Wnt signaling through phosphorylation of the co-receptor LRP6 [35]. Importantly, Wnt signaling is involved in the regulation of adipogenesis [36,37]. Therefore, future studies should pay attention to the relationship between CCNY and cell signaling pathways that regulate adipocyte differentiation.

CCNY mainly involves in regulating insulin signaling pathway of mouse liver

Insulin signaling controls metabolism of glucose and lipid in peripheral tissues of animals, particularly in the liver, white adipose tissue (WAT) and skeletal muscle. Importantly, insulin signaling regulates various components in those different tissues, e.g. insulin facilitates GLUT4 translocation from intracellular sites to the plasma membrane of fat or muscle cells, but promotes GLUT2 translocation in the liver cells [38]. The present study showed that insulin signaling pathway of both the Ccny-deficient HepG2 cells and the primary hepatocytes derived from the Ccny KO mice was impaired in response to the insulin stimulation, whereas no such defect of insulin signaling was detected in the Ccny-knockdown adipocytes (Fig 6A and 6B). The data that liver but not WAT and muscle from the Ccny KO mice presented insulin resistance (Fig 6C and 6D) further support this observation at the cell-level, suggesting that CCNY is mainly required for the functional insulin signaling in mouse liver. The future studies should focus on the molecular mechanism of CCNY regulating the function of insulin signaling in mouse liver, which might help us to understand how CCNY selectively functions in insulin signaling pathway of different peripheral tissues.

It is known that insulin signaling pathway controls the activity of a lipid regulator SREBP1 [8,39,40]. SREBP1 is regulated at three levels, the transcriptional level, the proteolytic cleavage level and the post-translational modification level [9, 41]. The present results indicate that CCNY in the hepatic cells or tissue regulates the mature forms of SREBP1 after proteolytic cleavage instead of regulating the expression level of pre-SREBP1 (Fig 7A and 7B). Because the administration of insulin only resulted in a significant increase in mature SREBP1 in CCNY-positive cells but not CCNY-deficient cells (Fig 7B), we propose that CCNY regulates the maturation of SREBP1 through activation of the insulin signaling pathway. This idea is supported by our present observations that CCNY deficiency resulted in partial inhibition of AKT and GSK3β (Fig 6A and 6B). However, the relationship between CCNY and insulin signaling requires further investigation. It is worth analyzing whether CCNY can co-localize with the insulin receptor and directly bind to members of the insulin signaling pathway. Although
insulin signaling was impaired in liver of the Ccny KO mice (Fig 6), the GTT and ITT showed no significant changes between the Ccny KO mice and the WT controls (Fig 1E and 1F). In addition, there was no difference in the fasting glucose and insulin levels between the Ccny KO mice and the WT controls (Fig 1D and 1E). Two identified features in the present study should be considered for this phenotype of normal glucose metabolism in the Ccny KO mice. Firstly, the CCNY-deficiency generated the impeded adipogenesis of WAT (Fig 1B and 1C; Fig 4), which might result in lean body mass (Fig 1B; S1 Fig) and resistant to the HFD treatment (Fig 3A and 3B; S4A and S4B Fig). Secondly, the CCNY-deficiency resulted in the up-regulation of BAT activity (Fig 2), which could elevate energy expenditure in the Ccny KO mice (S2 Fig), in addition to the increase of physical activity (S2D Fig).

In conclusion, the present studies indicate that CCNY might play diversified roles in the different peripheral tissues of mice, and the interactions among these CCNY-deficient peripheral tissues might compensate each other for regulating the glucose and lipid metabolism in the body.

Supporting Information

S1 Fig. The body fat content of Ccny KO mice is lower than that of WT mice.
(TIF)

S2 Fig. Elevated energy expenditure in Ccny KO mice.
(TIF)

S3 Fig. Female Ccny KO mice are resistant to high-fat diet (HFD)-induced obesity.
(TIF)

S4 Fig. Ccny knockdown deregulates the expression of adipogenesis marker proteins.
(TIF)

S5 Fig. Ccny knockdown has no influence on the cell cycle.
(TIF)

S1 Table. The primer pairs used in the text.
(DOCX)

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Author Contributions

Conceived and designed the experiments: WWA ZZZ JRW. Performed the experiments: WWA ZZZ YY. Analyzed the data: WWA JRW. Contributed reagents/materials/analysis tools: WWA LYZ YY. Wrote the paper: WWA JRW. Obtained permission for use of the Ccny KO mice: LYZ XLZ.

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