C1q/TNF-related protein 2 (CTRP2) deletion promotes adipose tissue lipolysis and hepatic triglyceride secretion

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The highly conserved C1q/TNF-related protein (CTRP) family of secreted hormones has emerged as important regulators of insulin action and of sugar and fat metabolisms. Among these, the specific biological function of CTRP2 remains elusive. Here, we show that the expression of human CTRP2 is positively correlated with body mass index (BMI) and is up-regulated in obesity. We used a knockout (KO) mouse model to determine CTRP2 function and found that Ctrp2-KO mice have significantly elevated metabolic rates and energy expenditure leading to lower body weights and lower adiposity. CTRP2 deficiency up-regulated the expression of lipolytic enzymes and protein kinase A signaling, resulting in enhanced adipose tissue lipolysis. In cultured adipocytes, CTRP2 treatment suppressed triglyceride (TG) hydrolysis, and its deficiency enhanced agonist-induced lipolysis in vivo. CTRP2-deficient mice also had altered hepatic and plasma lipid profiles. Liver size and hepatic TG content were significantly reduced, but plasma TG was elevated in KO mice. Both plasma and hepatic cholesterol levels, however, were reduced in KO mice. Loss of CTRP2 also enhanced hepatic TG secretion and contributed to impaired plasma lipid clearance following an oral lipid gavage. Liver metabolomic analysis revealed significant changes in diacylglycerols and phospholipids, suggesting that increased membrane remodeling may underlie the altered hepatic TG secretion we observed. Our results provide the first in vivo evidence that CTRP2 regulates lipid metabolism in adipose tissue and liver.

The global rise in obesity and associated comorbidities has sparked intense efforts to understand adipocyte biology (1, 2). White adipose tissue serves critical conserved functions as the major storage depot for excess fat and as a major source of numerous secretory proteins and hormones, collectively termed adipokines (3). Many of these adipokines (e.g. adiponectin, leptin, omentin, resistin, and RBP4) have endocrine roles in regulating food intake, sugar and fat metabolism, vascular homeostasis, and immune responses (4, 5). The circulating levels of many adipokines are frequently dysregulated in the context of obesity and diabetes (6).

Our recent efforts to better understand tissue crosstalk and endocrine control of metabolism led to the identification and characterization of a highly conserved family of secreted plasma proteins known as the C1q/TNF-related proteins (CTRP1–15) (7–13). Each of the C1q family members, currently comprising >30 members, each encoded by a distinct gene. All share a structurally homologous C-terminal globular C1q domain (14, 15). Each of the CTRP proteins has a distinct tissue expression profile and a few are highly expressed in adipose tissue (14). Genetic gain- and loss-of-function mouse models indicate overlapping and non-redundant metabolic functions for the C1q family members that have been characterized thus far (7, 8, 16–29).

Mouse Ctrp2 transcript is enriched in adipose tissue (12) and CTRP2 is mainly produced by stromal vascular compartment cells in adipose tissue. Its expression in adipose tissue is up-regulated in leptin-deficient (ob/ob) obese mice relative to lean controls (12). Of the C1q family members, CTRP2 shares one of the highest degrees of amino acid identity (43%) with adiponectin in the globular C1q domain. We previously showed that mouse CTRP2 can activate AMP-activated protein kinase (AMPK) and increase glycogen deposition and fat oxidation in cultured C2C12 myotubes (13). In a transgenic mouse model with elevated circulating plasma CTRP2, we observed an enhanced rate of lipid clearance from circulation following an acute lipid infusion (30). The level of locally produced CTRP2 in skeletal muscle is also elevated in aged rats (24 months old) compared with young rats (4 months old) (31). Aside from these limited studies, the biological function of CTRP2 in a normal physiological context is unknown.

To decipher the function and mechanisms of action of endogenous CTRP2, we used a genetic loss-of-function mouse model. This study provides the first evidence that CTRP2 is indeed a secreted regulator of lipid metabolism in vivo and is required for maintaining energy homeostasis.

**Results**

*Increased adipose expression of CTRP2 in human obesity*

Sequence alignments indicate a high degree of amino acid identity (94%) between full-length human and mouse CTRP2...
protein (Fig. 1A), suggesting conserved structure and function. We previously showed that Ctrp2 mRNA expression in visceral adipose tissue was significantly increased in C57BL/6J male mice fed a high-fat diet (HFD) for 12 weeks compared with mice fed a control low-fat diet (LFD) (30). To address whether adipose expression of CTRP2 is also altered in human obesity, we measured its mRNA levels in visceral (omental) and subcutaneous fat depots obtained from healthy lean controls as well as obese individuals with or without type 2 diabetes. Expression levels were normalized to 36B4 (also known as RPLP0) in each sample. *, p < 0.05; ***, p < 0.001. Error bars, S.E.

The abbreviations used are: HFD, high-fat diet; BMI, body mass index; BW, body weight; EE, energy expenditure; eWAT, epididymal white adipose tissue; GTT, glucose tolerance tests; HDL, high-density lipoprotein; ITT, insulin tolerance tests; iWAT, inguinal white adipose tissue; LDL, low-density lipoprotein; LFD, low-fat diet; LPL, lipoprotein lipase; OFTT, oral fat tolerance tests; PC, phosphatidylcholines; PE, phosphatidylethanolamines; RER, respiratory exchange ratio; T2D, type 2 diabetes; TG, triglyceride; TNF, tumor necrosis factor; VLDL, very low-density lipoprotein.
CTRP2 is required for lipid homeostasis

**A**

![Diagram](image)

**Figure 2. Ctrp2 KO mouse model.** A, schematic showing the gene targeting strategy used to generate Ctrp2 KO mice. B, PCR genotyping results showing the successful generation of WT (+/+), heterozygous (+/−), and homozygous (−/−) knock-out mice using the indicated primer pairs as shown in A. C, the absence of Ctrp2 mRNA in eWAT of KO mice was confirmed by real-time PCR using primer pairs specific for Ctrp2. Expression levels were normalized to β-actin in each sample. WT, n = 6; KO, n = 8. ND, not detected. *Errors bars* represent Mean ± S.E.

Expression of CTRP2 in both omental and subcutaneous fat depots was positively correlated with body mass index (BMI) in humans (Fig. 1, B and C). Compared with lean controls, CTRP2 expression was markedly up-regulated in both the omental and subcutaneous fat depots of obese individuals with and without type 2 diabetes (Fig. 1, D and E).

**Reduced body weight and increased energy expenditure in LFD- and HFD-fed Ctrp2 knockout mice**

A genetic loss-of-function mouse model was used to determine the metabolic role of CTRP2 in vivo (Fig. 2A). Two sets of primers were designed to amplify a sequence spanning intron 2 and exon 3 of the WT allele and a sequence spanning the downstream deletion site in the lacZ gene to confirm the genotype of WT and KO mice, respectively (Fig. 2B). As expected from targeted disruption of the gene, Ctrp2 mRNA was absent from the epididymal white adipose tissue (eWAT) of KO mice (Fig. 2C). Ctrp2 KO mice were born at the expected Mendelian ratio and appeared normal with no gross developmental abnormalities. Although Ctrp2 mRNA is expressed at low levels during embryonic development (12), its deletion is dispensable for mouse development.

To determine the contribution of CTRP2 to systemic energy metabolism in the normal and obese states, WT and Ctrp2 KO male and female mice were fed an HFD or a control LFD for 16 weeks, beginning at 5 weeks of age. In both LFD- and HFD-fed groups, we observed reduced body weight in male KO mice compared with WT littermates. However, there were no differences between female WT and KO mice on either diet (Fig. 3, A and B). For this reason, we chose to focus our metabolic analyses on male mice. In the LFD-fed group, Ctrp2 KO mice showed reduced whole-body fat mass accounting for the reduced body weight relative to WT littermates. However, in the HFD-fed group, reduced body weight of KO mice was because of a significant reduction in both fat and lean mass (Fig. 3C). No differences in food intake or total physical activity were seen between genotypes (Fig. 3, D and E). However, elevated metabolic rate, indicated by reduced respiratory exchange ratio (RER) and reflected by increased oxygen consumption rate ($V_{\text{CO2}}$) and increased fat oxidation, were observed in LFD-fed KO mice (Fig. 3, F and H). In contrast, increased CO₂ production rate ($V_{\text{CO2}}$) was only seen in HFD-fed KO mice (Fig. 3G). Accordingly, both LFD- and HFD-fed KO mice had significantly increased energy expenditure, and the effect was much more pronounced in the LFD-fed KO mice (Fig. 3, J and F). For this reason, we focused our functional studies on LFD-fed mice.

**Reduced adiposity and enhanced lipolysis in LFD-fed Ctrp2 KO mice**

Consistent with the Echo-MRI results, the weight and size of visceral (eWAT) and subcutaneous (iWAT) fat depots were markedly reduced in Ctrp2 KO male mice relative to WT littermates (Fig. 4, A–D). Histological evaluation of adipocyte morphology in eWAT showed no significant differences between genotypes (Fig. 4E). To determine whether altered lipolysis contributes to reduced adipose tissue weight, we examined the three key enzymes (ATGL, HSL, MGL) involved in triglyceride hydrolysis (32) in the fasted states, in which tissues were collected after food was removed for 4 h. Both mRNA and protein expression of these enzymes were significantly up-regulated in the KO mice (Fig. 4, F–H). In addition, phosphorylation of PKA substrates were also strikingly elevated in the KO mice, implicating enhanced PKA signaling in promoting adipose tissue lipolysis (Fig. 4F). Next, we tested whether stimulated in vitro lipolysis in response to β3-adrenergic receptor agonist (CL316243) was also elevated in the KO mice. Plasma free fatty acid (FFA) and glycerol levels were induced by CL316243 to a significantly greater extent in KO mice than in WT controls (Fig. 4, J and K). In differentiated 3T3-L1 adipocytes, forskolin can robustly increase cAMP accumulation and promote lipolysis (33). To test whether CTRP2 has a direct effect on forskolin-stimulated lipolysis, different concentrations of purified recombinant mouse CTRP2 protein were used to treat differentiated 3T3-L1 adipocytes. Both 5 μg/ml and 10 μg/ml of CTRP2 markedly suppressed adipocyte lipolysis in response to forskolin (Fig. 4L). Together, these results support a role for CTRP2 in regulating adipose tissue lipolysis in vitro and in vivo. In contrast to lipid metabolism, whole-body glucose metabolism was not appreciably affected by CTRP2 deficiency when mice were fed a control LFD; neither glucose nor insulin tolerance test results were different between genotypes (Fig. 4, M and N).

**Altered hepatic and plasma lipid profiles in LFD-fed Ctrp2 KO mice**

In the fasted state, plasma triglyceride (TG) levels were significantly higher in KO mice compared with WT littermates, and the TG content of very low-density lipoprotein (VLDL) particles was ~1.8-fold greater in the KO mice (Fig. 5, A and B). No differences in fecal TG content were noted between genotypes (Fig. 5C), indicating that elevated plasma TG levels could not be because of reduced fecal TG excretion. Although plasma
TG concentration was much higher, total plasma cholesterol was significantly reduced in KO mice (Fig. 5D). Cholesterol content was strikingly reduced in the low-density lipoprotein (LDL) and high-density lipoprotein (HDL) particle fractions (Fig. 5E). Fecal cholesterol content was also reduced, excluding fecal excretion as a cause of reduced plasma cholesterol levels (Fig. 5F). In striking contrast to the plasma lipid profile, the size of the mouse livers and hepatic TG content were both markedly reduced in Ctrp2 KO mice (Fig. 5G–J). Parallel to the reduction in plasma cholesterol, we also observed reduced hepatic cholesterol content in KO mice (Fig. 5K). In striking contrast to eWAT, the expression of lipolytic genes (Atgl, Hsl, Mgl) in liver was not different between WT and KO mice (Fig. 5L).

To better understand the altered TG metabolism observed in the KO mice, we performed oral fat tolerance tests (OFTT) on overnight-fasted mice to determine whether loss of CTRP2 impairs the ability of the mice to handle an acute lipid load. Following an oral lipid gavage, the Ctrp2 KO mice had a dramatic rise in serum TG compared with WT controls (Fig. 6A). When we administered lipid emulsion via intraperitoneal injection to bypass gut absorption, we still observed a significant increase in serum TG levels at 2 h post injection in the KO mice relative to WT controls (Fig. 6B), indicating that increased plasma TG following an acute lipid load is because of elevated hepatic VLDL-TG secretion or reduced lipid clearance from circulation by peripheral tissues. To test the former, we inhibited TG clearance from circulation in fasted mice by administering the nonionic surfactant poloxamer 407, an inhibitor of lipoprotein lipase (LPL) that blocks fatty acid uptake by peripheral tissues (34). In this case, circulating TG is mainly derived from VLDL-TG secreted by the liver. When lipid uptake was blocked in the fasted state, hepatic VLDL-TG secretion was significantly elevated in KO mice (Fig. 6C). LPL localized to the luminal surface of vascular endothelial cells is responsible for the bulk of TG clearance from circulation by hydrolyzing circulating TG to release FFAs for use in peripheral tissues. Heparin can be used to displace LPL from the vascular endothelium in mice and LPL activity can then be measured in post-heparin plasma. The postheparin plasma LPL activity was not significantly different between WT and KO mice (Fig. 6D). These experiments indicate that enhanced hepatic VLDL-TG secretion contributes to the marked increase in plasma TG levels in KO mice following an acute lipid load.

**Increased hepatic membrane remodeling in LFD-fed Ctrp2 KO mice**

To further examine alterations of metabolic pathways in the KO mouse liver, we performed unbiased metabolite profiling. In general, phospholipids, including phosphatidylcholines (PC) and phosphatidylethanolamines (PE), were significantly elevated, indicating increased phospholipid synthesis in the liver of KO mice (Fig. 7, A and B). Multiple intermediates of phos-
pholipid metabolism, including CMP, cytidine 5’-diphospho-
choline, glycerophosphorylcholine, cytidine 5’-diphosphoetha-
nolamine, and glycerophosphoethanolamine, were increased in
the livers of Ctrp2 KO mice (Fig. 7C). Numerous diacylglycerols
(also a precursor of phospholipids), such as palmitoyl-palmi-
toyl-glycerol, were significantly reduced in the livers of KO
mice, suggesting an increased utilization of these precursors in
phospholipid synthesis (Fig. 7D). Collectively, these data sug-
gest substantial cell membrane remodeling in the livers of
CTRP2-deficient mice (Fig. 7E) which may be needed for
enhanced VLDL assembly and TG secretion.

Discussion

Our findings provide insight into the biological function of
CTRP2, an enigmatic secretory protein highly conserved from
fish to human. The expression of CTRP2 was significantly up-
regulated in different fat depots of obese individuals relative to
healthy lean controls and was positively correlated with BMI.
This correlation prompted us to use a genetic mouse model to
assess the metabolic role of CTRP2 in a physiological context. It
should be noted that our human data are based on limited sam-
plies from mostly Caucasian subjects. Further confirmation is
needed in a larger cohort of human samples from multiple
racial and ethnic groups.

When fed either a control low-fat diet or a high-fat diet,
Ctrp2 KO male mice gained significantly less (~5 g less) body
weight relative to WT littermate controls. This body weight
phenotype appeared to be sex-dependent, as female mice lack-
ing CTRP2 were indistinguishable from WT littermates regard-
less of diet, reinforcing the importance of sex as a biological
variable contributing to phenotypic outcomes. Reduced body
weight and fat mass in male Ctrp2 KO mice was not because of
decreased food intake or increased physical activity; instead, it
was attributed to increased metabolic rate and energy expend-
Male mice lacking CTRP2 also had a lower respiratory exchange ratio, suggesting a preferential use of lipid substrates for oxidative metabolism. Loss of CTRP2 preferentially affected lipid, but not glucose, metabolism. Assessment of whole-body carbohydrate metabolism by glucose and insulin tolerance tests revealed no differences in glucose clearance and insulin sensitivity in Ctrp2 KO male mice fed a low-fat diet relative to WT littermates.

Several notable lipid phenotypes were observed in the Ctrp2 KO mice. In the absence of CTRP2, we observed enhanced expression of the major lipolytic enzymes (ATGL, HSL, and MGL) at both the mRNA and protein levels in adipose tissue. Whereas adipose tissue triglyceride lipase (ATGL) and hormone-sensitive lipase (HSL) expression are known to be regulated by anti-diabetic drugs, insulin, and stress hormones (e.g. glucocorticoids and ACTH) (35–40), much less is known about what signals regulate monoacylglycerol lipase (MGL) expression (41). Thus, the induction of all three lipolytic enzymes in KO mice suggests that CTRP2 may be involved in regulating the expression of these catabolic enzymes in adipose tissue. Mice lacking CTRP2 also had a lower respiratory exchange ratio, suggesting a preferential use of lipid substrates for oxidative metabolism. Loss of CTRP2 preferentially affected lipid, but not glucose, metabolism. Assessment of whole-body carbohydrate metabolism by glucose and insulin tolerance tests revealed no differences in glucose clearance and insulin sensitivity in Ctrp2 KO male mice fed a low-fat diet relative to WT littermates.

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Figure 6. Impaired lipid tolerance in LFD-fed Ctrp2 KO male mice. A, plasma TG levels measured at the indicated time points during OFTT. B, plasma TG measured during intraperitoneal fat tolerance test. C, plasma TG measured after administration of poloxamer 407 (an inhibitor of lipoprotein lipase activity) in overnight-fasted mice. D, LPL activity measured in post-heparin plasma. n = 6–8/group. **, p < 0.01; ***, p < 0.001. Error bars, S.E.

CTRP2 also had enhanced PKA signaling. Both increased expression of lipolytic enzymes, and PKA signaling contributed to the elevated adipose tissue lipolysis seen in the Ctrp2 KO mice in response to β3-adrenergic receptor agonist (CL316243) stimulation. Using cultured 3T3-L1 differentiated adipocytes in vitro, we demonstrated a direct role for CTRP2 in regulating TG hydrolysis. Treatment of adipocytes with recombinant CTRP2 robustly suppressed forskolin-induced lipolysis. Thus, our in vitro and in vivo data suggest that CTRP2 has anti-lipolytic action on adipocytes. In this context, the observed up-regulation of CTRP2 in obese human adipose tissue may be clinically relevant as obesity-linked insulin resistance promotes adipose tissue lipolysis (42). Interestingly, the related C1q family member adiponectin was also reported to inhibit basal and catecholamine-induced lipolysis in human and mouse adipocytes (43, 44), highlighting a shared biological function between the two related secretory proteins. Reduced fat mass seen in the KO mice could also be because of decreased lipid synthesis and/or increased fat oxidation in the adipose tissue. Assessment of a panel of lipid synthesis and oxidation genes in adipose tissue ruled out these possibilities (data not shown). Thus, lower adiposity in Ctrp2 KO mice is likely because of the combined effects of increased whole-body energy expenditure and elevated adipose tissue lipolysis.

In the absence of CTRP2, we observed a marked increase in fasting plasma TG and VLDL-TG levels in LFD-fed KO mice. This prompted us to examine whether CTRP2 is required for proper handling of an acute lipid load. We saw a striking impairment of lipid clearance from circulation following an oral lipid gavage in the Ctrp2 KO mice relative to WT littermates. Changes in plasma TG levels following an oral lipid load are generally attributed to TG associated with chylomicron and nonchylomicron fractions (chylomicron remnants, VLDL, and VLDL remnants) (45, 46). Because fecal TG content was not different between genotypes, we assumed that there was no difference in intestinal fat absorption or chylomicron secretion. Importantly, when we delivered the emulsified lipids via intraperitoneal injection to bypass gut absorption, we still observed significant differences in plasma TG levels in the Ctrp2 KO mice relative to WT controls. Therefore, increased plasma TG following lipid loading could be because of reduced lipid uptake into peripheral tissues or increased hepatic TG secretion in the form of VLDL-TG particles. LPL plays a critical role in lipid uptake from the circulation by peripheral tissues (47). We observed no differences in post-heparin LPL activity between WT and KO mice. In contrast, when we prevented tissue uptake of circulating TG by inhibiting LPL with poloxamer 407, we observed a marked increase in plasma TG in the Ctrp2 KO mice. The extent to which plasma TG rises in response to LPL inhibition (in the fasted state) largely reflects hepatic TG secretion in the form of VLDL-TG particles. Combined, our data suggest that CTRP2 modulates VLDL-TG secretion and that its absence promotes hepatic TG secretion leading to the elevated plasma TG and VLDL-TG seen in the KO mice.

Unlike the reciprocal changes in plasma and liver TG content, cholesterol levels in both liver and plasma were significantly reduced in Ctrp2 KO mice fed either a control low-fat diet or a high-fat diet. In mice, cholesterol turnover involved 1) cholesterol synthesis in hepatic and extrahepatic tissues, 2) cholesterol transport in plasma and uptake via LDL and HDL receptors, and 3) cholesterol excretion from the liver into the intestine in the form of bile acids (48). In LFD-fed mice, lower plasma cholesterol levels in KO mice were attributed to lower LDL-cholesterol and HDL-cholesterol. Because fecal cholesterol was significantly reduced in KO mice, this ruled out enhanced bile acid excretion as a cause of lower plasma cholesterol. Our data suggest that reduced hepatic cholesterol synthesis likely accounts, at least in part, for the lower plasma and liver cholesterol observed in the Ctrp2 KO mice. Alternatively, this phenotype could be because of reduced cholesterol uptake by the intestine in Ctrp2 KO mice.

Hepatic VLDL assembly and secretion can be significantly altered by de novo biosynthesis of phospholipids, such as PE and PC (49). PC is the major phospholipid component of VLDL and is located on the hydrophilic surface of the particles (50). Two pathways are involved in hepatic PC synthesis: 70% of total hepatic PC synthesis is derived from the CDP-choline pathway, whereas the remaining 30% is synthesized through the PE methylation pathway. Inhibiting PC biosynthesis by choline deprivation strongly reduces VLDL secretion in hepatocytes (51). Inhibiting PC synthesis via the PE methylation pathway also impairs the incorporation of lipids into VLDL in hepatocytes (52). In LFD-fed Ctrp2 KO mice, hepatic PC and PE levels were dramatically increased and diacylglycerol levels were reciprocally reduced. Because diacylglycerols provide the precursors for PE and PC synthesis, their reduction reflects greater consumption by the PC and PE synthesis pathways. Our metabolite profiling data suggest that increased membrane remodeling in
hepatocytes likely contributes to the enhanced postprandial VLDL-TG assembly and secretion seen in the Ctrp2 KO mice.

In summary, our study provides the first in vivo evidence that CTRP2 regulates lipid metabolism in adipose tissue and liver either directly or indirectly (Fig. 8). CTRP2 acted on adipocytes to suppress fat mobilization and played a role in restraining excessive TG hydrolysis. Although a direct action on liver still remains to be demonstrated, CTRP2 also appears to affect TG and cholesterol levels in liver, as well as membrane phospholipid turnover, thereby modulating systemic lipid profiles. Future studies will seek to identify the CTRP2 receptor (currently unknown) and signaling pathways engaged by CTRP2 to control fat metabolism in different organs and tissues.

Experimental procedures

Human tissue samples

Visceral (omenta) and subcutaneous adipose tissues were obtained from the Adipose Biology Core of the National Institutes of Health (NIH)-funded Mid-Atlantic Nutrition Obesity Research Center at the University of Maryland. Study protocols were approved by the Institutional Review Board for Human Subjects Research at the University of Maryland, and the studies abide by the Declaration of Helsinki principles.
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Table 1

| Gene       | Forward (5‘–3‘)                        | Reverse (5‘–3‘)                     |
|------------|----------------------------------------|------------------------------------|
| Human CTRP2| GTATAGACACTCCACCGGCCG                 | GAGGCTAGAGGCGCTCTACTCTG            |
| Human 36BP4 | AGCCGACACACTGTCCTC                    | ACTGAGATTCTCAAAGCTGCC              |
| Mouse CTRP2| GCCACCATGCTGTTGGAGGC                 | GCCAACAGTATTCTCCCTCTCTT            |
| Mouse β-Actin| AGGTGACGTTGAACTCCGTTA                 | GCCAACGACTGATTTCTCCCTCT            |
| Agl         | TGGGACCTCATCTCCCTCCTA                 | TGGTGATGGTTGACTGCT                |
| Hsl         | GCCACGCTGACTGACTGTT                    | GTGAACGGTAACTGCTCCAT              |
| Mgl         | CGGACTCCAGTTPGTTTGTGAG                | GACGCCAATGAGTGGAGCTG              |

informed consent was obtained from all human subjects. Type 2 diabetes (T2D) was determined by hemoglobin A1c values (>6.5% according to the World Health Organization criteria. Characteristics of the lean controls without type 2 diabetes (non-T2D), obese (non-T2D), and obese with T2D individuals have been described previously (16).

Knockout mouse model

The mouse strain used for this research project, B6;129S5-C1qtnf2-tm1Lex/Mmcd (stock number 032165-UCD), was obtained from the Mutant Mouse Regional Resource Center (MMRRC), a National Center for Research Resources funded strain repository, and was donated to the MMRRC by Genentech, Inc. To generate Ctrp2 KO mice, the entire exon 3 (260 bp on chromosome 11) was replaced with targeting cassette containing the β-gal reporter gene, lacZ. Genotyping primers for the WT allele were WT-F (5’-CAGTGGAGAAGAAGGTAAAGGAC-3’) and WT-R (5’-TACACAGGTAGTGATGAGT-GCTAGC-3’). The expected size of the WT band was 121 bp. Genotyping primers for the KO allele were KO-F (5’-CGCAGC-GACATCGGCTCTTATC-3’) and KO-R (5’-GATGACTCTTCTCAGAACATCC-3’). The expected size of the KO band was 543 bp. The absence of Ctrp2 mRNA resulting from the Ctrp2 gene disruption was confirmed by real-time PCR. The primers used to confirm the presence of Ctrp2 mRNA are listed in Table 1. Ctrp2 KO mice were initially generated on a mixed (129/Sv × C57BL/6) genetic background. We backcrossed the mice onto a C57BL/6 genetic background for more than six generations. For each cohort of mice, at least 10 breeding sets (1 heterozygous male and 2 heterozygous female mice) were set up at the same time. Only homozygous KO male and female mice (+/−) and WT littermate (+/+ controls with close dates of birth were used in our experiments. From 5 weeks of age, Ctrp2 KO and WT male and female mice were fed either a low-fat diet (10% kcal derived from fat, D12450B; Research Diets) or a high-fat diet (60% kcal derived from fat, D12492; Research Diets), and body weights were measured weekly. All mice were housed in polycarbonate cages under a 12:12-h light-dark photocycle and had access to water ad libitum throughout the study period. All animal experiments were approved by the Animal Care and Use Committee at the Johns Hopkins University School of Medicine (protocol no. MO16M431).

Body composition analysis

Body composition of Ctrp2 KO and WT mice (18 weeks old) was determined using a quantitative NMR instrument (Echo-MRI-100, Echo Medical Systems LLC) at the Johns Hopkins University School of Medicine mouse phenotyping core facility. Echo-MRI analyses measure total fat mass, lean mass, and water content.

Indirect calorimetry

Ctrp2 KO and WT mice fed an LFD or an HFD for 20 weeks were used for simultaneous assessments of daily body weight change, food intake (corrected for spillage), physical activity, and whole-body metabolic profiling in an open flow indirect calorimeter (Comprehensive Laboratory Animal Monitoring System (CLAMS), Columbus Instruments). Data were collected for 3 days to confirm that mice were acclimated to the calorimetry chambers (indicated by stable body weights, food intake, and diurnal metabolic patterns), and data were analyzed from the fourth day onward. Rates of oxygen consumption (V O2) and carbon dioxide production (V CO2) in each chamber were measured throughout the study. Respiratory exchange ratio (RER = V CO2/V O2) was calculated by CLAMS software (version 4.02) to estimate the relative oxidation of carbohydrates (RER = 1.0) versus fats (RER = 0.7), not accounting for protein oxidation. Energy expenditure (EE) was calculated as EE = V O2 × (3.815 + (1.232 × RER)). Physical activity was measured by IR beam breaks in the metabolic chamber. Average metabolic values were calculated per subject and averaged across subjects for statistical analysis.

Histological analysis of adipose tissue and liver

eWAT, iWAT, and liver from WT and KO mice (40 weeks old, fasted for 4 h) were removed and weighed after the mice were anesthetized with isoflurane. Formalin-fixed, paraffin-embedded tissues were stained with H&E at the Johns Hopkins University School of Medicine Reference Histology Laboratory. Images were captured with an AxioPlan upright microscope and an AxioCam color CCD camera (Carl Zeiss Microscopy).

RNA isolation and real-time PCR

Total RNA was isolated from tissue using TRIzol® Reagent and 2 μg of RNA was reverse-transcribed using GoScript™ Reverse Transcriptase (Promega). 1 ng/μl of cDNA from each sample was used in real-time PCR analysis using SYBR Green PCR Master Mix on a CFX Connect™ system (Bio-Rad). Results were analyzed using the 2–ΔΔCT method (53). Primer sequences are listed in Table 1.

Western blot analysis

Mouse tissues were homogenized in lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 0.5% Nonidet P-40, and 10% glycerol) containing PhosSTOP phosphatase inhibitor mixture (Roche Applied Science) and Protease Inhibitor mixture.
Liver and fecal TG and cholesterol measurements

After liver tissue (≈50 mg) was sonicated in 0.5-ml solution (5% Triton X-100 in water), the samples were slowly heated to 95 °C in a water bath until the Triton X-100 solution became cloudy and then cooled down to room temperature. The heating was repeated one more time to solubilize all TG. TG and cholesterol were measured using an Infinity™ kit (Thermo Fisher Scientific) after the samples were centrifuged at 20,000 × g for 2 min to remove any insoluble material and diluted 10-fold with dH2O for measurement. For the analysis of fecal lipids, feces were collected from mice housed individually over a 24-h period, cleaned, and dried for 1 h at 70 °C. 100 mg aliquots were added to 500 µl saline and homogenized. Then, 200 µl of sample was incubated with 1 ml of chloroform-methanol (2:1) for 30 min at 60 °C with constant agitation and centrifuged for 10 min at 1000 × g. The lower chloroform phase was then transferred to a new tube, and the sample was evaporated to dryness. Samples were then resuspended in 500 µl 1% Triton X-100 in pure chloroform, evaporated to dryness, and finally resuspended in 500 µl of water. TG and cholesterol were measured using an Infinity™ kit (Thermo Fisher Scientific) using standards diluted with 1% Triton X-100 in water.

Glucose and insulin tolerance tests

LFD-fed Ctrp2 KO male mice and WT littermates (22 weeks old) were subjected to intraperitoneal glucose tolerance tests (GTT) and insulin tolerance tests (ITT). For GTT, mice were fasted overnight before injection with glucose (1 g/kg BW). For ITT, food was removed 2 h before insulin injection (1 units/kg BW). Blood glucose was measured at the indicated time points using a glucometer (BD Pharmaningen).

Lipid tolerance test

For OFTT, overnight-fasted mice were given an olive oil gavage (5 ml/kg BW) and blood was collected for TG measurement. For intraperitoneal fat tolerance testing, overnight-fasted mice were injected i.p. with 5 ml/kg BW of 20% (v/v) emulsified intralipid (Sigma), and blood was collected for TG measurement. To measure hepatic TG production, overnight-fasted mice were injected with 1 g/kg BW poloxamer 407 and blood was collected at the indicated time points.

Metabolomic analysis

Liver samples from LFD-fed WT and KO male mice were sent to Metabolon, Inc. (Durham, NC) for metabolomic profiling analysis as described previously (54).

Statistical analysis

Spearman’s correlation coefficient analysis was used to analyze the associations between adipose expression of CTRP2 and BMI. Kruskal–Wallis analysis of variance with pairwise comparisons was used to determine differences among the three groups (lean, obese without T2D, and obese with T2D). Two-way analysis of variance (ANOVA) was used to compare differ-
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ences in body weight over time, energy expenditure, and lipid/glucose/insulin tolerance tests between WT and KO mice. Other comparisons were made using either a two-tailed Student’s t test for two groups or one-way ANOVA for multiple groups. Values are reported as mean ± S.E. p < 0.05 was considered statistically significant.

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