ADIPOSE TISSUE SECRETES LARGE numbers of polypeptide hormones and cytokines (collectively termed adipokines) that have local and/or systemic effects on carbohydrate and fat metabolism (8, 35). These secreted metabolic regulators modulate whole body insulin sensitivity and energy metabolism by acting directly on metabolic tissues (e.g., skeletal muscle and liver) or indirectly through regulatory roles in inflammatory processes (8, 35). The list of adipokines has grown considerably over the past 10 years, with leptin and adiponectin being the most widely studied and best understood (8, 10, 12).

Despite the pleiotropic function of adiponectin (12, 35), targeted disruption of the gene in mice results in surprisingly mild metabolic phenotypes (17, 21, 22, 27). While enhanced leptin sensitivity may partly compensate for the loss of adiponectin (51), other secreted factors that share overlapping function with adiponectin may play a role in ameliorating the metabolic dysfunction of adiponectin-null mice (7, 48). In fact, adiponectin belongs to the C1q family of proteins, which currently consists of more than 30 members, all of which possess the signature globular C1q domain (15). On the basis of shared sequence homology, we recently identified and characterized a family of 15 novel secreted proteins of the C1q family, designated as C1q/TNF-related proteins (CTRP1–15) (33, 37, 43, 45–49). Several of the CTRPs play roles in regulating glucose and fatty acid metabolism in vitro and/or in vivo (9, 28, 30–32, 37, 42–44).

Of the CTRPs, CTRP9 shares the highest degree of sequence identity (54%) with adiponectin at the presumed functional globular domain (47). In addition, CTRP9 and adiponectin share multiple common biochemical and structural features. These include adipose-selective expression, domain structure, formation of trimers, and posttranslational modifications (proline hydroxylation and lysine glycosylation). Intriguingly, CTRP9 and adiponectin also form heterotrimeric complexes in vitro and in vivo (47); however, the significance of this phenomenon remains unknown.

Recent work has shown that CTRP9 hormone plays a protective role in the heart against ischemia/reperfusion injury (14, 39), as well as attenuating neointima formation in response to vascular injury (41). However, the potential protective function and mechanism of action of CTRP9 in the context of diet-induced obesity and Type 2 diabetes remain unknown. Here, we provide in vivo and in vitro evidence that CTRP9 is, indeed, a novel adipokine with important metabolic functions.

MATERIALS AND METHODS

Antibodies and chemicals. Mouse monoclonal anti-FLAG M2 antibody was obtained from Sigma, and rat monoclonal anti-HA (clone 3F10) antibody was obtained from Roche. Rabbit antibodies recognizing phospho-AMPKα (Thr-172), AMPKα, and COX IV were obtained from Cell Signaling Technology. Mouse anti-GAPDH monoclonal antibody was obtained from Novus Biologicals. HRP-conjugated rabbit polyclonal anti-tubulin antibody was obtained from Abcam. Rabbit polyclonal antibody used to detect endogenous CTRP9 was generated previously (47).

Animals. C57BL/6J male mice (The Jackson Laboratory) were used to evaluate diet-induced changes in CTRP9 mRNA and circulating levels. Sera from wild-type male mice subjected to ad libitum fed, fasted, and refeed were collected, and CTRP9 levels were quantified by Western blot analysis. For ad libitum-fed group, serum samples were obtained at 2–3 h into the light cycle. For the fasted group, food was removed for 16 h (beginning at 10 h into the light cycle), and sera...
were collected at ~2–3 h into the light cycle. For the refed group, mice were fasted for 16 h and refed with chow pellets for 2 h before sera were collected. For all other experiments, mice were bred and weaned at The Johns Hopkins University School of Medicine animal facilities. Four-week-old CTRP9 transgenic mice and WT control littermates were housed in polycarbonate cages on a 12:12-h light-dark photocycle with ad libitum access to water throughout the study period. Male mice were used throughout the study. Mice were fed ad libitum a high-fat diet (HFD) (60% kcal derived from fat, Research Diets; D12492) or an isocaloric-matched low-fat diet (LFD) (10% kcal derived from fat, Research Diets; D12450B). HFD was provided for a period of 14 wk. Blood samples were collected for serum analysis. Tissues were collected, snap frozen in liquid nitrogen, and kept at −80°C. All animal protocols were approved by the Institutional Animal Care and Use Committee of the Johns Hopkins University School of Medicine.

Generation of CTRP9 transgenic mice. The C-terminal HA epitope-tagged CTRP9 was cloned into the XhoI site of pCAGGS vector (25). Expression of CTRP9 transgene was driven by the ubiquitous CAG promoter, which consists of a CMV enhancer element with a chicken β-actin promoter. The plasmid construct was digested with SalI and NotI restriction enzymes, and resulting DNA fragments (~3.5 and 2.5 kb) were separated on a 1% agarose gel. The ~3.5 kb linear DNA fragment containing the CAG promoter and enhancer, CTRP9-HA transgene, and the rabbit β-globin polyA adenylation signal were excised from the agarose gel, purified, and verified by DNA sequencing. Pronuclear injections were performed; several transgenic founder lines were obtained. Multiple founder lines were obtained, and all showed a lean phenotype. However, only one line was maintained and expanded for phenotypic analysis. The transgenic line was maintained on a (C57BL/6 × FVB) mixed genetic background. Transgene-negative littermates were used as WT control mice throughout the study.

Semiquantitative PCR analysis. Total RNAs from mouse tissues were isolated with TRIzol (Invitrogen). Two micrograms of total RNA were reverse-transcribed using Superscript II (Invitrogen). Thirty-cycle PCR was carried out using Hot Start Taq Blue polymerase (Denville); the cycling conditions were as follows: 15 s denaturation at 95°C, 15 s primer annealing at 60°C, and 45 s primer extension at 72°C. Primers used included the following: CTRP9-HA forward 5′-CCAGAT- GCAACCATTAATTCG-3′ and reverse 5′-TCAAGGCTGTCCTGGA ACCTGCTATGG-3′; and β-actin forward 5′-CGTGCATTAGGA GAAGCTGTC-3′ and reverse 5′-CTCAAGGAGCAATGATCTT- GAT-3′.

Mouse serum and blood chemistry analysis. Mouse serum samples were harvested by tail bleeding after overnight fast (~16 h) or at an indicated time point. Serum samples were separated using microvette CB 300 (Sarstedt). Glucose concentration was determined at time of collection with a glucometer (BD Bioscience). Serum samples were prepared according to manufacturer’s instructions for individual assay.

Fig. 1. Diet and metabolic state modulate circulating levels of CTRP9. A: quantitative real-time PCR analyses of Ctrp9 expression in adipose tissue isolated from 12-wk-old chow-fed male mice under fasted or fasted/refed conditions. B: quantitative Western blot analysis of CTRP9 serum levels in 12-wk-old chow-fed male mice under fasted, fasted/refed, or ad libitum conditions. Quantitative real-time PCR (C) and Western blot (D) analysis of CTRP9 mRNA and serum levels in male C57BL/6 mice fed a high-fat diet (HFD) or a low-fat diet (LFD) for 12 wk. Values shown are means ± SE; n = 8–10 mice per group. *P < 0.05. N.S., not significant.

Fig. 2. Generation of CTRP9 gain-of-function mouse model. A: schematic of CTRP9 transgene construct. HA epitope-tagged CTRP9 transgene is driven by the ubiquitous CAG promoter. B: semiquantitative RT-PCR analysis of CTRP9-HA transgene and β-actin expression in mouse tissues. C: Western blot analysis of CTRP9 in wild-type (WT) and transgenic (Tg) mouse sera. D: Western blot analysis of CTRP9-HA protein in mouse tissues.
or diluted 1:20 in SDS loading buffer (50 mM Tris-HCl, pH 7.4, 2% SDS wt/vol, 6% glycerol wt/vol, 1% 2-mercaptoethanol vol/vol, and 0.01% bromophenol blue wt/vol) and subjected to Western blot analysis. Serum/tissue triglyceride (ThermoFisher Scientific), nonesterified free fatty acid (NEFA; Wako), insulin, leptin, and adiponectin (Millipore) were determined according to kit manufacturer’s instructions.

**Intraperitoneal glucose and insulin tolerance tests.** Separate cohorts of 8–10 13-wk-old transgenic (Tg) and control littersmates were injected intraperitoneally with glucose (1 g/kg). Animals were fasted for 6 h prior to the glucose tolerance test (1). For the insulin tolerance test, food was removed 2 h prior to insulin injection. Serum samples were collected at the indicated time points shown in the **RESULTS.**

**Body composition analysis.** Body compositions of Tg and control littersmates were determined using a whole body NMR instrument (EchoMRI) at the metabolic phenotyping core facility at Johns Hopkins University School of Medicine. EchoMRI analysis provided values for fat mass, lean mass, and water content.

**Indirect calorimetry.** CTRP9 Tg mice and control littersmates (n = 7 or 8 per group) were used for simultaneous assessments of changes in daily body weight, energy intake (corrected for spillage), and whole body metabolic profile in an open-flow indirect calorimeter (Oxymax, Columbus Instruments), as described previously (30). LFD- and HFD-fed mice were evaluated in separate studies. Data were collected for 3 days to confirm acclimation to the calorimetry chambers (stable body weights and food intakes), and data from the fourth day in the Oxymax were analyzed. Rates of oxygen consumption (V\(\dot{O}_2\), ml·kg\(^{-1}\)·h\(^{-1}\)) and carbon dioxide production (V\(\dot{CO}_2\)) were measured for each chamber every 16 min throughout the studies. Respiratory exchange ratio (RER = V\(\dot{CO}_2\)/V\(\dot{O}_2\)) was calculated by Oxymax software (v. 4.02) to estimate relative oxidation of carbohydrate (RER = 1.0) vs. fat (RER approaching 0.7), not accounting for protein oxidation. Energy expenditure was calculated as EE = V\(\dot{O}_2\) × [3.815 + (1.232 × RER)] (20), and normalized to lean body mass (kcal·kg\(^{-1}\)·h\(^{-1}\)) as recommended by Butler and Kozak (4). Average metabolic values were calculated within subjects, then averaged across subjects for statistical analysis by Student’s t-test, with \(P \leq 0.05\), indicating significant group differences.

**Physical activity.** Mice (n = 8 per group) were tested in open-field chambers with infrared beam arrays to detect movement (photobeam...
activity system (PAS)-open field; San Diego Instruments, San Diego, CA). Locomotor activity was monitored for 24 h, in 12 bins of 2-h duration, and reported as total ambulatory activity (ambulatory-like beam break patterns in the center plus periphery).

Cell culture. GripTite HEK 293 cells (Invitrogen) were cultured in DMEM containing 10% FBS, 2 mM l-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin. Rat H4IIE hepatocytes and rat L6 myocytes were cultured in DMEM containing 10% FBS and antibiotics. L6 myotubes were differentiated as previously described (32). Each experiment represents the combined data from three independently performed experiments. For the in vitro assay, free fatty acid (palmitate)/BSA conjugates were prepared as previously described (19). Briefly, a 20-mM solution of free fatty acids in 0.01 M NaOH were incubated at 70°C for 30 min, and fatty acid soaps were then complexed with 5% BSA in PBS at an 8:1 ratio of fatty acid to BSA. The conjugates were administered overnight to cultured H4IIE hepatocytes at concentrations indicated.

Fatty acid oxidation. To measure fatty acid oxidation, we adapted the protocol as described by Buzzai et al. (5), in which oxidation of [9,10-3H]-palmitic acid results in formation of [3H]H2O. In brief, rat L6 myotubes were incubated for 2 h in serum-free DMEM containing 0.2% BSA with recombinant CTRP9 (5 μg/ml) or vehicle buffer. Next, 0.2 μCi/ml [9,10-3H]palmitic acid (Moravek Biochemical) were added to the media and incubated for 60 min. The tritiated palmitate was oxidized to CO2 and [3H]H2O. After incubation, the medium was transferred to a tube containing equal volume of chilled (4°C) 10% trichloroacetic acid. Samples were mixed and incubated for 10 min at 4°C, and then centrifuged for 30 min at 4°C. After centrifugation, 400 μl of the supernatant were collected and combined with 55 μl of 6 N NaOH, and then transferred to a Micro Bio-spin chromatography column (Bio-Rad; cat. no. 732-6204), containing 0.5 g Dowex ion exchange resin (Sigma 217-425). The [3H]H2O would be selectively retained by the resin, while the hydrophobic, nonoxidized [9,10-3H]palmitic acid in the supernatant would pass through the column. The bound [3H]H2O in the column was eluted with 1 ml dH2O, and the elution was transferred to a liquid scintillation vial. The amount of [3H] radioactivity was determined with a Beckman Coulter counter (model LS6000SC). The amount of [3H]H2O collected indicates the extent of fatty acid oxidation.

Western blot analysis. Tissue lysates were prepared in T-PER buffer (Pierce Biotechnology, Rockford, IL) with phosphatase and protease inhibitors added (Calbiochem). Protein concentrations were determined using the Coomassie Plus protein assay reagent (Thermo Scientific). Ten micrograms of protein from tissue lysates or 1 μl of serum were loaded and separated on a 10% bis-Tris NuPAGE gel (Invitrogen). Western blotting and quantification were carried out as previously described (43).

Statistical analysis. All results are expressed as means ± SE. Statistical analysis was performed with Prism 5 software (GraphPad). Blood chemistry data were analyzed with two-tailed Student’s t-tests between CTRP9 Tg and control littermates. Repeated-measures ANOVA were performed on body weights, as well as serum glucose and insulin measurements in various tolerance tests. Values were considered to be significant at \( P < 0.05 \).

Fig. 4. Reduced adiposity and adipocyte size in CTRP9 Tg mice. A: quantification of subcutaneous (inguinal) fat pad mass in WT and Tg mice. B: representative tissue sections of inguinal fat pad of WT and Tg mice. C: quantification of visceral (gonadal) fat pad mass in WT and Tg mice. D: representative tissue sections of gonadal fat pad of WT and Tg mice. Values shown are expressed as means ± SE; \( n = 8–10 \) mice per group. *\( P < 0.05 \) vs. WT.
RESULTS

Metabolic state affects circulating levels of CTRP9. Expression and circulating levels of many adipokines change in response to alterations in energy state. Therefore, we first determined whether changes in nutritional states acutely affect circulating levels of CTRP9. Mice fed ad libitum and mice that were fasted/refed had three-fold higher transcript levels of CTRP9 in adipose tissue and four-fold higher circulating levels compared with overnight fasted animals (Fig. 1, A and B). In diet-induced obese male mice fed an HFD for 12 wk, we observed a ~50% reduction in circulating levels of CTRP9 (Fig. 1D). However, reduced serum levels of CTRP9 did not result from a reduction in mRNA expression in the epididymal (visceral) adipose tissue (Fig. 1C). This suggests that diet-induced alteration in serum CTRP9 levels may be mediated by a posttranscriptional mechanism. Together, these data indicate that short-term changes in nutritional state, as well as chronic metabolic stress induced by a high-fat diet, alter circulating levels of CTRP9.

Generation of CTRP9 transgenic mice. To address the in vivo metabolic function of CTRP9, we generated a Tg mouse model overexpressing HA epitope-tagged CTRP9. In mice, endogenous CTRP9 mRNA is expressed predominantly by adipose tissue, with lower expression levels in other tissues. Within adipose tissue, both adipocytes and cells of the stromal vascular fraction express CTRP9 mRNA (47). Therefore, expression of the CTRP9 transgene was driven by a ubiquitous promoter (Fig. 2A). In the Tg mouse line, CTRP9 expression was detected in the adipose tissue, skeletal muscle, heart, brain, and kidney (Fig. 2B). Despite comparable expression of CTRP9 transgene mRNA in various mouse tissues, a substantially higher level of CTRP9-HA protein was detected in the skeletal muscle and heart compared with other tissues (Fig. 2D). As expected, overexpression also resulted in a significant increase in circulating levels of CTRP9, ~5-fold greater than baseline serum levels in wild-type mice (Fig. 2C). This gain-of-function mouse model enabled us to explore the long-term metabolic consequences of elevated circulating levels of CTRP9.

CTRP9 Tg mice are lean and resistant to diet-induced weight gain. Although comparable in body weight at weaning (4 wk of age), Tg mice fed an LFD consistently gained less body weight over time (Fig. 3A). By 18 wk of age, Tg mice fed an LFD were ~22% lighter than control littermates. Remarkably, when fed an HFD, the body weight phenotype of the animals became overtly pronounced. While wild-type control littermates became progressively obese over time on an HFD as expected, Tg mice were resistant to weight gain (Fig. 3, B and C). In fact, by 18 wk of age, Tg mice (26.9 ± 2.4 g) were just half the body weight of control littermates (50.4 ± 3.7 g). Strikingly, Tg mice on an HFD gained less body weight and accumulated similar amounts of fat mass (percent of body

Fig. 5. Indirect calorimetry analysis of CTRP9 Tg mice fed a low-fat diet. A: food intake analysis in WT and Tg mice on a LFD. B: 24-h ambulatory activity of WT and Tg mice on an LFD. Data were binned into 2-h segments. C–F: oxygen consumption (VO2; C), carbon dioxide release (VCO2; D), respiratory exchange ratio (RER = VCO2/VO2; E), and energy expenditure (F) of WT and Tg mice on an LFD, as determined by indirect calorimetry. Values shown are expressed as means ± SE; n = 6 mice per group.
weight) compared with Tg mice on an LFD (Fig. 3, A and B). These data have been independently confirmed in three separate cohorts of Tg mice and their WT littermate controls. Thus, the beneficial and protective metabolic function of CTRP9 was revealed when mice were challenged with an HFD to induce metabolic stress.

Reduced adiposity and adipocyte size in CTRP9 Tg mice. Differences in body weight between WT and Tg mice fed an HFD could result from reduced accumulation of fat mass. Indeed, body composition analysis using quantitative NMR revealed that Tg mice had significantly less fat mass compared with WT controls (Fig. 3D), accounting for the lower body weight of Tg mice. In contrast, we observed an increase in percent lean mass (~9%) in Tg mice fed an HFD relative to littermate controls (Fig. 3E). Reduction in adiposity appeared more striking in subcutaneous (inguinal fat pad) compared with visceral fat depots (gonadal fat pad) in Tg mice relative to littermate controls (Fig. 4, A and C). Further, the size of adipocytes was significantly smaller in Tg mice (Fig. 4, B and D); again, the difference was more pronounced in subcutaneous fat pad. We quantified the number of adipocytes in a given random ×20 magnification field. In the gonadal fat pad, there were 79 ± 14 (WT) vs. 183 ± 27 (Tg) adipocytes per ×20 field (n = 9 for WT and n = 6 for Tg mice). In the subcutaneous fat pad, there were 101 ± 9 (WT) vs. 346 ± 39 (Tg) adipocytes per ×20 field (n = 9 for WT and n = 6 for Tg mice). On the basis of these data, we estimated that the adipocytes found in the gonadal and subcutaneous fat depots of Tg mice are, on average, 2.3 and 3.4 times smaller than the WT counterparts, respectively. Smaller adipocytes are associated with improved metabolic profile and insulin sensitivity of white adipose tissue (6, 16, 34, 38). We also looked for potential “browning” of the adipose compartment; we observed no differences in the mRNA levels of key marker genes (UCP-1, Elovl3, Otop1, Cox7a1) previously shown to be associated with brown adipocyte-like cells within white adipose tissue (3).

CTRP9 Tg mice show enhanced energy expenditure. Several mechanisms could account for the lean phenotype of CTRP9 Tg mice, such as differences in food intake, voluntary physical activity levels, and/or energy expenditure. Indirect calorimetry analyses were carried out to determine the consequences of CTRP9 overexpression on whole-body energy balance. On an LFD, WT and Tg mice consumed comparable amounts of food pellets and showed no differences in physical activity levels, VO2, RER, and energy expenditure (Fig. 5). However, Tg mice fed an HFD consumed fewer calories (Fig. 6A) and were physically less active during the dark photocycle compared with littermate controls (Fig. 6B). Further, Tg mice fed an HFD had increased oxygen consumption (Fig. 6C) and increased...
carbon dioxide production (Fig. 6D) relative to littermate controls, indicating an enhanced metabolic rate. As expected, mice fed an HFD had lower RER compared with mice fed an LFD, due to a greater oxidation of lipid over carbohydrate substrates. Within the HFD group, CTRP9 Tg mice had a lower RER compared with littermate controls (Fig. 6E), indicating greater fat oxidation. Because of increased metabolic rate, Tg mice fed an HFD had lower RER compared with mice fed an LFD, due to a greater oxidation of lipid over carbohydrate substrates. Within the HFD group, CTRP9 Tg mice had a lower RER compared with littermate controls (Fig. 6E), indicating greater fat oxidation. Because of increased metabolic rate, Tg mice fed an HFD had a modest overall increase in whole body energy expenditure compared with WT mice (Fig. 6F). These data suggest that a combination of reduced food intake and increased basal metabolism over a 14-wk period on HFD could account for the remarkable differences in body weight and percent fat and lean mass between WT and CTRP9 Tg mice (Fig. 3).

CTRP9 enhances AMPK activation and promotes skeletal muscle fat oxidation. To uncover the mechanism by which CTRP9 promotes energy expenditure, we examined possible signaling pathways and enzymes that regulate fat oxidation in skeletal muscle. Consistent with enhanced fat oxidation, we observed a three-fold increase in the expression of fatty acid oxidation enzyme genes (Lcad and Mcad) in the skeletal muscle of Tg mice relative to control littermates (Fig. 7A). Further, there was over a twofold increase in the expression of mitochondrion-specific genes (CoxII and CytoB) in the skeletal muscle of Tg mice (Fig. 7B). As with the mRNA expression data, mitochondrion-specific protein (e.g., COX IV) levels were also significantly increased (approximately twofold) (Fig. 7C), indicating greater mitochondrial content in the skeletal muscle of Tg mice on an HFD. Increased fat oxidation consequently resulted in lower triglyceride content in the skeletal muscle of Tg mice relative to control littermates fed an HFD (Fig. 7D).

AMPK phosphorylation (at Thr-172) and activation are known to increase mitochondrial biogenesis and muscle fat oxidation (50, 53). In the skeletal muscle of CTRP9 Tg mice, AMPKα (the catalytic subunit) was hyperphosphorylated at Thr-172 relative to WT controls (Fig. 8A), indicating enhanced AMPK activation. In accordance with a direct effect of CTRP9 in promoting muscle fat oxidation in vivo, purified recombinant CTRP9 likewise induced AMPKα (Thr-172) phosphorylation (Fig. 8B) and enhanced fatty acid oxidation in differentiated rat L6 myotubes (Fig. 8C). Treatment of myotubes with compound C, an AMPK inhibitor (52), abolished the ability of CTRP9 to enhance fatty acid oxidation (Fig. 8C), confirming that CTRP9 activates AMPK signaling to control fat oxidation. Together, these data indicate that CTRP9 promotes energy expenditure in vivo by enhancing skeletal muscle fat oxidation via AMPK activation and by increasing mitochondrial content.

It has been increasingly appreciated that brown adipose tissue also plays an important role in whole body energy expenditure by promoting fat oxidation (36). Although the white adipose tissue mass was greatly reduced in Tg mice (Fig. 3D), we did not observe any changes in interscapular brown adipose tissue mass (normalized to body weight) nor any differences in the expression levels of uncoupling protein-1 (UCP-1) mRNA and protein in brown fat isolated from WT and Tg mice fed an HFD (data not shown), indicating minimal contribution by brown fat to overall differences in energy expenditure observed between WT and Tg mice.

CTRP9 decreases hepatic lipid accumulation. While HFD leads to hepatic triglyceride accumulation and, consequently, fatty liver (hepatic steatosis) in WT mice, overexpressing CTRP9 substantially reduced hepatic triglyceride levels by ~30% (Fig. 9, A and B). Circulating ketone levels, a product of
hepatic fatty acid oxidation, were elevated in Tg mice relative to WT controls (Table 1), indicating enhanced fat oxidation in the liver. This may account, in part, for lower hepatic triglyceride content in Tg mice. Consistent with the in vivo data, recombinant CTRP9 treatment reduced basal, as well as palmi-
tate-induced lipid accumulation in cultured H4IIE hepatocytes (Fig. 9C). Together, these results suggest that skeletal muscle and liver are target tissues of CTRP9 in vivo and are likely responsible for oxidizing a substantial amount of the available lipid substrates.

**Improved metabolic profiles of mice overexpressing CTRP9.** Enhanced fat oxidation coupled with reduced hepatic and skeletal muscle triglyceride levels are predicted to improve the metabolic profiles of CTRP9 Tg mice. Indeed, on an HFD, overnight-fasted Tg mice had markedly reduced insulin and glucose levels compared with littermate controls (Table 1), indicating enhanced insulin sensitivity in Tg animals. As expected from a reduction in fat mass, serum leptin levels in Tg mice were correspondingly decreased relative to WT controls. Serum glucagon levels were also reduced in Tg mice, likely a consequence of decreased insulin levels. On an HFD, Tg mice have higher circulating levels of triglycerides and no signifi-
cant difference in NEFA. Circulating levels of adiponectin were comparable between WT and Tg mice. However, for the LFD-fed mice, only fasting insulin levels were different between WT and Tg mice (Table 1).

**Fig. 8. CTRP9 activates AMPK signaling in vivo and in vitro.** A: quantitative Western blot analysis of AMPKα (Thr-172) phosphorylation in the skeletal muscle of WT and Tg mice fed an HFD. Phospho-protein levels were normalized to total AMPKα levels. B: Western blot analysis of AMPKα phosphorylation in rat L6 myotubes stimulated with vehicle control or recom-
binant CTRP9 (5 μg/ml). C: fatty acid (palmitate) oxidation was measured in L6 myotubes treated with vehicle control or recombinant CTRP9 (5 μg/ml). Values shown are expressed as means ± SE. Representative gels are shown here. *P < 0.05 vs. WT. n = 8–10 mice per group for in vivo studies; n = 6 for in vitro experiments, representing three independent experiments.

**Fig. 9. Reduced hepatic triglyceride accumulation in Tg mice.** A: representative images of WT and Tg liver sections stained with oil red O. B: quantification of hepatic triglyceride levels in WT and Tg mice fed an HFD. C: lipid accumulation in rat H4IIE hepatocytes treated overnight with vehicle control or CTRP9 (5 μg/ml) in the presence or absence of 100 μM palmitate. All data shown are expressed as means ± SE; n = 8–10 mice per group for in vivo studies; n = 6 in vitro experiments, representing three independent experi-
ments. *P < 0.05 vs. WT.
Table 1. Blood chemistry analysis

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Values are expressed as means ± SE. Sera were collected from overnight-fastened WT and Tg mice (n = 6 for LFD and n = 8–10 for HFD group) when they were 18 wk old and had been on a low-fat diet (LFD) or high-fat diet (HFD) for the previous 14 wk. NEFA, nonesterified fatty acids; ns, not significant.

CTR9 prevents HFD-induced insulin resistance. Assessment of whole-body insulin sensitivity using the homeostatic model assessment insulin resistance index (23) revealed a substantial reduction in insulin resistance in CTR9 Tg mice relative to WT controls fed an HFD (data not shown). To confirm that Tg mice were indeed, more insulin-sensitive, an intraperitoneal glucose tolerance test (GTT) was performed. Mice overexpressing CTR9 were much better at handling a glucose load, with a significantly higher rate of glucose disposal in the peripheral tissues (Fig. 10, E and F). The rate of glucose excursion in GTT is dependent on the magnitude of insulin secretion from the pancreatic β-cells in response to glucose challenge, as well as peripheral tissue insulin sensitivity. Therefore, insulin levels were measured. The magnitude of insulin secretion in Tg mice in response to the GTT was significantly lower (Fig. 10, G and H), despite a much greater rate of glucose disposal in these animals. These results indicate that CTR9 Tg mice do not reduce to HFD are, indeed, more insulin-sensitive compared with littermate controls. Interestingly, although both Tg and WT mice fed an LFD showed comparable glucose disposal rate in GTT (Fig. 10, A and B), Tg mice did so with reduced insulin secretion during GTT (Fig. 10, C and D), indicative of enhanced insulin sensitivity in the peripheral tissues of these animals.


discussion

We provide in vivo evidence that CTR9 plays an important role in regulating whole-body metabolism. While endogenous CTR9 is predominantly produced by adipose tissue, transgene expression driven by a ubiquitous and not an adipose tissue-specific promoter is a limitation of the present study. Nonetheless, transgenic mice with an elevated circulating level of CTR9 have a remarkable ability to handle HFD-mediated metabolic challenges. These mice are resistant to HFD-induced weight gain, the development of insulin resistance, and hepatic steatosis. Reduced food intake partially accounts for the differences in body weight between Tg and WT mice in response to HFD, suggesting that CTR9 may be acting in the hypothalamus to modulate food intake. The substantially improved metabolic profiles of Tg mice are due to reduced adiposity and enhanced basal metabolism resulting from greater fat oxidation in the skeletal muscle and liver. Mechanistically, CTR9 activates AMPK signaling to promote muscle fat oxidation. This in vivo effect appeared direct; treatment of myotubes with recombinant CTR9 in vitro enhanced fatty acid oxidation, an effect abrogated by the AMPK inhibitor, compound C. In skeletal muscle, AMPK activation results in decreased synthesis of malonyl-CoA, an allosteric inhibitor of carnitine palmitoyltransferase (CPT) (24). This promotes fatty acyl-CoA import into mitochondria for β-oxidation (25, 40). Further, chronic activation of AMPK also increased mitochondrial biogenesis (2, 53), and this likely accounts for the twofold increase in mitochondrial content seen in the skeletal muscle of Tg mice.

Regulation of whole body energy balance involves the coordinated actions of multiple tissues and organs, and their intimate crosstalk is mediated by secreted hormones. Thus, in a broader context, our present study, along with recent findings (30, 32, 37, 43), highlights the importance of CTRPs as a novel family of secreted metabolic regulators that mediate the integrated control of whole-body metabolism. Because all CTRPs characterized to date circulate in plasma and, in principle, can function as endocrine hormones, questions naturally arise regarding their target tissue(s) in vivo and the metabolic processes they regulate. We have recently shown that CTRP1 promotes skeletal muscle, but not liver, fat oxidation (30). Our current study suggests that CTRP9 acts on skeletal muscle and liver to control systemic energy balance. Transgenic mice with elevated circulating levels of CTRP9 are strikingly resistant to HFD-induced obesity, insulin resistance, and hepatic steatosis. In contrast, overexpressing CTRP1 in mice provides only modest protection against HFD-induced weight gain and glucose intolerance, with no apparent effect on the development of fatty liver (30). Unlike CTRP1 and CTRP9, CTRP3 functions primarily in the liver to regulate hepatic glucose output via Akt activation, with no apparent effect on fat oxidation, at least in cultured myotubes (32). In mice, CTRP12 improves insulin signaling and lowers blood glucose by suppressing gluconeogenesis in liver and promoting glucose uptake in adipocytes (43), as well as by dampening adipose tissue inflammation (9). While CTRP1, CTRP3, CTRP9, and CTRP12 are considered adipokines due to their adipose-selective expression, myonectin/CTRP15 is a skeletal muscle-derived myokine that functions to regulate lipid homeostasis in the liver and adipose tissue (37). Although much remains to be uncovered, recent studies indicate unique, as well as overlapping, biological functions for each of the CTRPs. Along with other secreted adipokines (8), myokines (3, 29), and hepatokines (11, 18, 26), CTRP9 and several of its related CTRP family members can be considered as integral components of the complex hormonal circuits governing intertissue crosstalk and systemic energy balance.

Adiponectin is a widely studied adipokine with pleiotropic functions, well known for its antidiabetic, antiatherogenic, and anti-inflammatory properties (13). However, targeted disruption of the adiponectin gene in mice reveals surprisingly mild metabolic phenotypes (17, 21, 22, 27), attributable only, in part, to enhanced leptin sensitivity in these animals (51) or other compensatory mechanisms (7, 48). Since CTRPs and adiponectin belong to the C1q protein family with shared biochemical features and overlapping functions, CTRPs may
prevent more overt metabolic phenotypes in adiponectin-deficient animals. Future studies using CTRP9 or CTRP9/adiponectin compound knockout mice may provide further insight into the contributions of these secreted hormones to overall energy homeostasis.

In summary, we provide the first in vivo evidence linking CTRP9 to regulation of fat metabolism in liver and skeletal muscle via AMPK signaling pathway and highlight its protective metabolic function in the context of HFD-mediated metabolic insults. Thus, CTRP9 can be considered a novel component of the metabolic network that links adipose tissue to systemic energy balance.

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Fig. 10. Improved insulin sensitivity in CTRP9 transgenic mice. Intraperitoneal glucose tolerance test (GTT) for mice fed a low-fat diet (LFD; A) or a high-fat diet (HFD; E). Quantification of the cumulative glucose clearance in GTT by integration of area under the curve (AUC) for LFD-fed (B) and HFD-fed mice (F). Insulin levels during the course of GTT for LFD-fed (C) and HFD-fed mice (G). Quantification of the cumulative insulin release in GTT by integration of AUC for LFD-fed (D) and HFD-fed mice (H). All mice were 13 wk old and had been on LFD or HFD for the previous 9 wk. Values are expressed as means ± SE; n = 6 for LFD group and n = 8–10 mice for HFD group. *P < 0.05 vs. WT.
REFERENCES


DISCLOSURES

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