

RGC32 deficiency protects against high-fat diet-induced obesity and insulin resistance in mice

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Abstract

Obesity is an important independent risk factor for type 2 diabetes, cardiovascular diseases and many other chronic diseases. Adipose tissue inflammation is a critical link between obesity and insulin resistance and type 2 diabetes and a contributor to disease susceptibility and progression. The objective of this study was to determine the role of response gene to complement 32 (RGC32) in the development of obesity and insulin resistance. WT and RGC32 knockout (*Rgc32*^{-/-} (*Rgcc*)) mice were fed normal chow or high-fat diet (HFD) for 12 weeks. Metabolic, biochemical, and histologic analyses were performed. 3T3-L1 preadipocytes were used to study the role of RGC32 in adipocytes *in vitro*. *Rgc32*^{-/-} mice fed with HFD exhibited a lean phenotype with reduced epididymal fat weight compared with WT controls. Blood biochemical analysis and insulin tolerance test showed that RGC32 deficiency improved HFD-induced dyslipidemia and insulin resistance. Although it had no effect on adipocyte differentiation, RGC32 deficiency ameliorated adipose tissue and systemic inflammation. Moreover, *Rgc32*^{-/-} induced browning of adipose tissues and increased energy expenditure. Our data indicated that RGC32 plays an important role in diet-induced obesity and insulin resistance, and thus it may serve as a potential novel drug target for developing therapeutics to treat obesity and metabolic disorders.

Key Words

- ▶ response gene to complement 32
- ▶ obesity
- ▶ insulin resistance
- ▶ adipose tissue

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Introduction

Obesity is an important independent risk factor for type 2 diabetes and cardiovascular diseases, and it is associated with high morbidity and mortality (Friedman 2003). Although a range of therapies have been developed, these pharmaceutical therapies often have adverse side effects or limited efficacy (Bandyopadhyay 2009, Distefano & Watanabe 2010). Therefore, a continued effort is required to identify novel drug targets and develop more effective therapeutics for the treatment of obesity.

Adipose tissue plays a critical role in energy homeostasis, not only in storing triglycerides but also in secreting hormones and biologically active molecules that regulate cellular lipid storage capacity, tissue and systemic insulin sensitivity, and metabolic energy balance (Chatterjee *et al.* 2014). Although the underlying mechanism is not fully understood, adipose tissue inflammation has been acknowledged as a critical link between obesity and insulin resistance and type 2 diabetes, and as a contributor

to disease susceptibility and progression (Mori *et al.* 2010). Adipose tissue inflammation, characterized by increased secretion of proinflammatory cytokines and chemokines including interleukin 6 (*IL6*), tumor necrosis factor α (*TNF α*), and *IL12*, contributes to low-grade systemic inflammation, insulin resistance, and metabolic disorders (Xu *et al.* 2003, Hotamisligil 2006). Blocking the function of proinflammatory cytokines or chemokines results in improved insulin sensitivity and glucose homeostasis (Uysal *et al.* 1997, Kanda *et al.* 2006). Therefore, genes or molecules that control obesity or adipose tissue inflammation are promising therapeutic targets for insulin resistance, type 2 diabetes, and cardiovascular complications.

Response gene to complement 32 (RGC32) is expressed in numerous human organs and tissues including placenta, kidney, liver, heart, and brain (Badea *et al.* 1998). Functionally, RGC32 plays an important role in cell proliferation, differentiation (Li *et al.* 2007, Fosbrink *et al.* 2009, Wang *et al.* 2011), fibrosis (Li *et al.* 2011a), and cancer (Fosbrink *et al.* 2005, Vlaicu *et al.* 2010, Kim *et al.* 2011). Recently, we have generated RGC32 knockout (*Rgc32*^{-/-} (*Rgcc*)) mice and found that *Rgc32*^{-/-} mice were born smaller than their WT littermates because of the impaired placental angiogenesis (Cui *et al.* 2013). However, it is unknown if RGC32 plays a role in postnatal metabolism. In this study, we demonstrate that high-fat diet (HFD) dramatically induces RGC32 expression in the adipose tissue. RGC32 deficiency attenuates HFD-induced obesity and insulin resistance in mice. The beneficial effect of RGC32 deficiency is due to the decreased adipose tissue content and systemic inflammation and increased energy expenditure of adipose tissue. This is the first report showing that RGC32 deficiency prevents diet-induced obesity and insulin resistance in mice. Therefore, RGC32 may serve as a potential novel drug target for preventing obesity and type 2 diabetes.

Materials and methods

Animals and diets

Rgc32^{-/-} mice on the C57BL/6 background were generated and genotyped as described previously (Cui *et al.* 2013). The parallel line WT C57BL/6 mice were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). The age-matched WT and *Rgc32*^{-/-} male mice were maintained on normal chow for 8 weeks, after which they were fed with either normal chow (25% protein, 62% carbohydrate, and 13% fat; 3.07 kcal/g; 5053, LabDiet, St. Louis, MO, USA) or

HFD (20% protein, 40% carbohydrate, and 40% fat; 4.5 kcal/g; D12108C, Research Diets, New Brunswick, NJ, USA) for an additional 12 weeks. The mice were fasted overnight and anesthetized (2.0% isoflurane), and blood was collected by direct cardiac puncture. Epididymal fat was carefully removed and weighed. A portion of the epididymal fat was fixed in 4% paraformaldehyde for histological analysis, whereas the other portion was stored at -80 °C for RNA and protein preparation. All animals were housed under conventional conditions in the animal care facilities and received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals. All experimental procedures were approved by Institutional Animal Care and Use Committee (IACUC) of University of Georgia.

Body weight and metabolic studies

Mice were weighed every 4 weeks. For metabolic studies, the mice were housed individually in metabolic cages (model 3600M021, Techniplast, Buguggiate, VA, Italy) with free access to food and water. After a 5-day period of acclimatization, daily food and water intakes were recorded, and fecal pellets and urine were weighed. The mice were observed for 3 days in the metabolic cage and then were put back in their original cages. To assess the energy expenditure, the mice were fasted overnight and weighed. This body weight was used as a baseline. The mice were fasted for an additional 8 h and weighed, and then the percentage change in the body weight from the baseline was calculated.

Glucose and insulin tolerance tests

For the glucose tolerance test (GTT), mice were fasted overnight followed by an i.p. glucose injection (1 g/kg body weight). Blood glucose was measured by tail bleeding using the One-Touch AccuChek Glucometer (Roche) at indicated times. For the insulin tolerance test (ITT), mice without fast were injected intraperitoneally with insulin (Sigma-Aldrich) at a dose of 1.5 IU/kg body weight, and blood glucose was measured at indicated times.

Blood biochemical analysis

Serum samples were analyzed for adiponectin, leptin, insulin, triglyceride, and cholesterol using the Adiponectin Mouse ELISA Kit (Abcam, Cambridge, MA, USA), Leptin Mouse ELISA Kit (Abcam), Rat/Mouse Insulin ELISA Kit (Millipore, Billerica, MA, USA), Triglyceride Quantification

Kit (Abcam), and HDL and LDL/VLDL Cholesterol Assay Kit (Abcam).

Cytometric bead array immunoassay

Serum from individual mouse was assayed for inflammatory markers using the Cytometric Bead Array Mouse Inflammation Kit (BD Biosciences, San Jose, CA, USA). Data were collected by FACSCalibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ, USA).

3T3-L1 cell culture and differentiation

3T3-L1 preadipocytes (ATCC, Manassas, VA, USA) were maintained in DMEM (Invitrogen) supplemented with 10% FBS (Sigma). At confluence, adipocyte differentiation was induced by treatment with 1 $\mu\text{mol/l}$ dexamethasone (Sigma), 500 $\mu\text{mol/l}$ isobutylmethylxanthine (Sigma), 10 $\mu\text{mol/l}$ pioglitazone, and 100 nmol/l insulin (Sigma) for 2 days. The cells were then incubated in 100 nmol/l insulin-containing medium for 6 days to induce lipid accumulation. The medium was replaced every other day.

Oil Red O staining

Culture plates were washed by PBS, and cells were fixed in 4% formaldehyde for 1 h, followed by staining with Oil Red O (Sigma) for 1 h. Oil Red O was prepared by diluting a stock solution (0.5% in isopropanol) with water (60:40 vol/vol) followed by filtration. After staining, the plates were washed twice in water and photographed. The dye was then extracted with 100% isopropanol, and the absorbance was determined at 520 nm.

RNA extraction and real-time quantitative RT-PCR

Quantitative RT-PCR (qPCR) was performed in a Mx3005P qPCR machine using SYBR Green master mix (Agilent Technologies, Santa Clara, CA, USA) as described previously (Li *et al.* 2011b). Each sample was amplified in triplicate. The expression of each gene was normalized with cyclophilin. Primer sequences are summarized in Table 1.

Western blotting analysis

Western blotting was performed as described previously (Cui *et al.* 2012). Antibodies against hormone-sensitive lipase (LIPE, Cell Signaling Technology, 4107S, Danvers, MA, USA), peroxisome proliferator-activated receptor α (PPAR α), (Abcam, ab8934), PPAR gamma coactivator 1 α (PGC1 α)

Table 1 Primer sequences utilized for qPCR evaluation of gene expression

Gene	Primer sequence
Cyclophilin	F: 5'-GTG GTC TTT GGG AAG GTG AA-3' R: 5'-TTA CAG GAC ATT GCG AGC AG-3'
Rgc32	F: 5'-CCG ATC TGG ACA GGA CCT TA-3' R: 5'-AGC TTC ACT CTC CGA ACT GC-3'
Ppar γ	F: 5'-ATCCCTGGTTTCATTAACCT-3' R: 5'-GCTCCATAAAGTCACCAAAG-3'
Ppar α	F: 5'-GGATGTCACACAATGCAATTG-3' R: 5'-TCACAGAACGGCTTCCTCAGGT-3'
C/Ebp α	F: 5'-CAAGAACAGCAACGAGTACCG-3' R: 5'-GTCAGTGGTCAACTCCAGCAC-3'
Adiponectin	F: 5'-TGTTCCCTTAATCCTGCCCA-3' R: 5'-CCAACCTGCACAAGTTCCTT-3'
Leptin	F: 5'-GTGGCTTTGGTCTATCTGTC-3' R: 5'-CGTGTGTGAAATGTCATTGATCC-3'
LipE	F: 5'-AAGGACTTGAGCAACTCAGA-3' R: 5'-TTGACTATGGGTGACGTGTA-3'
Pgc1 α	F: 5'-ATACCGCAAAGAGCAGAGAA-3' R: 5'-CTAAGAGCAGCGAAAGCGTCA-3'
Il6	F: 5'-CTGCAAGAGACTTCCATCCAG-3' R: 5'-AGTGGTATAGACAGGTCTGTTGG-3'
Il12	F: 5'-TGTTTGGCATCGTTTGGCTG-3' R: 5'-ACAGGTGAGGTTCACTGTTTCT-3'
Tnf α	F: 5'-CAGGCGGTGCCTATGTCTC-3' R: 5'-CGATCACCCCGAAGTTCAGTAG-3'
Ucp1	F: 5'-TATCATCACCTTCCCGCTG-3' R: 5'-GTCATATGTACCAGCTCTG-3'
Prdm16	F: 5'-AGCCTCGCCACAACCTG-3' R: 5'-TGACCCCGGCTTCCGTTCA-3'

(Abcam, ab54481), RGC32, and α -tubulin (Cell Signaling Technology, 9099S) were used. All the antibodies were applied at 1:1000 dilution. Protein expressions were detected using an enhanced chemiluminescence kit (Millipore).

Histological analysis

After fixing in 4% paraformaldehyde overnight, the epididymal fat was dehydrated and embedded in paraffin. The sections (5 μm) were cut with a microtome. The sections were deparaffinized and stained with hematoxylin and eosin (H&E). Images were captured by a Nikon microscope. For quantitative analysis of adipocyte area, eight images of H&E-stained sections were acquired from each animal, and the cross-sectional area of each adipocyte was measured using ImageJ software.

Statistical analyses

Data are presented as means \pm s.d., and the numbers of independent experiments are indicated for each data set. For statistical analysis, the two groups were compared using two-tailed Student's *t*-tests, and the four groups were

evaluated by two-way ANOVA followed by Bonferroni *post hoc* tests for multiple comparisons, whereas the three and five groups were evaluated by one-way ANOVA followed by Tukey's multiple comparisons using GraphPad Prism 5.0 software (La Jolla, CA, USA). $P < 0.05$ was considered statistically significant.

Results

RGC32 deficiency prevented HFD-induced obesity

Our previous study has shown that the body size of *Rgc32*^{-/-} mice is smaller compared with their WT littermates when they are born (Cui *et al.* 2013). Interestingly, the difference is not as dramatic as they age, suggesting that RGC32 has little effect on the post-natal growth on the regular chow diet. It is unknown, however, whether RGC32 affects HFD-induced obesity. To test this, we fed WT mice with HFD for 12 weeks and then detected RGC32 expression in adipose tissue. We found that RGC32 expression was dramatically upregulated by the HFD (Fig. 1A). To investigate the potential role of RGC32 in obesity, the WT and *Rgc32*^{-/-} mice were fed with HFD for 12 weeks. The HFD-fed WT mice gained

significantly more weight than the normal chow controls. However, *Rgc32*^{-/-} appeared to diminish the weight gain (Fig. 1B). The weight of epididymal fat pads was also markedly lower in HFD-fed *Rgc32*^{-/-} mice as compared to HFD-fed WT mice, although it was increased compared with the normal chow controls (Fig. 1C). Histological analysis of epididymal fat showed that HFD induced a significant adipocyte hypertrophy (more than fivefold) in WT mice. However, this effect was significantly reduced in *Rgc32*^{-/-} mice (Fig. 1D and E). To determine whether the lean phenotype of *Rgc32*^{-/-} mice was due to a reduced energy intake, we housed the mice individually in metabolic cages and monitored the food intake. As shown in Fig. 1F, the energy intake of WT and *Rgc32*^{-/-} mice fed with HFD was increased compared with the normal chow controls, while there was no difference between WT and *Rgc32*^{-/-} mice fed on either normal chow or HFD. There were also no significant differences in the water intake, urine, and feces (data not shown). To assess the energy expenditure, we measured the body weight before and after an 8-h fast. In the absence of energy intake, greater loss of body weight indicates increased energy expenditure. After fasting, although there was no significant difference between WT and *Rgc32*^{-/-} mice under chow conditions,

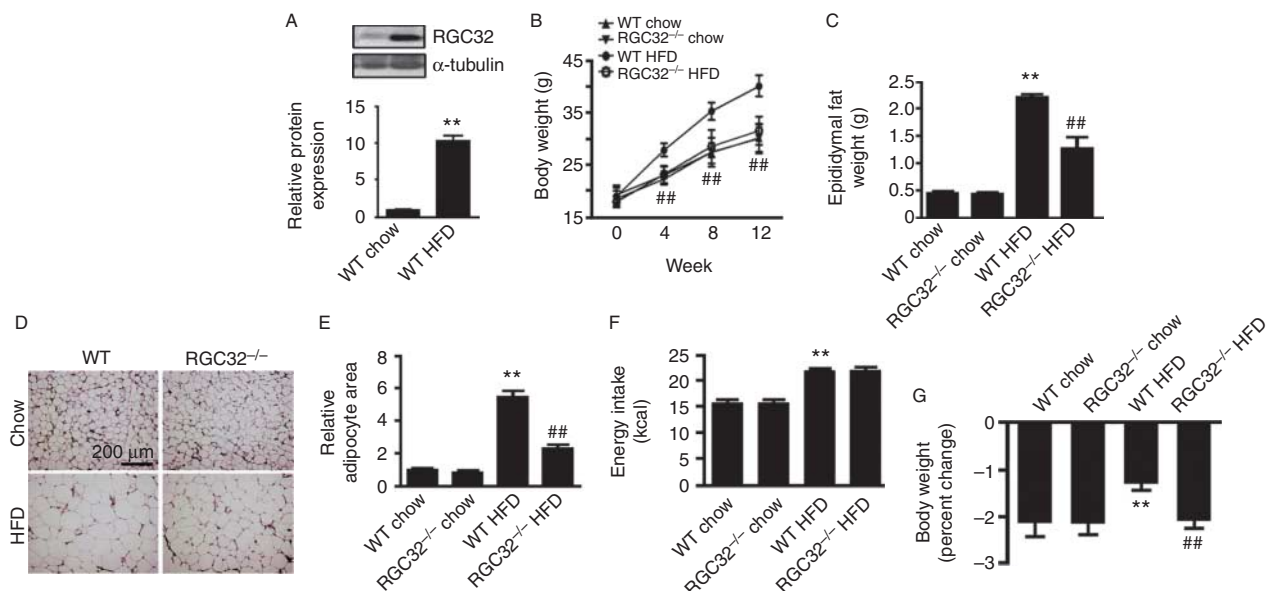


Figure 1

RGC32 deficiency prevented HFD-induced obesity. (A) RGC32 expression in adipose tissue of WT mice fed with normal chow or a 12-week high-fat diet (HFD) were detected by western blotting and normalized to α -tubulin ($n = 3$). (B) Body weight of WT and *Rgc32*^{-/-} mice fed with normal chow ($n = 6$) or HFD ($n = 10$). (C) Weights of epididymal fat from WT and *Rgc32*^{-/-} mice fed on normal chow and HFD. (D) Representative H&E-stained images of epididymal fat from WT and *Rgc32*^{-/-} mice fed on normal chow

and HFD. (E) Quantitative analysis of the mean adipocyte area. The areas were normalized to the mean adipocyte area of WT mice fed on normal chow. (F) Energy intake of WT and *Rgc32*^{-/-} mice fed on normal chow and HFD. (G) Body weight change of WT and *Rgc32*^{-/-} mice after an 8-h fast. ** $P < 0.01$ compared with WT chow group, ## $P < 0.01$ compared with WT HFD group. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-14-0548>.

HFD-fed *Rgc32*^{-/-} mice lost more body weight than HFD-fed WT mice (Fig. 1G), suggesting that the energy expenditure was increased in HFD-fed *Rgc32*^{-/-} mice, which may, at least partially, be responsible for the lean phenotype of HFD-fed *Rgc32*^{-/-} mice.

RGC32 deficiency improved metabolic homeostasis in HFD-fed mice

Diet-induced obesity is typically accompanied by dyslipidemia and insulin resistance. Therefore, we measured serum triglyceride and cholesterol concentrations. No difference was observed between WT and *Rgc32*^{-/-} mice on normal chow (Fig. 2A and B). However, on HFD, WT mice exhibited significantly increased serum concentrations of triglyceride, HDL cholesterol, and LDL/VLDL cholesterol (Fig. 2A and B). Importantly, *Rgc32*^{-/-} mice appeared to be resistant to the HFD-induced increase in serum triglyceride and cholesterol. The serum triglyceride and LDL/VLDL cholesterol concentrations in *Rgc32*^{-/-} mice were not altered by the HFD feeding and thus were much lower compared with the HFD-fed WT control. HDL cholesterol was slightly lower in HFD-fed *Rgc32*^{-/-} mice

than the WT control, although it was increased compared with *Rgc32*^{-/-} mice fed with normal chow (Fig. 2A and B).

To determine whether RGC32 affects insulin sensitivity, blood glucose and serum insulin levels were detected. *Rgc32*^{-/-} mice showed similar fasting blood glucose and insulin levels compared with WT mice fed with normal chow (Fig. 2C and D). Thus, the homeostasis model assessment-insulin resistance (HOMA-IR) scores had no difference (Fig. 2E). HFD significantly increased fasting blood glucose level, insulin concentration, and HOMA-IR score in WT mice but not in *Rgc32*^{-/-} mice (Fig. 2C, D, and E), suggesting that insulin sensitivity is improved due to RGC32 deficiency. Improved insulin sensitivity in HFD-fed *Rgc32*^{-/-} mice was also confirmed using ITT, although there was no difference between WT and *Rgc32*^{-/-} mice fed with normal chow (Fig. 3A, B, and C). Furthermore, GTT showed that on normal chow, blood glucose levels appeared to be improved at 15 and 30 min (Fig. 3D), and the area under the curve (AUC) was significantly lower in *Rgc32*^{-/-} mice compared with WT controls (Fig. 3F), indicating that the glucose tolerance was improved in *Rgc32*^{-/-} mice under chow conditions. HFD-fed WT mice developed severe glucose intolerance,

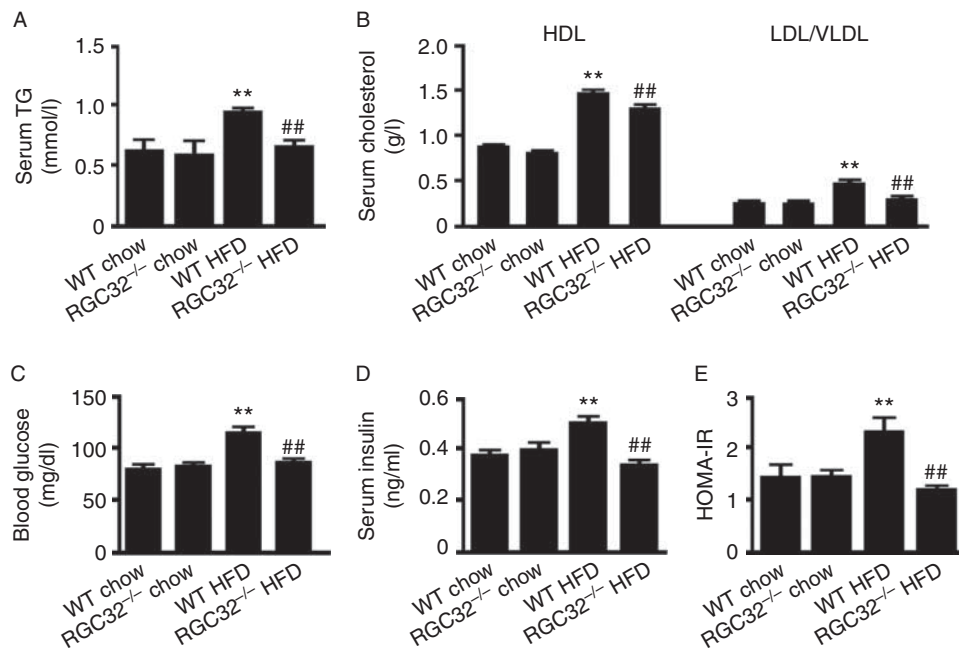


Figure 2

RGC32 deficiency improved metabolic homeostasis in HFD-fed mice. (A) Serum triglyceride (TG), (B) HDL cholesterol, and LDL/VLDL cholesterol concentrations in WT and *Rgc32*^{-/-} mice fed on normal chow and HFD ($n=6$). (C) Fasting blood glucose, (D) insulin concentration, and

(E) homeostasis model assessment-insulin resistance (HOMA-IR = fasting glucose \times fasting insulin/22.5) in WT and *Rgc32*^{-/-} mice fed on normal chow and HFD ($n=6$). ** $P < 0.01$ compared with WT chow group, ## $P < 0.01$ compared with WT HFD group.

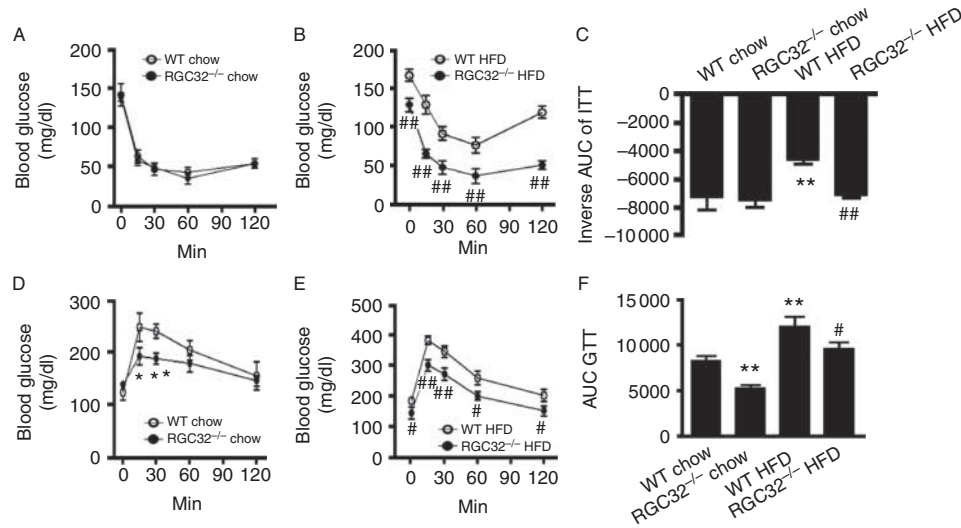


Figure 3

RGC32 deficiency prevents HFD-induced insulin resistance in mice. (A and B) Insulin tolerance test (ITT) in WT and *Rgc32*^{-/-} mice fed on normal chow (A) and HFD (B) ($n=6$). (C) Inverse area under the curve (AUC) of ITT. (D and E) Glucose tolerance test (GTT) in WT and *Rgc32*^{-/-} mice fed on

normal chow (D) and HFD (E) ($n=6$). (F) Quantification of the AUC of GTT. * $P<0.05$, ** $P<0.01$ compared with WT chow group, # $P<0.05$, ## $P<0.01$ compared with WT HFD group.

whereas *Rgc32*^{-/-} mice showed a significantly improved glucose tolerance compared with the HFD-fed WT mice (Fig. 3E and F). These data demonstrate that RGC32 deficiency improves glucose tolerance and protects mice from HFD-induced insulin resistance.

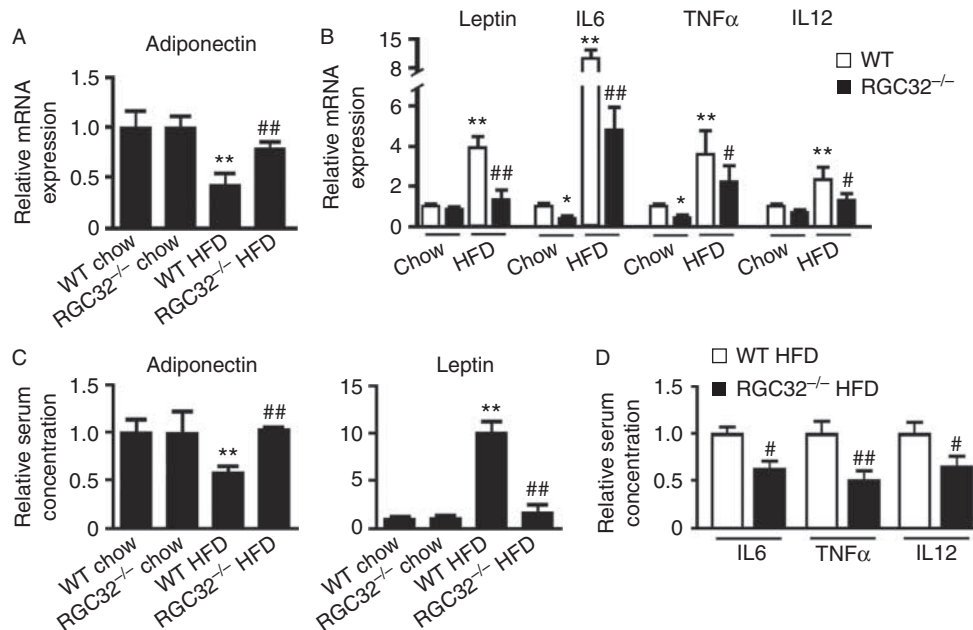
RGC32 deficiency attenuated adipose tissue and systemic inflammation in HFD-fed mice

Since adipose tissue inflammation and low levels of systemic inflammation are important factors contributing to the development of obesity, we detected adipose tissue and serum inflammatory status of the mice. RGC32 deficiency appeared to decrease *Il6* and *Tnf α* mRNA expression in the fat tissue under chow conditions, while had no effect on other adipokines such as adiponectin, leptin, and IL12 (Fig. 4A and B). HFD dramatically decreased adiponectin, an anti-inflammatory adipokine, while increased the expression of pro-inflammatory adipokines leptin, IL6, TNF α , and IL12 in the fat tissue of WT mice. However, these effects were alleviated in *Rgc32*^{-/-} mice (Fig. 4A and B). Similar results were observed with the circulating adipokines (Fig. 4C and D), although the circulating IL6, TNF α , and IL12 were undetectable under chow conditions (data not shown). These data demonstrate that RGC32 deficiency attenuates adipose tissue and systemic inflammation in HFD-fed mice.

RGC32 deficiency increased the expression of metabolic genes in adipose tissues

Since HFD-fed *Rgc32*^{-/-} mice had reduced fat mass and improved adipose tissue inflammation, we further assessed the expression of metabolic genes related to β -oxidation, lipolysis, and thermogenesis in adipose tissue. As shown in Fig. 5A, RGC32 deficiency increased the mRNA expression of *Ppara*, *Lipec*, and *Pgc1 α* in epididymal fat tissue as compared with WT controls both under normal chow and HFD conditions. Since the alterations in HFD groups were greater than those in the normal chow groups, and the body weight difference was observed in HFD groups, we confirmed the protein expression of these genes in the epididymal adipose tissue of HFD-fed mice (Fig. 5B and C).

It is known that PGC1 α can induce browning of subcutaneous adipose tissue through regulation of uncoupling protein 1 (UCP1) (Bostrom *et al.* 2012). We further determined adipose tissue browning in interscapular and inguinal fat tissues of WT and *Rgc32*^{-/-} mice. As shown in Fig. 5D and E, PGC1 α , UCP1, and PRDM16 were all increased in both interscapular and inguinal fat tissues of *Rgc32*^{-/-} mice compared with WT controls under both normal chow and HFD conditions. These data suggest that RGC32 deficiency induces browning of adipose tissues, leading to increased energy expenditure.

**Figure 4**

RGC32 deficiency attenuated adipose tissue and systemic inflammation in HFD-fed mice. (A and B) mRNA expression of adiponectin, leptin, interleukin 6 (IL6), tumor necrosis factor α (TNF α), and IL12 in epididymal adipose tissue from WT and *Rgc32*^{-/-} mice ($n=6$) was measured by qPCR.

(C and D) Protein concentration of adiponectin, leptin, IL6, TNF α , and IL12 in the serum from WT and *Rgc32*^{-/-} mice ($n=6$) was measured by cytometric bead array immunoassay. * $P<0.05$, ** $P<0.01$ compared with WT chow group, # $P<0.05$, ## $P<0.01$ compared with WT HFD group.

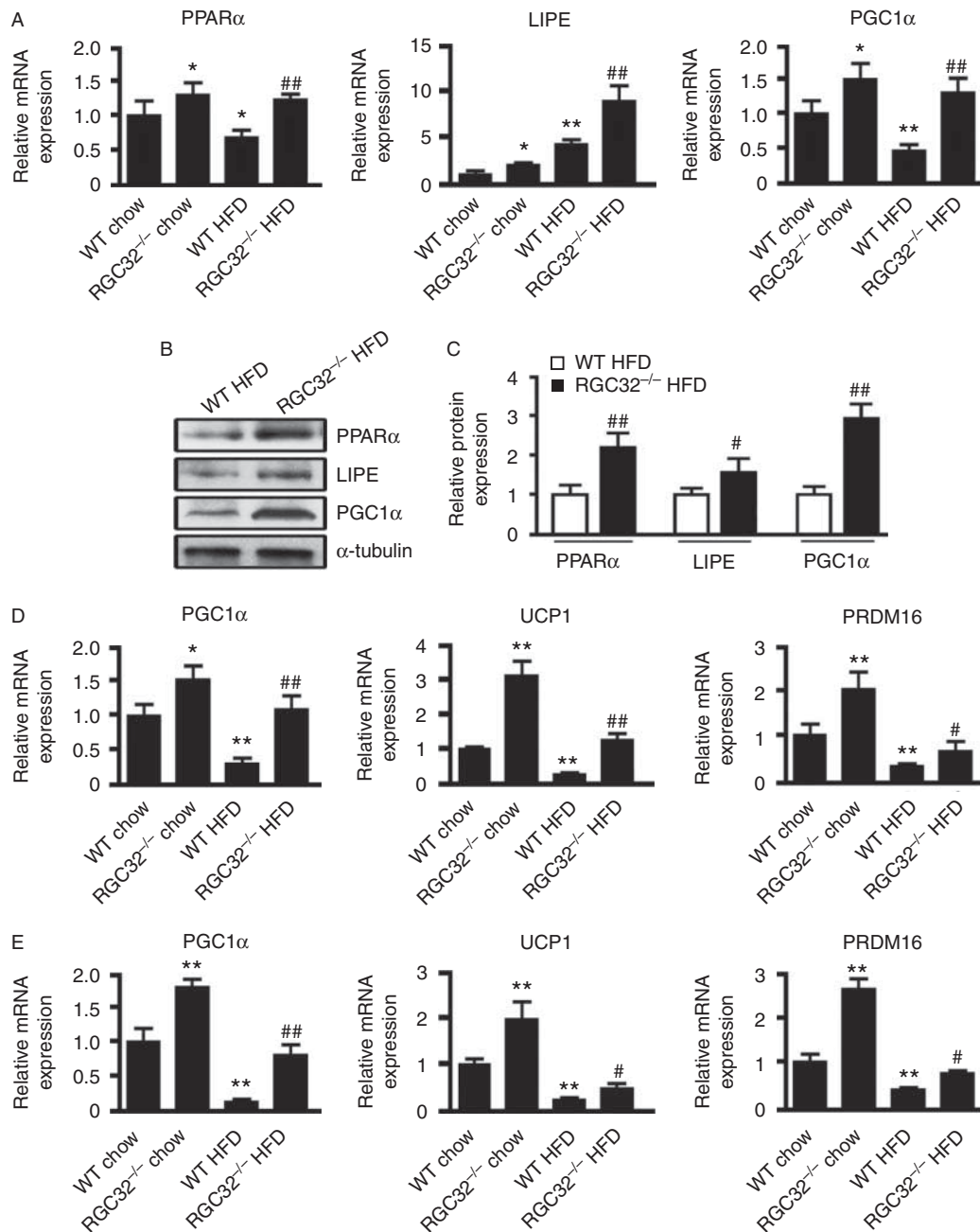
RGC32 had no effect on adipocyte differentiation

Since HFD induced a significant adipocyte hypertrophy in WT mice (Fig. 1D and E), we sought to determine whether RGC32 promotes adipocyte differentiation. RGC32 has been shown to induce smooth muscle cell differentiation (Li *et al.* 2007). In the differentiation of 3T3-L1 preadipocytes, RGC32 expression was dramatically increased accompanied with elevated expression of adipogenic differentiation specific genes *Ppar γ* and *C/Ebp α* (Fig. 6A, B, and C). To test RGC32 function in this process, we knocked down or overexpressed RGC32 in 3T3-L1 cells followed by induction of adipocyte differentiation and found that neither knockdown nor overexpression of RGC32 had any effect on the expression of adipogenic-specific genes (Fig. 6D) or lipid accumulation (Fig. 6E and F). These data indicate that the role of RGC32 in obesity was not due to an increased adipocyte differentiation. To confirm the regulation of energy expenditure-related genes by RGC32 as observed in the fat tissue (shown in Fig. 5A, B, and C), we detected the protein expression of PPAR α , LIPE, and PGC1 α in adipocytes differentiated from 3T3-L1 cells. Consistently, all of these genes were increased when RGC32 was knocked down (Fig. 6G and H). The expression of *Lipe*

and *Pgc1 α* was decreased when RGC32 was overexpressed. *Ppar α* expression appeared not to be dramatically affected by RGC32 overexpression (Fig. 6G and H). These data indicate that RGC32 deficiency prevents mice from developing diet-induced obesity through the upregulation of energy expenditure-related genes in adipose tissues.

Discussion

Our present study demonstrates for the first time that RGC32 plays an important role in HFD-induced obesity. RGC32 is strongly upregulated in adipose tissue of HFD-fed mice. RGC32 deficiency prevents the development of HFD-induced obesity and insulin resistance because *Rgc32*^{-/-} mice fed a HFD gain less weight without change in energy intake and have a reduced fat mass. Interestingly, HFD increases the fat mass of *Rgc32*^{-/-} mice without altering their body weight, which is probably because the increase in the fat mass in these mice accounts for a relatively small portion of the whole body mass and thus does not cause a significant change in their body weight (as shown in Fig. 1B). HFD-fed *Rgc32*^{-/-} mice also exhibit significantly improved insulin sensitivity as indicated by improved HOMA-IR and insulin tolerance. Our results are consistent with previous studies showing that HFD causes

**Figure 5**

RGC32 deficiency increased the expression of metabolic genes in adipose tissues. (A) mRNA expression of *Ppar α* , *LiPE*, and *Pgc1 α* in the epididymal adipose tissues of WT and *Rgc32*^{-/-} mice ($n=6$). (B and C) PPAR α , LIPE, and PGC1 α protein expression in the epididymal adipose tissues of WT and *Rgc32*^{-/-} mice were detected by western blotting analysis and normalized

to α -tubulin ($n=6$). (D and E) mRNA expression of *Pgc1 α* , *Ucp1*, and *Prdm16* in the interscapular (D) and inguinal (E) fat tissues of WT and *Rgc32*^{-/-} mice ($n=6$). * $P<0.05$, ** $P<0.01$ compared with WT chow group, # $P<0.05$, ## $P<0.01$ compared with WT HFD group.

obesity and insulin resistance (Winzell & Ahren 2004), which is a characteristics of type 2 diabetes and several cardiometabolic syndromes including hypertension and dyslipidemia (Kim *et al.* 2008). Although adipocyte differentiation is involved in diet-induced obesity (Berry

et al. 2012, Federico *et al.* 2012), it may not be involved in RGC32-mediated obesity because either overexpression or knockdown of RGC32 has no effect on the expression of adipogenic differentiation-specific genes *Ppar γ* and *C/Ebpa* and lipid accumulation of the adipocytes.

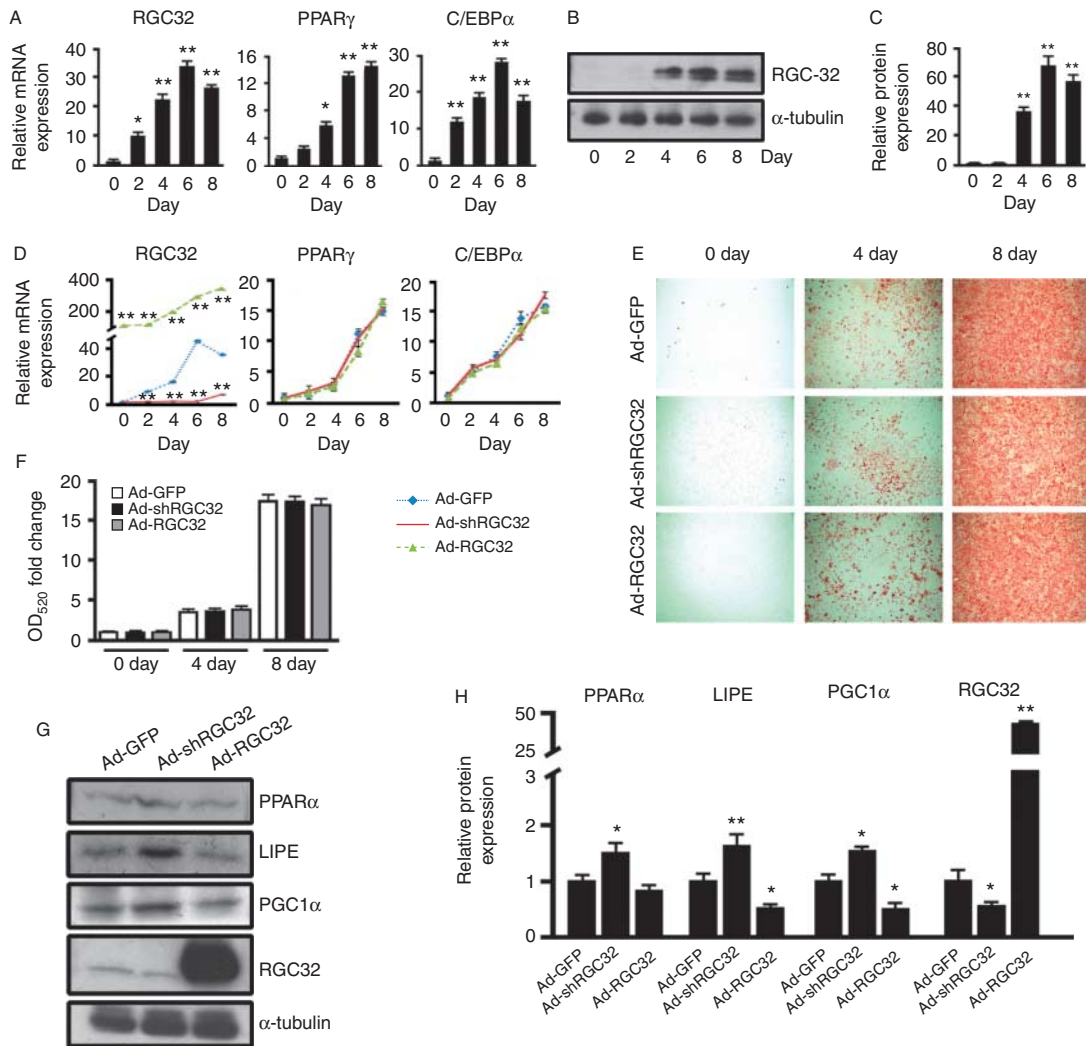


Figure 6

RGC32 had no effect on adipocyte differentiation. (A) mRNA expression of *Rgc32*, *Pparγ*, and *C/EBPα* during 3T3-L1 preadipocyte differentiation at the indicated times. (B and C) RGC32 protein expression during 3T3-L1 preadipocyte differentiation was detected by western blotting analysis and normalized to α -tubulin. * $P < 0.05$, ** $P < 0.01$ compared with vehicle-treated group (0 day). (D, E, F, G, and H) 3T3-L1 preadipocyte was transduced with Ad-GFP, Ad-shRGC32, or Ad-RGC32 for 24 h and then was induced for adipocyte differentiation. (D) mRNA expression of *Rgc32*,

Pparγ, and *C/EBPα* and (E and F) lipid droplet accumulation was determined at the indicated times. (G and H) PPAR α , LIPE, PGC1 α , and RGC32 expression was detected by western blotting analysis and normalized to α -tubulin. * $P < 0.05$, ** $P < 0.01$ compared with Ad-GFP group. All results are representatives of at least three independent experiments. A full colour version of this figure is available at <http://dx.doi.org/10.1530/JOE-14-0548>.

The protection from diet-induced obesity in *Rgc32*^{-/-} mice is linked to the attenuated adipose tissue and systemic inflammation and increased adipose tissue energy expenditure. Recent data have shown that adipose tissue inflammation is a key mechanism leading to obesity and insulin resistance (Chakrabarti *et al.* 2009, Lee *et al.* 2010). RGC32 is essential for C5b-9-induced cell cycle activation (Fosbrink *et al.* 2009), indicating an important role in the regulation of inflammation. Indeed, RGC32 deficiency suppresses adipose tissue and systemic

inflammation in HFD-fed mice, as evidenced by the reduced production of proinflammatory adipokines including leptin, IL6, TNF α , and IL12 and increased anti-inflammatory adipokine adiponectin. Dietary excess and obesity have been shown to cause lipid accumulation in adipocytes, initiating a state of cellular stress and activation of NF κ B signaling pathway (Shoelson *et al.* 2006), leading to an increased adipocyte production of proinflammatory cytokines. Our previous study has shown that RGC32 activates NF κ B in vascular endothelial

cells (Cui *et al.* 2013). Therefore, RGC32 may increase inflammation of adipose tissue through activating NF- κ B signaling pathway, which may be studied in the future.

RGC32 appears to regulate obesity development by influencing the thermogenesis. Particularly, RGC32 may suppress the expression of metabolic genes *PPAR α* , *LiPE*, and *PGC1 α* because dramatic elevation of *PPAR α* , *LIPE*, and *PGC1 α* is observed in the adipose tissue of HFD-fed *Rgc32*^{-/-} mice. *LIPE* is a rate-limiting enzyme to cleave fatty acids from the triglyceride molecule (Schweiger *et al.* 2006). *PPAR α* promotes uptake, utilization, and catabolism of fatty acids by upregulating the genes involved in fatty acid transport; fatty acid binding and activation; and peroxisomal and mitochondrial fatty acid β -oxidation (Rakhshandehroo *et al.* 2010). *PGC1 α* enhances thermogenesis and oxidative metabolism of adipose tissue (Liang & Ward 2006, Jun *et al.* 2014). Indeed, RGC32 deficiency promotes the browning of adipose tissues. Brown adipose tissue is known to dissipate chemical energy and protect against obesity through a process termed as nonshivering thermogenesis (Bi *et al.* 2014). Active brown adipose tissue burns lipids to produce heat, resulting in an increase in energy expenditure. *PGC1 α* and *UCP1* are highly expressed in brown adipose tissue (Fisher *et al.* 2012), and *PRDM16* is a brown adipose determination factor (Seale *et al.* 2011). Increased expression of these genes in interscapular and inguinal fat tissues may have collectively caused the increased energy expenditure and the lean phenotype of HFD-fed *Rgc32*^{-/-} mice. Besides adipose tissue, defective energy expenditure of skeletal muscle also contributes to diet-induced obesity and insulin resistance. However, since RGC32 expression is undetectable in skeletal muscle, contribution of skeletal muscle energy expenditure to the lean phenotype of HFD-fed *Rgc32*^{-/-} mice is likely to be minimal.

In summary, our study demonstrates that RGC32 mediates HFD-induced obesity through enhancing inflammation while decreasing energy expenditure in adipose tissues.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

X-B C conceived and carried out the experiments including data collection, analysis and interpretation, and wrote the manuscript. J-N L performed the qPCR, western blotting analysis and adipocyte differentiation experiments. J Y offered critical logistical advice on experimental design and data interpretation and critically read the manuscript. S-Y C is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis. All authors had final approval of the submitted and published versions.

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