Targeted deletion of C1q/TNF-related protein 9 increases food intake, decreases insulin sensitivity, and promotes hepatic steatosis in mice

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1Department of Physiology, Johns Hopkins University School of Medicine, Baltimore, Maryland; 2Department of Neuroscience, Johns Hopkins University School of Medicine, Baltimore, Maryland; and 3Center for Metabolism and Obesity Research, Johns Hopkins University School of Medicine, Baltimore, Maryland

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Wei Z, Lei X, Petersen PS, Aja S, Wong GW. Targeted deletion of C1q/TNF-related protein 9 increases food intake, decreases insulin sensitivity, and promotes hepatic steatosis in mice. Am J Physiol Endocrinol Metab 306: E779–E790, 2014. First published January 28, 2014; doi:10.1152/ajpendo.00593.2013.—Transgenic overexpression of CTRP9, a secreted hormone downregulated in obesity, confers striking protection against diet-induced obesity and type 2 diabetes. However, the physiological relevance of this adiponectin-related plasma protein remains undefined. Here, we used gene targeting to establish the metabolic function of CTRP9 in a physiological context. Mice lacking CTRP9 were obese and gained significantly more body weight when fed standard laboratory chow. Increased food intake, due in part to upregulated expression of hypothalamic orexigenic neuropeptides, contributed to greater adiposity in CTRP9 knockout mice. Although the frequency of food intake remained unchanged, CTRP9 knockout mice increased caloric intake by increasing meal size and decreasing satiety ratios. The absence of CTRP9 also resulted in peripheral tissue insulin resistance, leading to increased fasting insulin levels, impaired hepatic insulin signaling, and reduced insulin tolerance. Increased expression of lipogenic genes, combined with enhanced caloric intake, contributed to hepatic steatosis in CTRP9 knockout mice. Loss of CTRP9 also resulted in reduced skeletal muscle AMPK activation and mitochondrial content. Together, these results provide the genetic evidence for a physiological role of CTRP9 in controlling energy balance via central and peripheral mechanisms.

C1QTNF-RELATED PROTEINS (CTRP1–15) are a family of 15 secreted proteins of the C1q family (27). Each possesses the signature globular C1q domain, and most are found circulating in the blood as potential endocrine hormones (26, 34–39). Several CTRPs have been shown recently to have salutary metabolic (2, 17, 18, 19, 26, 33–35, 37, 38), cardioprotective (7, 29, 30), and vasculoprotective (32) functions.

Of the CTRPs, CTRP9 is the closest paralog of adiponectin, an anti-diabetic adipokine with pleiotropic functions (6, 31). At the sequence level, CTRP9 and adiponectin share 54% amino acid identity in the globular domain (37). They also share the following multiple common biochemical features: predominant expression by adipose tissue, circulation in plasma as endocrine hormones, formation of higher-order oligomeric complexes, similar posttranslational modifications that include proline hydroxylation and lysine glycosylation, and downregulated plasma levels in obesity. Although the metabolic function and regulation of adiponectin is well described (6, 31), the role of CTRP9 in regulating metabolic and cardiovascular functions has only begun to be elucidated (7, 19, 29, 32, 37).

Recent CTRP9 studies have employed the use of recombinant protein administration, adenosinergic-mediated overexpression, and/or transgenic overexpression in mice. In this context, CTRP9 has the remarkable ability to protect mice against metabolic insulin from high-fat feeding (19), cardiac injury induced by ischemia-reperfusion (7, 29), and vascular injury induced by steel wire (32). Whether and how CTRP9 regulates energy homeostasis in a physiological context has not been established. Therefore, we addressed these questions using a loss-of-function mouse model.

EXPERIMENTAL PROCEDURES

CTRP9 knockout mice. The CTRP9 knockout (KO) mouse strain was generated from C57BL/6-derived embryonic stem (ES) cells with a targeted deletion of Ctrp9/C1qtnf9. The ES clone was obtained from the National Institutes of Health-funded Knock-Out Mouse Project (KOMP) repository, and CTRP9 KO mice were generated by VeloGene (Regeneron, Tarrytown, NY). Genotyping primers for the Ctrp9 wild-type (WT) allele were: forward, 5′-CCCCAGATGCAC-CATATAAATTGC-3′; and reverse, 5′-CTCTTGCACTGGGCTT-CACAGAGG-3′. Primers for the Ctrp9 null allele were provided by KOMP: sequence specific upstream (SU), 5′-CCTTCTCTGATGTCGCT-GTGG-3′; and LacInZRev, 5′-GTCTTCTAGTCTCT-CCTAGG-3′. CTRP9 KO mice were generated on a C57BL/6 genetic background, and no back-crossing was necessary. Male and female CTRP9 KO mice and WT littermate controls were housed in polycarbonate cages on a 12:12-h light-dark photocycle and had access to water and ad libitum throughout the study period. Unless otherwise noted, mice were fed an ad libitum standard laboratory chow diet (chow, 18% kcal from fat, 2018SX; Teklad Global Rodent Diets) or a high-fat diet (HFD; 60% kcal from fat, D12492; Research Diets). All animal protocols were approved by the Institutional Animal Care and Use Committee of The Johns Hopkins University School of Medicine.

Antibodies and chemicals. Mouse monoclonal anti-FLAG M2 antibody was obtained from Sigma-Aldrich (St. Louis, MO). Rabbit antibodies that recognize phospho-Akt (Thr308) and Akt were obtained from Cell Signaling Technology (Beverly, MA). Anti-CTRP9 antibody was developed as described previously (37). Recombinant insulin was obtained from Sigma-Aldrich.

Western blot. For CTRP9 expression analyses in male WT and CTRP9 KO mice, epididymal fat pads were homogenized and prepared in tissue protein extraction buffer (Thermo Scientific, Waltham, MA) supplemented with protease inhibitors (Roche Applied Science, Indianapolis, IN). For insulin-signaling analyses, animals were fasted for 7 h before being given an intraperitoneal (ip) injection of insulin (1 U/kg body wt). Muscle, liver, and epididymal adipose tissues were obtained and processed immediately in ice-cold tissue protein extraction buffer (Thermo Scientific) containing protease inhibitors (Roche

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E779
Table 1. Real-time PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5' → 3')</th>
<th>Reverse Primer (5' → 3')</th>
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<td>Agrp</td>
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<td>CAGAAGTTAGACCTGGAGACTCT</td>
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<td>Ctrp12</td>
<td>CGATTACAGGCCGGAGGAC</td>
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Agrp, agouti-related protein; Cart, cocaine- and amphetamine-regulated transcript; Npy, neuropeptide Y; Pomc, proopiomelanocortin; Acc, acetyl-CoA carboxylase; Fasn, fatty acid synthase; Srebplc, sterol regulatory element-binding protein-1c; Ctrp1, -2, and -3, C1q/tumor necrosis factor-related protein 1, 2, and 3, respectively.
circulating CTRP9 levels are lean when fed a low-fat diet and resist weight gain when metabolically challenged with an HFD (19). Male CTRP9 KO mice gained significantly more body weight compared with WT controls when fed a standard chow diet (Fig. 2A). At the end of the study, male CTRP9 KO mice averaged 43 ± 1.5 g, and male WT mice averaged 32.7 ± 1.23 g in weight, representing a 31.5% difference in body weight between males of different genotypes (Fig. 2A, left). Female CTRP9 KO mice also gained more body weight compared with WT controls, although differences in body weight became apparent only at ~9 mo of age (Fig. 2A, right). At the end of the study, female CTRP9 KO mice averaged 28.9 ± 1.3 g, and female WT controls averaged 25.9 ± 0.9 g in weight, representing an 11.6% difference in body weight between female mice. These results indicate that CTRP9 plays a role in body weight regulation. Since female CTRP9 KO mice had a modest weight gain compared with WT controls, subsequent metabolic analyses focused on male CTRP9 KO mice.

To examine whether CTRP9 KO mice had increased lean and fat mass, whole body composition analyses were performed. EchoMRI analyzed chow-fed WT and CTRP9 KO mice at 36 wk of age. Male WT mice had 6.26 ± 0.67 g of total body fat mass, whereas CTRP9 KO mice had 11.45 ± 1.33 g of total body fat mass. Lean mass also increased slightly from 19.89 ± 0.22 g in WT controls to 22.30 ± 0.72 g in CTRP9 KO mice. When tissue mass was normalized to body weight, CTRP9 KO mice had an increase in fat mass by ~10% and a decrease in lean mass by ~8% (Fig. 2B, right). These results indicate that body weight differences between WT and CTRP9 KO mice can be attributed to differences in fat mass.

Effects of HFD on CTRP9 KO mice. Because CTRP9 KO mice gained more weight on a chow diet, we investigated how these animals handle metabolic stress induced by high-fat feeding. Surprisingly, when CTRP9 KO mice were fed an HFD, male and female mice gained weight at a rate similar to WT controls (Fig. 2C). At 41 wk of age, male CTRP9 KO mice averaged 52.4 ± 0.5 g, and male WT controls averaged 51.4 ± 0.8 g in weight. At 45 wk of age, female CTRP9 KO mice averaged 37.4 ± 4.0 g, and female WT controls averaged 38.5 ± 1.3 g in weight. Consistently, no differences were observed in lean or fat mass between HFD-fed WT and CTRP9 KO mice (Fig. 2D). These results indicate that body weight and adiposity of WT and CTRP9 KO mice are dependent on diet. For this reason, all subsequent analyses used chow-fed male mice.

Altered food intake and meal size in CTRP9 KO mice. Differences in body weight and adiposity between chow-fed WT and CTRP9 KO mice prompted us to examine food intake. Real-time cumulative food intake measurements in the CLAMS revealed that CTRP9 KO mice consumed more chow and that most of the increased food intake occurred in the second half of the dark phase of the photocycle (Fig. 3, A and B). No difference in total food intake in the light phase was observed (Fig. 3B).

We next analyzed meal patterns by defining a meal as the consumption of 0.04 g or more of chow and requiring the interval between two meals to exceed 10 min. A food intake event was considered a meal only when both criteria were met. CTRP9 KO mice consumed more chow per meal (Fig. 3C). The average meal size over a 24-h period was 0.14 ± 0.002 g for WT and 0.21 ± 0.016 g for CTRP9 KO mice. Differences in meal size were more pronounced when the analysis was restricted to chow consumed during the dark phase of the photocycle, where the average meal size was 0.16 ± 0.007 g for WT and 0.26 ± 0.04 g for CTRP9 KO mice. When meal number was analyzed over 24 h, no difference between WT and CTRP9 KO mice was observed during either the light or dark phase of the photocycle (Fig. 3D). Time between consecutive meals, or intermeal interval, also did not differ between WT and CTRP9 KO mice over a 24-h period (Fig. 3E). However, CTRP9 KO mice had a decreased satiety ratio, defined as intermeal interval divided by meal size (min/g), during the dark phase of the photocycle (Fig. 3F); these results are consistent with increased chow consumption and meal size during this period. Together, these results

Fig. 1. Generation of C1q/TNF-related protein 9 (CTRP9) knockout (KO) mice. A: schematic showing the strategy for generating CTRP9 KO mice by targeted deletion of the mouse Ctrp9 allele and replacement with a lacZ reporter and a neomycin resistance cassette. B: genotyping results indicate the successful generation of wild-type (WT), heterozygous, and homozygous KO alleles using the primer set (forward and reverse arrows) indicated in A. C: Western blot analysis of white adipose tissue lysates from WT and KO mice indicates the absence of detectable CTRP9 in KO mice. BacVec, bacterial artificial chromosome vector.
indicate that increased food intake in mice lacking CTRP9 resulted from increased meal size and decreased satiety.

Altered expression of orexigenic neuropeptides in the hypothalami of CTRP9 KO mice. To explore the molecular mechanism responsible for increased food intake, we examined the expression of appetite-regulating neuropeptides in the hypothalami of CTRP9 KO mice. Quantitative real-time PCR analyses revealed that hypothalamic expression of neuropeptide Y (Npy) and agouti-related peptide (Agrp), two orexigenic neuropeptides that promote food intake (25), was upregulated in CTRP9 KO mice relative to WT controls (Fig. 3G). In contrast, hypothalamic expression of cocaine- and amphetamine-regulated transcript (Cart) and proopiomelanocortin (Pomc), two anorexigenic neuropeptides that suppress food intake, did not differ significantly between WT and CTRP9 KO mice (Fig. 3G). However, Cart and Pomc had an increased trend in CTRP9 KO mice, which may represent a compensatory response to increased food intake and the consequent positive energy balance (25). These results suggest that CTRP9 regulates the hypothalamic expression of neuropeptides to modulate food intake, and its absence in CTRP9 KO mice.
Fig. 3. Ingestive behavior of chow-fed CTRP9 WT and KO mice. A: real-time cumulative food intake in WT and KO mice over a 24-h period (n = 4–5; 35-wk-old mice). Error bars (SE) are indicated by the thickness of the line. B: food intake of WT and KO mice (n = 4–5) over a 24-h period or in the dark or light phase of the photocycle. C–F: meal size (C), meal number (D), intermeal interval (E), and satiety ratio (F) of WT and KO mice (n = 4–5) over a 24-h period or in the dark or light phase of the photocycle. G: real-time PCR analyses of neuropeptide gene expression in the hypothalami of WT and KO mice (n = 4–5). Expression levels were normalized to 18S rRNA. Statistical analyses were performed using 2-tailed Student’s t-test. All data are expressed as means ± SE. *P < 0.05; **P < 0.01; #P < 0.001. Agrp, agouti-related peptide; Pomc, proopiomelanocortin; Npy, neuropeptide Y; Cart, cocaine- and amphetamine-regulated transcript.
Fig. 4. Energy expenditure (EE) and physical activity levels of chow-fed WT and CTRP9 KO mice. A–D: oxygen consumption (V\textsubscript{O\textsubscript{2}}; A), CO\textsubscript{2} production (V\textsubscript{CO\textsubscript{2}}; B), respiratory exchange ratio (RER; C), and EE (D) of WT and KO mice (n = 4–5; 35-wk-old mice) over a 24-h period in the dark and the light phases of the photocycle. E: ambulatory activity levels of WT and KO mice (n = 4–5) over a 24-h period. Inset indicates the breakdown of ambulatory activities of WT and KO mice over a 24-h period in the dark and light phases of the photocycle. F: total physical activity levels of WT and KO mice (n = 4–5) over a 24-h period. Inset indicates the breakdown of total physical activity levels of WT and KO mice over a 24-h period in the dark and light phases of the photocycle.
animals promotes food intake due to altered expression of orexigenic neuropeptides.

When HFD-fed WT and CTRP9 KO mice were examined for ingestive behavior, no differences were observed in food intake, meal size, meal number, or satiety ratio (data not shown). Appetite-regulating gene expression (Npy, Agrp, Pomc, and Cart) was also unchanged in the hypothalami of HFD-fed WT and CTRP9 KO mice (data not shown). These results correlate with the absence of body weight differences between HFD-fed WT and CTRP9 KO mice.

EE and physical activity of CTRP9 KO mice. To uncover potential additional mechanisms by which chow-fed CTRP9 KO mice demonstrated increased body weight, we conducted indirect calorimetry of WT and CTRP9 KO mice. VO₂ (Fig. 4A), VCO₂ (Fig. 4B), and EE (Fig. 4D) did not differ between WT and CTRP9 KO mice when these parameters were normalized to lean body mass. Similarly, these measurements did not differ by genotype during the light or dark phases of the photocycle (Fig. 4, A, B, and D). Further, the RER was similar between the WT and CTRP9 KO mice (Fig. 4C), reflecting similar fuel mixture catabolism.

Ambulatory (horizontal plane movements) and total physical activity levels did not differ significantly between WT and CTRP9 KO mice (Fig. 4, E and F), although 24-h cumulative levels had a decreased trend in CTRP9 KO mice (Fig. 4, E and F, insets). When HFD-fed WT and CTRP9 KO mice were subjected to similar indirect calorimetry analyses, we also observed no differences in any metabolic parameters (data not shown). These data indicate that the absence of CTRP9 in mice had no discernable impact on EE or physical activity levels.

CTRP9 KO mice are insulin resistant. We next addressed whether the obese phenotype of chow-fed CTRP9 KO mice affects whole body glucose metabolism and insulin sensitivity. Fasting (7-h) glucose levels did not differ between WT and CTRP9 KO mice (Fig. 5A); however, fasting serum insulin levels increased twofold in CTRP9 KO mice compared with WT controls (Fig. 5B). GTT assessed the ability of WT and CTRP9 KO mice to handle a glucose challenge and showed no difference in glucose disposal rate over time (Fig. 5C). However, CTRP9 KO mice had much higher insulin levels throughout the GTT (Fig. 5D), suggesting that CTRP9 KO mice had decreased insulin sensitivity.

Therefore, whole body insulin sensitivity was assessed with HOMA-IR (13). HOMA-IR revealed increased insulin resistance in CTRP9 KO mice relative to WT controls (Fig. 5E). To confirm these results, insulin tolerance test (ITT) demonstrated

![Graphs showing glucose and insulin levels](image-url1)

**Fig. 5.** Whole body glucose metabolism in chow-fed male WT and CTRP9 KO mice. A and B: fasting blood glucose (A) and serum insulin levels (B) of WT and KO mice (n = 4–5). Male mice (31 wk old) were fasted for 7 h prior to blood glucose measurements. C: blood glucose levels of WT and KO mice (n = 4–5) during the glucose tolerance test (GTT). D: serum insulin levels of WT and KO mice during GTT (n = 4–5). E: homeostasis model assessment of insulin resistance (HOMA-IR) index of WT and KO mice (n = 4–5). F: blood glucose levels of WT and KO mice (n = 4–5) during the insulin tolerance test (ITT). All data are expressed as means ± SE. *P < 0.05; **P < 0.01.
E786

CTRP9 MODULATES FOOD INTAKE AND ENERGY BALANCE

that CTRP9 KO mice have decreased glucose clearance rates in peripheral tissues in response to insulin, resulting in higher blood glucose levels during the test (Fig. 5F). These data indicate that CTRP9 KO mice were insulin resistant, which is likely a reflection of increased adiposity. To compensate for peripheral insulin resistance, CTRP9 KO mice increased fasting insulin levels and insulin secretion in response to glucose challenges.

**CTRP9 KO mice have impaired insulin signaling in peripheral tissues.** To more closely assess the insulin resistance of CTRP9 KO mice, we examined insulin signaling in three major metabolic tissues: adipose, skeletal muscle, and liver. Chow-fed WT and CTRP9 KO mice were fasted (7 h) and then injected ip with a bolus of insulin (1 U/kg body wt). Insulin robustly stimulates the phosphorylation of serine/threonine protein kinase B/Akt (12). Administration of insulin increased Akt (Thr308) phosphorylation in the adipose tissue of WT and CTRP9 KO mice to the same extent (Fig. 6, A and B). These results indicate that insulin signaling is largely unaffected in the adipose tissue of CTRP9 KO mice. Similarly, insulin signaling, as judged by Akt phosphorylation, remained largely intact in CTRP9 KO skeletal muscles (Fig. 6, C and D). In contrast, insulin-stimulated Akt phosphorylation was decreased in CTRP9 KO livers (Fig. 6, E and F), suggesting that hepatic insulin signaling was impaired; whereas insulin-induced Akt phosphorylation increased fivefold in WT livers, this phosphorylation increased only 1.3-fold in CTRP9 KO livers. These results indicate that the liver, but not adipose tissue or skeletal muscle, of CTRP9 KO mice exhibited insulin resistance.

**CTRP9 KO mice develop hepatic steatosis.** Since CTRP9 is expressed predominantly by adipose tissue (37), we explored whether CTRP9 KO mice had an altered adipose tissue morphology. Tissue sections from the epididymal adipose depot of chow-fed WT and CTRP9 KO mice (43 wk old) revealed similar adipocyte cell size (Fig. 7A). Given the presence of hepatic insulin resistance, we also histologically stained liver tissue sections of WT and CTRP9 KO mice. H & E stained sections revealed the presence of lipid droplets, indicated by vacuolar space, in WT livers (Fig. 7B, left). CTRP9 KO livers revealed pronounced accumulation of lipid droplets (Fig. 7B, right), which likely reflect the increased body weight and adiposity of these mice.

**Lipid profiles of WT and CTRP9 KO mice.** The presence of steatosis in CTRP9 KO mice led us to examine their serum lipid profiles. WT and CTRP9 KO mice had similar levels of serum NEFA (Fig. 8A). Serum TG levels were similar between WT and CTRP9 KO mice, with a trend toward increased serum TG in CTRP9 KO mice (Fig. 8B). Although liver weight was comparable between both groups (Fig. 8C), CTRP9 KO mice had substantially greater hepatic TG content (Fig. 8D). Whereas WT liver had 34.88 ± 5.67 mg/g tissue of TG, CTRP9 KO liver had 52.33 ± 6.20 mg/g tissue of TG; this change represents a nearly 50% increase in hepatic TG content of CTRP9 KO mice compared with WT controls.

We then performed quantitative real-time PCR to assess whether the expression of lipid synthesis genes was altered in CTRP9 KO mice. Hepatic expression of Srebp-1c, a major transcriptional activator of lipogenic gene expression (4), was increased in CTRP9 KO mice (Fig. 8E). Hepatic expression of

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Fig. 6. Insulin signaling in adipose, skeletal muscle, and liver tissues of chow-fed WT and CTRP9 KO mice. WT and KO mice (38 wk old) were fasted for 7 h and then injected ip with a bolus of insulin (1 U/kg body wt). A: epididymal adipose tissues were collected, and baseline or insulin-stimulated phosphorylation of Akt (Ser308) from WT and KO mice (n = 4) was analyzed by Western blot. Each lane represents a sample from an individual mouse. B: quantification of phosphorylation of Akt (Ser308) in epididymal adipose tissues of WT and KO mice (n = 4) was analyzed by Western blot. Each lane represents a sample from an individual mouse. C: skeletal muscle tissues were collected, and baseline or insulin-stimulated phosphorylation of Akt (Ser308) from WT and KO mice (n = 4) was analyzed by Western blot. Each lane represents a sample from an individual mouse. D: quantification of phosphorylation of Akt (Ser308) in skeletal muscle tissues of WT and KO mice. E: liver tissues were collected, and baseline or insulin-stimulated phosphorylation of Akt (Ser308) from WT and KO mice (n = 4) was analyzed by Western blot. Each lane represents a sample from an individual mouse. F: quantification of phosphorylation of Akt (Ser308) in liver tissues of WT and KO mice. All data are expressed as means ± SE. *P < 0.05; #P < 0.001.
acetyl-CoA carboxylase (Acc), a downstream target of SREBP-1c, was also increased in CTRP9 KO mice. However, fatty acid synthase (Fasn) expression did not differ between WT and CTRP9 KO mice (Fig. 8E). Expression of genes involved in fatty acid oxidation (Lcad and Mcad) also did not differ between WT and KO mice (Fig. 8F). These results indicate that increased caloric intake and lipogenesis likely contributed to the fatty liver phenotype of CTRP9 KO mice.

Reduced skeletal muscle AMPK activation and mitochondrial content in CTRP9 KO mice. Previously, we showed that transgenic overexpression of CTRP9 results in increased skeletal muscle AMPK activation and mitochondrial content (19).

Fig. 8. Serum lipid profiles of chow-fed WT and CTRP9 KO mice. A–D: serum nonesterified fatty acids (NEFA; A), triacylglycerol (TG; B), liver weight (C), and liver TG content (D) were measured from chow-fed WT and KO mice (n = 4–5). E: real-time PCR analysis of lipogenic gene expression in the livers of WT and KO mice (n = 4–5). Expression levels of acetyl-CoA carboxylase (Acc), fatty acid synthase (Fasn), and sterol regulatory element-binding protein-1c (Srebp1c) were normalized to β-actin. F: real-time PCR analysis of fatty acid oxidation genes in the livers of WT and KO mice (n = 4–5). Expression levels of long-chain acyl-CoA dehydrogenase (Lcad) and medium-chain acetyl-CoA dehydrogenase (Mcad) were normalized to β-actin. All data are expressed as means ± SE. *P < 0.05. NS, not significant.
In contrast, the loss of CTRP9 resulted in decreased skeletal muscle AMPK activation, as judged by the level of AMPKα (Thr172) phosphorylation (Fig. 9C). Consistent with the role of AMPK in regulating mitochondrial biogenesis (22), CTRP9 KO mice had reduced skeletal muscle mitochondrial content, as indicated by the expression levels of mitochondria-specific marker genes (CoxII and CytoB; Fig. 9B).

Recent functional studies have shown that CTRP1, CTRP3, and CTRP12 also play positive roles in regulating glucose and lipid metabolism (17–19, 34); however, these related CTRP family members did not appear to compensate for the metabolic deficits seen in the CTRP9 KO mice. Indeed, we did not observe any alteration in the expression of Ctrp1, Ctrp3, or Ctrp12 in adipose tissue of WT and CTRP9 KO mice (Fig. 9C).

**Serum adipokine profiles of WT and CTRP9 KO mice.** In the obese state, adipose tissue and its resident macrophages secrete a variety of adipokines and cytokines that promote low-grade inflammation and insulin resistance (5). Therefore, we evaluated whether CTRP9 KO mice had altered serum adipokine profiles. Serum leptin levels increased threefold in CTRP9 KO mice compared with WT controls (Table 2), consistent with increased fat mass and body weight of these animals. Furthermore, levels of serum resistin, an adipokine that contributes to insulin resistance (28), were significantly upregulated (−2fold) in CTRP9 KO mice (Table 2). Elevated serum resistin levels may contribute in part to the insulin resistance phenotype of CTRP9 KO animals. Serum levels of other adipokines, including adiponectin, MCP-1, IL-6, and TNFα, were similar between WT and CTRP9 KO mice.

**DISCUSSION**

Using CTRP9 KO mouse model, we have established the importance of the hormone CTRP9 in regulating energy balance and insulin sensitivity. Our previous work focused on the metabolic function of CTRP9 by using transgenic mice in which the Ctrp9 transgene is driven by a ubiquitous promoter and showed that CTRP9 overexpression confers striking protection against HFD-induced obesity and insulin resistance (19). Whereas the phenotypes of CTRP9 transgenic mice suggest a pharmacological potential of CTRP9 as an anti-obesity and anti-diabetic agent, the impact and physiological significance of CTRP9 in whole body metabolism remains uncertain. Here, a loss-of-function mouse model allowed us to address directly the metabolic function of CTRP9 in a physiological context.

The phenotypes of our CTRP9 KO mouse model suggest that CTRP9 controls systemic energy balance via central and peripheral mechanisms. CTRP9 KO mice fed a chow diet gained significantly more body weight and adiposity due largely to increased food intake (Fig. 2). A closer look at the ingestive behavior of CTRP9 KO mice indicates that these animals consumed the same average number of meals over a 24-h period but consumed a larger portion of food at each meal (Fig. 3). The increased meal size of CTRP9 KO mice reflects a reduction in satiety ratio driven in part by the increased expression of orexigenic neuropeptides (Npy and Agrp) in their hypothalami. The fact that CTRP9 KO mice consumed more food in the dark phase of the photocycle but did not change food intake frequency (i.e., meal number) suggests that disrupting Ctrp9 leads to specific alterations in neurocircuitry controlling ingestive physiology. A role for CTRP9 in food intake regulation is also supported by our previous results showing that refeeding after fasting leads to increased expression of Ctrp9 transcripts in adipose tissue and a consequent increase in circulation (19). Refeeding leads to a positive energy balance, which then activates central pathways to reduce food intake (25); thus, increased expression and circulat-

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**Table 2. Serum adipokine levels in chow-fed WT and CTRP9 KO mice**

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<tr>
<th>Adipokine</th>
<th>WT</th>
<th>KO</th>
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<td>Adiponectin, pg/ml</td>
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<td>&gt;50,000</td>
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<tr>
<td>Leptin, pg/ml</td>
<td>7,999.74 ± 2,288.02</td>
<td>25,147.63 ± 4,129.23**</td>
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<td>MCP-1, pg/ml</td>
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<td>Resistin, pg/ml</td>
<td>2,300.56 ± 21.42</td>
<td>4,306.58 ± 370.94*</td>
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All data are expressed as means ± SE. WT, wild type; CTRP9, C1q/tumor necrosis factor-related protein 9; KO, knockout; MCP-1, monocyte chemoattractant protein 1. *P < 0.05; **P < 0.01.
ing CTRP9 levels upon refeeding suggest a role in food intake regulation. Importantly, CTRP9 transgenic mice are lean and resist HFD-induced obesity, which is due partly to decreased food intake (19). Thus, both gain- and loss-of-function mouse models implicate CTRP9 in modulating food intake via central mechanisms. Whether CTRP9 acts directly in the central nervous system or indirectly via other mechanisms to modulate ingestive behavior remains to be determined.

Reduced fasting insulin levels and impaired GTT and ITT indicate that chow-fed CTRP9 KO mice are insulin resistant (Fig. 5). Direct assessment of insulin signaling in mice by insulin administration suggests that insulin resistance occurs in the liver. We observed no impairment of insulin-induced Akt phosphorylation in skeletal muscle or adipose tissues, whereas the hepatic response of CTRP9 KO mice was clearly diminished (Fig. 6). Further supporting a role of CTRP9 in regulating insulin sensitivity in peripheral tissues, CTRP9 transgenic mice exhibit improved glucose tolerance and reduced fasting insulin levels (19). Thus, increased CTRP9 expression promotes, whereas its deficiency reduces, insulin sensitivity in mice. Interestingly, serum resistin levels are increased in CTRP9 KO mice (Table 2). Since this adipokine induces hepatic insulin resistance (14, 21, 24), higher serum resistin levels may contribute partly to the observed insulin resistance phenotype of CTRP9 KO mice.

Chow-fed CTRP9 KO mice develop hepatic steatosis (Fig. 7). The fatty liver phenotype is likely attributed to a combined effect of obesity due to increased caloric intake and increased hepatic lipogenesis (Figs. 3 and 8). Conversely, CTRP9 transgenic mice are resistant to steatosis (19). The complementary gain- and loss-of-function results support a role for CTRP9 in regulating hepatic lipid metabolism. Previously, we have shown that recombinant CTRP9 directly reduces basal as well as palmitate-induced lipid accumulation in cultured H4IIE hepatocytes (19), suggesting that CTRP9 acts directly on hepatocytes in vivo. Although hepatic TG content is often inversely correlated with hepatic insulin sensitivity of WT mice (23), the causal link between hepatic lipid content and insulin resistance remains uncertain (1, 3, 15).

Interestingly, CTRP9 KO mice fed an HFD had metabolic profiles (i.e., body weight, food intake, and insulin sensitivity) indistinguishable from WT controls. Metabolic dysfunction induced by high-fat feeding may mask any potential adverse effects resulting from CTRP9 deficiency, highlighting the impact of diet on metabolic outcome. Our previous study demonstrated that diet-induced obese (DIO) male mice fed an HFD for 12 wk had an ~50% reduction in the circulating levels of CTRP9 compared with lean controls (19). Thus, the HFD-fed DIO mice mimic a state of CTRP9 deficiency analogous to the loss of CTRP9 in the KO mouse model; this provides a possible explanation to reconcile the lack of food intake and body weight phenotype between WT and KO mice fed an HFD. Indeed, CTRP9 KO mice fed an HFD had food intake similar (2.34 ± 0.08 g/day) to WT controls (2.23 ± 0.24 g/day). The differential regulation of orexigenic genes seen in the hypothalamus of chow-fed KO mice relative to WT controls was also absent in the HFD-fed KO mice (data not shown).

Although CTRP9 is the closest paralog of adiponectin, this study indicates that CTRP9 and adiponectin have nonredundant functions. Four independent adiponectin KO mouse models were generated with variable metabolic phenotypes (8, 10, 11, 16); one model had surprisingly increased fat oxidation without any other overt metabolic phenotypes (10), whereas three other models had varying degrees of insulin resistance when challenged with an HFD (8, 11, 16). However, only one adiponectin KO mouse model developed mild insulin resistance on a standard chow diet (8); the other three adiponectin KO models showed no alteration in peripheral insulin sensitivity (10, 11, 16). There were also no differences in food intake and/or body weight between chow-fed WT and adiponectin KO mice (8, 11). The metabolic phenotypes we observed in chow-fed CTRP9 KO mice were largely absent in adiponectin KO mice, providing evidence that CTRP9 regulates physiological function distinctively from adiponectin. Furthermore, the metabolic deficits observed in CTRP9 KO mice did not appear to be compensated by other related CTRP family members, CTRP1, CTRP3, or CTRP12, known to positively regulate glucose and lipid metabolism (17–19, 34). This observation suggests a nonredundant function for different CTRP family members.

In summary, our results establish the importance and physiological relevance of CTRP9 as a component of the complex hormonal circuits that orchestrate energy homeostasis and modulate insulin sensitivity. Results from our current study using CTRP9 KO mice and previous study using transgenic CTRP9 mice (19) suggest that pharmacologically elevating circulating CTRP9 levels may have therapeutic value in treating obesity and obesity-associated insulin resistance.

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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

REFERENCES


