**Crif1 Deficiency Reduces Adipose OXPHOS Capacity and Triggers Inflammation and Insulin Resistance in Mice**

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Abstract

Impaired mitochondrial oxidative phosphorylation (OXPHOS) has been proposed as an etiological mechanism underlying insulin resistance. However, the initiating organ of OXPHOS dysfunction during the development of systemic insulin resistance has yet to be identified. To determine whether adipose OXPHOS deficiency plays an etiological role in systemic insulin resistance, the metabolic phenotype of mice with OXPHOS–deficient adipose tissue was examined. Crif1 is a protein required for the intramitochondrial production of mtDNA–encoded OXPHOS subunits; therefore, Crif1 haploinsufficient deficiency in mice results in a mild, but specific, failure of OXPHOS capacity in vivo. Although adipose-specific Crif1-haploinsufficient mice showed normal growth and development, they became insulin-resistant. Crif1-silenced adipocytes showed higher expression of chemokines, the expression of which is dependent upon stress kinases and antioxidant. Accordingly, examination of adipose tissue from Crif1-haploinsufficient mice revealed increased secretion of MCP1 and TNFα, as well as marked infiltration by macrophages. These findings indicate that the OXPHOS status of adipose tissue determines its metabolic and inflammatory responses, and may cause systemic inflammation and insulin resistance.


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Introduction

White adipose tissue (WAT) determines whole-body energy metabolism by controlling lipid storage and by releasing adipokines, which may directly or indirectly affect the physiological functions of almost all cell types (for a review, see [1,2]). These adipocyte functions are perturbed by genetic and environmental factors, which lead to adipocyte dysfunction characterized by hypertrophy, hypoxia and inflammatory process within adipose tissue [3]. Adipocyte dysfunction is further characterized by impaired insulin sensitivity, which is associated with changes in cellular composition or organelle dysfunction, particularly of the endoplasmic reticulum (ER) and mitochondria. An emerging concept to explain insulin resistance in obese individuals is maladaptive responses within the ER, which are prominent in adipose tissue (for a review, see [4–6]).

Besides the ER, the mitochondria in white adipocytes are linked with adipocyte differentiation and with the function of mature adipocytes. Recent studies show that drastic increases in mitochondrial biogenesis and reactive oxygen species (ROS) production via the OXPHOS complex play a crucial role in adipocyte differentiation. In addition, the mitochondria in differentiating adipocytes support high energy-consuming lipogenic processes to maintain mature adipocyte function [5,7]. Therefore, it is suggested that the contribution of adipocyte mitochondria to whole-body energy metabolism or adipocyte plasticity may depend on the mitochondrial OXPHOS capacity of the adipose tissue [6]. Consistent with this, decreased mitochondrial capacity in adipocytes may also alter their insulin sensitivity and/or function due to the high energy requirements of fatty acid storage, adipokine secretion, insulin signaling, and glucose uptake [8,9].

It is interesting that a marked decrease in the level of transcripts for nuclear-encoded mitochondrial genes in cells derived from the epididymal fat pads of ob/ob mice accompanies the onset of obesity [10]. In db/db and diet-induced obese mice, the expression of OXPHOS genes was markedly reduced compared with that in db/+ mice and control mice fed a standard-fat diet, respectively [11]. In humans, the mtDNA copy number is enriched in adipocytes in adipose tissue, but it decreases slightly with age and increasing BMI, and shows a strong positive correlation with basal and insulin-stimulated lipogenesis in fat cells [12]. More interestingly, suppression of OXPHOS genes is prominent in the visceral adipose tissue of humans with type 2 diabetes independent of...
Author Summary

Type 2 diabetes is one of the most challenging health problems in the 21st century. Although insulin resistance is regarded as a fundamental defect that precedes the development of type 2 diabetes, the nature and cause of insulin resistance remain unknown. Adipose tissue is an important organ that determines whole-body energy metabolism, and its dysfunction is a critical element in the development of systemic insulin resistance. Adipose mitochondrial function is suppressed in the insulin-resistant state, and increased adipose mitochondrial biogenesis is associated with the reversal of insulin resistance by a PPARγ agonist. However, despite these important observations, little is known about how mitochondrial respiratory dysfunction in white adipose tissue (WAT) causes insulin resistance. To determine whether adipose deficiency of mitochondrial respiratory capacity plays an etiological role in systemic insulin resistance, the metabolic phenotype of mice with mitochondrial OXPHOS (oxidative phosphorylation)–deficient adipose tissue was examined. Crif1 is a protein required for the translation of mtDNA–encoded OXPHOS subunits. Interestingly, mice haploinsufficient for Crif1 in adipose tissue showed reduced OXPHOS capacity and developed marked insulin resistance.

Obesity [13]. Agonists of peroxisome proliferator-activated receptor-gamma (PPARγ) increase the number of mitochondria and induce mitochondrial remodeling in adipocytes [10,11,14], and significantly increase the mitochondrial copy number and expression of factors involved in mitochondrial biogenesis, including PPARγ coactivator-lalpha (PGC1α) and mitochondrial transcription factor A (TFAM), which are required for mitochondrial transcription of OXPHOS genes in humans [15]. These observations in rodent models and human subjects suggest that the OXPHOS capacity of adipose tissue may affect the changes in adipocyte plasticity, which controls insulin sensitivity and may determine the therapeutic responsiveness to antidiabetic agents such as thiazolidinediones and CB1 receptor blockers that affect the mitochondrial content of adipocytes [10,16].

Here, we demonstrate that primary OXPHOS dysfunction in adipose tissue causes insulin resistance and a diabetic phenotype in mice with a Crif1 loss-of-function mutation. Crif1 is a mitochondrial protein that associates with large mitoribosomal subunits, which are located close to the polypeptide exit tunnel, and the elimination of Crif1 led to both aberrant synthesis and defective insertion of mtDNA-encoded nascent OXPHOS polypeptides into the inner membrane [17]. Targeted elimination of the Crif1 gene resulted in a phenotype characterized by organ-specific failure of OXPHOS function; therefore, we attempted to identify the adipose tissue phenotypes of adipose-specific Crif1–deficient mice. Consistently, mice lacking Crif1 in adipose tissue showed reduced OXPHOS capacity and developed marked insulin resistance.

Results

Homoyzgotic loss of Crif1 causes marked impairment of WAT development

Crif1 is a mitochondrial protein that specifically interacts with the protein components of the large subunit of the mitochondrial ribosome [17]. It specifically regulates the translation and insertion of the 13 polypeptide subunits that comprise mitochondrial OXPHOS complexes I, III, IV and V. Homozygous Crif1–null mouse embryonic fibroblasts (MEFs) showed a profound failure in translation and expression of these subunits, along with markedly low levels of basal and stimulated (CCCP-treated) mitochondrial oxygen consumption [17]. Disruption of the mouse Crif1 gene consistently resulted in a profound OXPHOS deficiency characterized by the loss of OXPHOS complex subunits and respiratory complexes in vivo.

Crif1 mRNA is ubiquitously expressed, and it is highly expressed in brain, heart, liver kidney and skeletal muscle (Figure S1A). Two types of adipose tissues, brown (BAT) and white (WAT), contained substantial amounts of Crif1 mRNA (Figure S1A). Crif1 mRNA levels were decreased in the WAT, BAT and liver of db/db and ob/ob mice compared to db/+ and ob/+ mice, respectively (Figure S1B). Interestingly, Crif1 mRNA expression in WAT of C57BL/6 mice was downregulated when they were fed a high fat diet (HFD) for 8 weeks (Figure S1C). These findings indicate that Crif1 expression correlates with the nutritional status in adipose tissue.

To identify the roles of Crif1 and mitochondrial OXPHOSs in adipose tissue, we tried to induce primary OXPHOS deficiency in adipose tissue in vivo using conditional Crif1 knockout mice. We crossed conditional Crif1 mice (Crif1flox/flox) [18] with mice expressing a Cre recombinase gene under the control of the fatty acid binding protein-4 (Fabp4) promoter (Fabp4-Cre) and the adiponectin promoter (Adipoq-Cre). The resulting pups were born healthy and viable, and showed a normal Mendelian ratio. However, these homozygous Crif1f/f,Fabp4Cre mice showed delayed weight gain and poor development of adipose tissue (Figure 1A–1C). Unlike the control (Crif1+/+,Fabp4Cre) and Crif1 heterozygous (Crif1+/+,Fabp4Cre) mice, Crif1f/f,Fabp4Cre mice showed uniform lethality within 24 days of birth (median survival = 19.4 days) (Figure 1D).

The perirenal, subcutaneous and epididymal fat pads of Crif1f/f,Fabp4Cre mice comprised small adipocytes with dystrophic changes (Figure 1E). To verify any mitochondrial abnormalities, the adipose tissues of Crif1f/f,Fabp4Cre mice were examined by transmission electron microscopy (TEM). The adipocytes of these mice contained mitochondria with ultrastructural abnormalities, such as swollen and distorted cristae, but mitochondrial number was unaffected (Figure 1F and 1G). In heterozygous Crif1f/f,Fabp4Cre mice, hematoyxin and cosin (H&E) staining of adipose tissue showed no evidence of histological abnormalities compared with the controls (Figure 1E). Consistent with the results of H&E staining, the mitochondria of Crif1f/f,Fabp4Cre mice showed no morphological or numerical abnormalities of mitochondria in TEM (Figure 1F and 1G). Collectively, this comprehensive analysis of the adipose tissues in Crif1f/f,Fabp4Cre mice indicated that loss of Crif1 results in a marked failure of WAT and BAT development.

Characterization of BAT in Crif1f/f,Fabp4Cre mice

The Fabp4-Cre transgene is expressed and localized within the dorsal root ganglion, centrum of the vertebra and the carpals of the embryo from the mid-gestation stage [19]. Neonatal Crif1−/−,Fabp4Cre or Crif1f/f,Fabp4Cre mice did not show developmental abnormalities when compared with control mice. Therefore, embryonic expression of the Fabp4-Cre transgene may not affect the development of Crif1−/−,Fabp4Cre and Crif1f/f,Fabp4Cre mice, and may not be a plausible reason for observed lethality at around postnatal Week 3. Mice are normally weaned at post-natal Week 3, at which point the rate of lipogenesis and UCP1 expression in the BAT rises sharply and reaches maximal levels to enhance thermogenesis [20]. The Fabp4-Cre transgene was uniformly detected in BAT from the early post-natal period (Day 7), the
Figure 1. Marked failure of adipose tissue development in Crif1<sup>f/f</sup>,Fabp4<sup>mice</sup>. Mice were generated by breeding two mouse lines, a Crif1<sup>lox/lox</sup> transgenic mouse line and a Fabp4-Cre recombinase transgenic mouse line. Data from Crif1<sup>f/f</sup>,Fabp4<sup>mice</sup> were obtained at 3 weeks-of-age. (A) Gross characteristics of control mice (Crif1<sup>+/+</sup>,Fabp4<sup>+</sup>), adipose-specific Crif1 heterozygous mice (Crif1<sup>f/+</sup>,Fabp4<sup>+</sup>), and adipose-specific Crif1 homozygous knockout mice (Crif1<sup>f/f</sup>,Fabp4<sup>+</sup>), TS, testis. (B and C) Body weight and tissue weight relative to control mice (n = 10). Values are means + SD, *p < 0.05 versus control mice. BAT, brown adipose tissue; WAT, white adipose tissue. (D) Crif1<sup>f/f</sup>,Fabp4<sup>mice</sup> showed markedly reduced survival rates after 2 weeks-of-age (n = 20). (E) Hematoxylin & eosin (H&E) staining of perirenal WAT (pWAT). Scale: 100 μm. (F) Transmission electron microscopy (TEM) of subcutaneous WAT (sWAT) revealed that the mitochondria of Crif1<sup>f/f</sup>,Fabp4<sup>mice</sup> developed swollen cristae (red arrows). L, lipid droplet. Scale: 6,000 nm. (G) Mitochondria number per area in sWAT (n = 10). Values are means + SD. n.s, not significant.
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Crif1 protein and OXPHOS complex subunits are downregulated in the BAT of 3-week-old mice (Table S1). As shown in Figure 1C, Crif1+/+;Fabp4−/− mice had less BAT at Day 21, but histological examination of inter-scapular BAT showed normal histological findings (Figure S2A). Crif1+/+;Fabp4−/− mice had fewer mitochondria than control mice, but these were larger in size and were characteristically disorganized and swollen, suggesting OXPHOS defects (Figure S2B and S2C). Consistent with these findings, Crif1+/+;Fabp4−/− mice showed a low body temperature under ambient conditions (23°C) and rapidly reached a fatally low rectal temperature within 5 minutes of immersion in cold water (4°C) (Figure S2D). However, although the mass of BAT was reduced, the level of UCP1 expression was not altered in Crif1+/+;Fabp4−/− mice (data not shown). When Crif1+/+;Fabp4−/− mice were housed at thermoneutrality (30°C), the median survival rate was increased and mortality was reduced (Figure S2E). This indicates that thermal stress caused by mitochondrial OXPHOS dysfunction in BAT following Crif1 ablation may be a critical factor in the premature death of Crif1+/+;Fabp4−/− mice.

By contrast, the BAT of Crif1+/+;Fabp4−/− mice showed normal development and histological and ultrastructural findings (Figure S2A–S2C). Furthermore, the response of Crif1+/+;Fabp4−/− mice (in terms of core temperature) to a cold environment were identical to those of control mice (Figure S2D). These results showed that Fabp4−/− driven haploinsufficiency of Crif1 may not affect the physiological function of BAT.

Characterization of mitochondrial OXPHOS function in Crif1+/+;Fabp4−/− mice

A previous study revealed that Crif1-deficient (−/−) MEFs prepared from Crif1−/− mice showed marked OXPHOS defects due to a profound failure of translation and insertion of the newly-synthesized OXPHOS polypeptides encoded by the mtDNA. Also, Crif1+/− MEFs showed increased anaerobic glycolysis, which eventually led to accelerated cell death [17]. Similar to Crif1−/− MEFs, loss of Crif1 in adipose-derived stem cells (ADSCs) (Crif1−/−) resulted in marked impairment of differentiation and accelerated cell death, which prevented functional analysis of the mitochondria (data not shown). However, control (Crif1+/+) and Crif1-haplinsufficient ADSCs (Crif1+/−) prepared from Crif1+/+;Fabp4−/− and Crif1+/+;Fabp4−/− mice showed identical levels of cell viability and differentiation to those of control cells (Figure 2A and 2B). Interestingly, Crif1+/− ADSCs showed lower expression of OXPHOS subunits (ND1, NDUFA9, UQCRCC2 and COX4) and assembled OXPHOS complex I on Western blot and Blue Native PAGE (BN-PAGE) analysis, respectively (Figure 2C and 2D). Crif1+/− ADSCs consumed less oxygen under basal conditions and showed reduced maximal OXPHOS capacity (Figure 2E). Taken together, Crif1 haploinsufficiency in adipocytes resulted in normal differentiation but reduced genetically-determined OXPHOS capacity.

Several experimental criteria have been proposed to test whether a primary in vivo OXPHOS deficiency plays a causal role in insulin resistance [21]. One of these criteria is that perturbations in OXPHOS gene expression and function must be as modest as possible [21]. Thus, we analyzed Crif1 and OXPHOS gene expression to test whether Crif1+/+;Fabp4−/− mice were suitable for our proposed experiments. Compared with Crif1+/+;Fabp4−/− mice, Crif1+/+;Fabp4−/− mice showed about 50% of the Crif1 mRNA and protein expression in epididymal WAT (eWAT) (Figure 2F and 2H). Although basal ATP levels in eWAT were not affected by Crif1 haploinsufficiency (Figure 2G), the expression levels of OXPHOS complex I, III and IV subunits were reduced in the epididymal fat pads of Crif1+/+;Fabp4−/− mice (Figure 2H). BN-PAGE analysis showed that the levels of Complex I and IV and supercomplex in WAT were approximately 20%, 40% and 50% lower, respectively, in Crif1+/+;Fabp4−/− mice compared to control mice (Figure 2I and 2J). However, normal levels of Crif1 and OXPHOS complexes were expressed in the liver and heart of Crif1+/+;Fabp4−/− mice (Figure S3A–S3C). In contrast to homozygous Crif1+/+;Fabp4−/− mice, heterozygous Crif1+/+;Fabp4−/− mice exhibited normal levels of OXPHOS subunits in BAT and mitochondrial morphology was normal (Figure 2H–2J and Figure S2B).

Food intake and weight gain were comparable in Crif1+/+;Fabp4−/− and Crif1+/+;Fabp4−/− mice when fed a normal chow diet (NCD) (Figure S4A and S4B). MR images of control and Crif1+/+;Fabp4−/− mice fed a NCD or a HFD showed a similar pattern of adipose distribution (Figure S4C). Triglyceride levels in the liver and plasma of Crif1+/+;Fabp4−/− mice were the same as those in control mice, regardless of whether they were fed a NCD or a HFD. Serum free fatty acid (FFA) levels tended to be higher in Crif1+/+;Fabp4−/− mice, but were not significantly different from those in control mice (Figure S4D–S4F). Taken together, these results show that Crif1+/+;Fabp4−/− mice have mildly reduced primary OXPHOS deficiency in adipose tissue but, unlike the lipodystrophic model, they show no defects in adipose tissue development, and no hyperlipidemia or ectopic lipid accumulation.

Insulin resistance in Crif1+/+;Fabp4−/− mice

To identify the relationship between insulin resistance and reduced OXPHOS capacity in adipocytes in vivo, control and Crif1+/+;Fabp4−/− mice were subjected to glucose tolerance tests after 8 weeks or 14 weeks on a NCD or HFD. Neither control nor Crif1+/+;Fabp4−/− mice fed a NCD for 8 weeks showed any differences in glucose tolerance following an intraperitoneal injection of glucose (IPGTT, 2 g/kg body weight) (Figure 3A). However, Crif1+/+;Fabp4−/− mice fed a NCD for 14 weeks developed glucose intolerance (Figure 3B). More impressively, Crif1+/+;Fabp4−/− mice fed a HFD for 8 weeks showed an earlier onset of glucose intolerance, which was characterized by higher peak glucose levels than those measured in control mice in the intraperitoneal glucose tolerance tests (Figure 3C). Crif1+/+;Fabp4−/− mice fed a HFD for 14 weeks showed more advanced glucose intolerance, with higher basal (168.8±13.2 mg/dL vs 131.3±8 mg/dL) and peak (516.2±34.8 mg/dL vs 420.4±52.3 mg/dL) plasma glucose levels (Figure 3D). Therefore, regardless of the caloric state, mice with Crif1 haploinsufficiency showed reduced glucose tolerance. Crif1+/+;Fabp4−/− mice fed a HFD for 14 weeks showed decreased Akt phosphorylation in the liver and muscle and a reduced glucose disposal rate after an intraperitoneal insulin challenge (Figure 3E and 3F). Furthermore, suppression of hepatic glucose production (HGP) by insulin was not different between the two groups, but the glucose infusion rate (GIR) and glucose uptake rate decreased by approximately 18.6% and 14.7%, respectively, during hyperinsulinemic euglycemic clamping after 14 weeks on a HFD (Figure 3G); these data supported the insulin tolerance tests (ITT) results. These findings indicate that Crif1+/+;Fabp4−/− mice, which have limited OXPHOS capacity in their adipose tissue, may show exacerbated diabetic mechanisms, which are characterized by insulin resistance.

The levels of saturated fatty acids and ceramides in WAT, muscle and liver were not significantly altered in Crif1+/+;Fabp4−/− mice (Figure S4G and S4H). Thus, abnormal accumulation of ceramides and saturated fatty acids in insulin sensitive tissues does not appear to underlie the insulin resistance of Crif1+/+;Fabp4−/− mice (Figure S4G and S4H).

Dysregulation of chemokines in Crif1-deficient differentiated adipocytes in vitro

To determine the molecular pathways that are dysregulated by mitochondrial OXPHOS dysfunction following Crif1 knockdown...
Figure 2. Dysfunctional mitochondria in Crif1 haploinsufficient adipocytes. (A) Adipose derived stem cells (ADSCs) were isolated from eWAT of Crif1+/+ and Crif1+/− mice. The number of Crif1+/+ and Crif1+/− ADSCs was counted in 96 well plates after 10 days of differentiation (n = 6). Values are means ± SD. n.s, not significant. (B) Oil red O staining of Crif1+/+ and Crif1+/− ADSCs. (C) OXPHOS complex subunits were detected in Adipose OXPHOS Deficiency and Insulin Resistance.
by western blotting with the appropriate antibodies. Anti-ND1 and anti-NDUF4A9 were used to detect OXPHOS complex I, anti-UQRC2 was used for complex III, and anti-COX4 was used for complex IV. (D) BN-PAGE analysis using a mixture of anti-OXPHOS antibodies to detect assembled OXPHOS complexes. (E) Oxygen consumption rates (OCR) measured by Seahorse XF-24 flux analyzer in Crif1+/+ and Crif1−/− ADSCs (n = 6). Values are means ± SD, *p < 0.05. CCCP, carbonyl cyanide m-chlorophenyl hydrazine. (F) Crif1 mRNA expression in eWAT, BAT, liver and heart of Crif1+/+Fabp4 and Crif1−/−Fabp4 mice (n = 8). Values are means ± SD, *p < 0.05. (G) Level of ATP in eWAT of Crif1+/+Fabp4 and Crif1−/−Fabp4 mice (n = 8). Values are means ± SD. n.s, not significant. (H) Western blots analysis of Crif1 and OXPHOS subunits from eWAT and BAT of mice. ND1 and NDUF4A9, subunit of OXPHOS complex I; UQRC2, subunit of OXPHOS complex III; COX4, subunit of OXPHOS complex IV. (I) BN-PAGE analysis of assembled OXPHOS complexes (I, II, III, IV and VI). Percentage of band intensities are presented in the graph (n = 4). Values are means ± SD. *p < 0.05. (J) BN-PAGE analysis of supercomplex (I+II2+III1+IV+III2+I+II).

The chemokines (Mcp1, Ip10 and Rantes) upregulated in Crif1 siRNA-treated adipocytes are thought to be critical for attracting macrophages and T lymphocytes into adipose tissue in obese subjects [30]. Therefore, we wondered whether Crif1 silenced 3T3-L1 cells would trigger the migration of macrophages. As shown in Figure 4G, Crif1 silenced 3T3-L1 cells enhanced the migration of RAW 264.7 cells and NAC treatment inhibited the migration of RAW 264.7 cells. Thus, our in vitro studies show that OXPHOS deficiency induced in differentiated cultured 3T3-L1 adipocytes by Crif1 silencing results in the upregulated expression of chemokines, which then recruit or activate macrophages, ROS and stress kinase dependently.

Macrophage infiltration and systemic inflammatory responses in Crif1−/−Fabp4 mice

To observe ROS stress associated with abnormal chemokine responses in adipose tissues in vivo, we measured lipid peroxidation (TBAR assays), stress kinase activation and cytokine expression in WAT of Crif1−/−Fabp4 mice fed a NCD or a HFD for 8 weeks. Consistent with the in vitro studies, lipid peroxidation in WAT and plasma was increased in Crif1−/−Fabp4 mice fed a HFD for 8 weeks compared to control mice (Figure 7A). Levels of p38 MAPK and JNK phosphorylation were higher in WAT of Crif1−/−Fabp4 mice fed a HFD for 8 weeks than in control mice (Figure 5A). Furthermore, the expression of Mcp1, Ip10 and Rantes was higher in adipose tissue from Crif1−/−Fabp4 mice than in control mice (Figure 5B). In addition, the level of secreted MCP1, but not IP10, were higher in the serum of Crif1−/−Fabp4 mice than in control mice (Figure 7B). The results showing dysregulation of chemokines in the absence of Crif1 suggest that mitochondrial OXPHOS dysfunction may trigger immune cell recruitment in adipose tissue.

To observe inflammation in the adipose tissue of Crif1−/−Fabp4 mice directly, the eWAT were stained with anti-F4/80, an antibody that detects macrophages. Increased F4/80 reactivity was observed in the eWAT of Crif1−/−Fabp4 mice fed a NCD for 8 weeks. Aging and a HFD had an even more pronounced effect (Figure 5C). Based on the quantitative real-time PCR results, the relative expression of proinflammatory M1 macrophage markers (Cd11c, Cd11b and Tnfα) increased significantly; however, the relative gene expression of an anti-inflammatory M2 macrophage marker (arginase I) did not change (Figure 5D). To quantify the number of macrophages in the adipose tissue, multi-parameter flow cytometry was performed with anti-F4/80, anti-Cd11c and anti-Cd206 antibodies using isolated stromal vascular fractions (SVF). F4/80+/Cd11c+/Cd206- M1 macrophages were predominant in Crif1−/−Fabp4 mice compared with control mice. The proportion of F4/80+/Cd11c+/Cd206+ M2 macrophages tended to be higher in Crif1−/−Fabp4 mice, but this did not reach statistical significance (Figure 5E). Taken together, the results suggested that the infiltrating macrophages were skewed towards the M1 phenotype in Crif1−/−Fabp4 mice. Recent studies show that B cell-mediated CD4+ and CD8+ T cell activation is required to induce inflammation and insulin resistance [31,32]. The present
Figure 3. Metabolic phenotypes and insulin resistance in Crif1+/−,Fabp4 mice. (A–D) An intraperitoneal glucose tolerance test (IPGTT) was performed with 1 g/kg glucose, after a 16 h fast in NCD or HFD mice (n = 8). Values are means ± SD, *p < 0.05 versus control mice. (E) Western blot analysis of p-Akt in the gastrocnemius muscle and liver after injecting 1 U/kg insulin in control and Crif1+/−,Fabp4 mice with 14 weeks of HFD. Data are
study found no difference between the numbers of CD4+ and CD8+ T cells in Cre1f/f,Fabp4 and Cre1f/+Fabp4 mice (data not shown).

Adipocytes in adipose tissue secrete adipokines, such as adiponectin, leptin, IL-6 and TNFα, which are involved in the control of whole-body insulin sensitivity. However, proinflammatory TNFα is released by dysfunctional adipocytes and amplifies local immune responses by recruiting macrophages to WAT [33,34]. Serum levels of TNFα were consistently higher in Cre1f/f,Fabp4 mice fed a HFD than in control mice (Figure 3F). This indicates that TNFα may be a crucial mediator of inflammation in WAT and whole-body insulin resistance of Cre1f/f,Fabp4 mice.

The Fabp4 gene is expressed in macrophages [35], but no Cre expression or activity was detected in macrophages isolated from Cre1f/f,Fabp4 mice. As shown in Figure 8A, the expression levels of Crif1 and macrophage markers (Cd11c, Tnfα, Cd11b, and Arg1) were not reduced in peritoneal macrophages obtained from Cre1f/f,Fabp4 mice (Figure 8A). Homologous recombination using PCR [36] identified Cre recombinase activity in WAT and BAT, but not in peritoneal macrophages in Cre1f/+Fabp4 mice at 20 weeks-of-age (Figure 8B and 8C).

Discussion

Mitochondrial dysfunction, characterized by reduced OXPHOS function in liver and skeletal muscle, is thought to be one of the underlying causes of insulin resistance and type 2 diabetes (for a review, see [39,40]). In addition, reduced hepatic OXPHOS function is closely related to hepatic lipid accumulation and insulin resistance [41]. Collectively, these studies provide evidence of a role for mitochondrial OXPHOS dysfunction in the development of human insulin resistance and type 2 diabetes. However, animal models of OXPHOS dysfunction in skeletal muscle and liver do not exhibit the human insulin resistance and type 2 diabetes.
phenotype [21,42,43]. The absence of insulin resistance in mice with homozygous or heterozygous Crif1 deletion in the liver (Crif1fl/fl, Albumin-Cre) or skeletal muscle (Crif1fl/fl,MitC-Cre) is in agreement with previous findings that hepatic and skeletal mitochondrial dysfunction does not cause insulin resistance (Figure S10). Therefore, whether or how mitochondrial OXPHOS contributes to the pathogenesis of insulin resistance remains to be resolved. It is reported that adipose OXPHOS capacity is controlled by both genetic and diet-induced obesity [10,44,45], which potentially contribute to adipose tissue dysfunction and exacerbation of insulin resistance. However, whether changes in adipose OXPHOS capacity are a cause or a consequence of complications associated with insulin resistance has not been clarified in vivo.

In this study, we have shown an association between limited mitochondrial OXPHOS capacity and adipose tissue inflammation and insulin resistance in a Crif1 haploinsufficiency animal model. Mitochondria play a key role in the differentiation and maturation of adipocytes. It is reported that marked mitochondrial biogenesis is observed during the adipocyte differentiation process in vitro. In fact, the concentration of mitochondrial proteins in differentiated 3T3-L1 adipocytes showed a 20- or 30-fold increase compared with that in pre-adipocytes [14,46]. Notably, chemical inhibition of respiratory chain function, for example by rotenone treatment, suppresses adipogenesis and induces changes in the expression levels of the key transcription factors, C/EBPz, PPARγ, and SREBP-1c [47]. However, the role played by mitochondria during adipogenesis has mostly been investigated in vitro by inhibiting or knocking down the genes encoding the OXPHOS complex. This study showed that homozygous Crif1 null mice generated by both Fabp4-Cre and Adipoq-Cre recombinase have defects in WAT and BAT development. These observations support that notion that intact OXPHOS function is critical for adipogenesis in vitro. By contrast, our own observations show that heterozygous Crif1 knockout mice do not have defects in adipogenesis and maturation under NCD and HFD conditions. This finding indicates that Crif1 haploinsufficiency and mildly reduced OXPHOS capacity do not cause the apparent failure of adipogenesis in WAT and BAT. Consistently, plasma and liver lipid levels were not increased in heterozygous Crif1 knockout mice, suggesting that the mice do not have the lipodystrophy phenotype. Furthermore, it is reported that insulin resistance in a mouse model of lipodystrophy was not relieved by controlling phenotype. Furthermore, it is reported that insulin resistance in a mice, suggesting that the mice do not have the lipodystrophy perturbed in homozygous sufficient knockout mice may not be related to lipodystrophic lipid levels were not increased in heterozygous in WAT and BAT. Consistently, plasma and liver ROS, p38 and JNK activation) need to be addressed by causing or eliminating these events while studying abnormal increases in ROS production and activation of p38 and JNK in adipose tissue are common denominators that respond to cellular stresses. Unexpectedly, haploinsufficient heterozygous Crif1fl/+;Fabp4+/− or control mice exhibited similar levels of adipose Akt phosphorylation in response to insulin injection. This indicates that insulin signaling in adipose tissue may not be the principal cause of the systemic glucose intolerance of Crif1fl/+;Fabp4+/− mice. Therefore, the relative importance of these factors (increased ROS, p38 and JNK activation) need to be addressed by suppressing or eliminating these events while studying abnormal chemokine responses and systemic insulin resistance.

We showed that the WAT in Crif1-deficient mice is predominantly infiltrated by macrophages, regardless of excessive adiposity. An increase in the number of adipose tissue macrophages (ATM) is a prominent feature associated with excessive adiposity [51,52]. Increased expression of chemokines, especially MCP1, is responsible for recruiting macrophages into the WAT [30]. The ATM infiltration of epidymal fat pads in Crif1-deficient mice showed several characteristic features. First, it was present in fat pads with normal adiposity. This finding suggests that macrophage recruitment to adipose tissue caused by impaired OXPHOS capacity may also develop independently of excessive adiposity, but is accentuated in cases of increased adiposity. Mitochondrial OXPHOS dysfunction in the adipose tissue of...
Figure 5. Macrophage infiltration and inflammation in adipose tissue of Crif1f/+;Fabp4−/− mice. (A) Western blotting for p38 MAPK and JNK phosphorylation in WAT of Crif1f/+;Fabp4−/− mice fed a HFD for 8 weeks. p38 MAPK, p38 mitogen-activated protein kinase; JNK, c-Jun N-terminal kinase. (B) Mcp1, Ip10 and Rantes mRNA levels in eWAT from control mice fed a NCD or a HFD for 8 weeks (n=8). Values are means ± SD, *p<0.05 versus control mice. (C) Immunohistochemistry with anti-F4/80 and hematoxylin staining reveals macrophage accumulation in eWAT. Scale: 200 μm. (D) Expression of the M1 macrophage and M2 macrophage-specific genes (Cd11c, Cd11b, Tnfα and arginase1) in eWAT (n=8). Values are means ± SD, *p<0.05 versus control mice, n.s, not significant. (E) Percentage of proinflammatory M1 macrophages and anti-inflammatory M2 macrophages assessed by counting CD11c-PE-A- and CD206-FITC-stained cells in the SVF in eWAT of 14 weeks HFD feeding mice (n=8). Values are means ± SD, *p<0.05 versus control mice. MΦ, Macrophage. (F) Serum IL4 and TNFα levels in mice fed HFD, as measured by Multiplex (n=8). Values are means ± SD, *p<0.05 versus control mice, n.s, not significant.
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Figure 6. Recruitment of macrophages and T cells to adipose tissue in Crif1f/f,Adipoq mice. Control (Crif1+/+,Adipoq) adipose-specific Crif1 heterozygous (Crif1f/+,Adipoq) and homozygous (Crif1f/f,Adipoq) knockout mice were generated by cross-breeding with Adipoq-Cre recombinase transgenic mice. Phenotypic analysis all of these mice fed with a NCD was performed at 8 weeks-of-age. (A) Secreted MCP1 and IP10 levels in the serum in the three strains of mice (n = 6). Values are means ± SD, *p < 0.05 versus control mice. (B) Immunohistochemical staining with an anti-F4/80 antibody shows severe macrophage accumulation in eWAT of Crif1f/f,Adipoq mice. Scale: 200 μm. (C) Percentage of proinflammatory M1 macrophages (CD11c-PE-A-) and anti-inflammatory M2 macrophages (CD206-FITC-) (n = 6). Values are means ± SD, *p < 0.05. MΦ, Macrophage. (D) Percentage of CD8+ T cells and CD4+ T cells in the SVF in eWAT. Quantification of the CD8/CD4 ratio by counting cells stained with CD8-FITC and CD4-PE-cy7 (n = 6). Values are means ± SD, *p < 0.05. (E) IPGTT was performed in NCD mice after a 16 h fast with 1 g/kg of glucose (n = 6). Values are means ± SD, *p < 0.05 versus control.
Figure 7. Depletion of macrophages in adipose tissue by clodronate. The clodronate study was performed after feeding Crif1+/+Fabp4 and Crif1f+/+,Fabp4 mice a HFD for 8 weeks. Two intraperitoneal injections of clodronate were given with a 3 day interval between each. IPGTT and ITT were performed 6 days after the first injection. (A) Immunohistochemistry with anti-F4/80 after macrophage depletion by liposomal clodronate in Crif1+/+Fabp4 and Crif1f+/+,Fabp4 mice fed HFD. Scale: 200 μm. (B) Real-time PCR using primers for the macrophage marker, Cd68 (n = 8). Values are means + SD, *p < 0.05. (C and D) IPGTT and ITT after macrophage depletion by intraperitoneal injection of liposomal clodronate or PBS control (n = 8). Values are means + SD, *p < 0.05, Crif1f+/+,Fabp4 mice versus control mice; + p < 0.05, clodronate versus PBS in control mice.

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Figure 8. A model of the systemic insulin resistance developed by Crif1-haploinsufficient mice, which shows limited adipose OXPHOS capacity.

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Csf1r−/−/Fabp4−/− mice fed a NCD for 8 weeks resulted in macrophage recruitment; however, the mice showed normal glucose tolerance. This suggests that a threshold level of macrophage recruitment or activation is required for the development of insulin resistance. Phenotypic analysis of ATM in Csf1r-deficient mice demonstrated that the proportion of both M1 and M2 macrophages tended to be increased under NCD and HFD conditions. However, a phenotypic shift toward M1 macrophages was observed in the adipose tissue of Csf1r-deficient mice. Thus, these features of macrophage recruitment in WAT were similar to those observed in a mouse model of diet-induced obesity [33]. Our data provide novel insights into the relationship between adipose inflammation and insulin resistance. This study supports the idea that adiposity overwhelms the genetically-determined OXPHOS capacity in adipose tissue, provoking an inflammatory response and insulin resistance. Therefore, it is possible that adipose mitochondrial OXPHOS capacity is an independent factor determining the risk of adipose inflammation and systemic insulin resistance in obese and even in non-obese subjects.

Materials and Methods

Cell culture

3T3-L1 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% bovine calf serum (Gibco BRL). Forty-eight hours post-confluence, the cells were differentiated with IBMX (0.5 mM), dexamethasone (1 μM), insulin (10 μg/ml) and 10% fetal bovine serum (Gibco BRL) [54]. Csf1r siRNA (GGA GUG CUC CGU UCC AGG AAC UAU U) was transfected by using Lipofectamine RNAiMAX reagent (Invitrogen) into 3T3-L1 adipocytes on day 4 of differentiation. Migration of Raw264.7 cells was examined in 8.0 μm Transwell filters (Corning Corp). Raw 264.7 cells were maintained on the top well, with the media from 3T3-L1 adipocytes in the bottom well. After twenty-four hours, the Raw 264.7 cells that had not migrated to the filter were removed, and the cells that had migrated through the filter were collected and stained with trypsin-blue. ADSCs were cultured as previously described [55]. ADSCs were differentiated into adipocytes using IBMX (0.5 mM), dexamethasone (1 μM), insulin (10 μg/ml) and rosiglitazone (0.5 μM) in M199 medium (Gibco BRL) supplemented with 10% fetal bovine serum (Gibco BRL). After induction of differentiation, lipid accumulation was detected with Oil red O staining. ADSCs were fixed with 10% neutralized formalin, washed with water, and then stained with freshly prepared 0.2% Oil red O solution.

Western blot analysis

Primary antibodies against OXPHOS complex subunits (NDUFA9, SDHA, UQRC2, and ATP5A1) were purchased from Invitrogen. Anti-COX4 (#ab4444) antibody was purchased from Cell Signaling. Anti-ND1 antibody (sc-65237) was purchased from Santa Cruz Biotechnology. Secondary antibodies (goat anti-mouse and goat anti-rabbit) were obtained from Cell Signaling. Anti-p38 antibody, anti-phospho-p38 antibody, anti-JNK-antibody, anti-phospho-JNK antibody, anti-phospho-Akt and total-Akt antibodies were obtained from Cell Signaling and anti-beta-actin, α-tubulin antibody was obtained from Sigma-Aldrich. Anti-UCP1 antibody was obtained from Abcam.

Northern blot analysis and real-time PCR

Total RNA was isolated using Trizol (Invitrogen). For Northern blot analysis, 10-20 μg of total RNA was loaded onto a 1.5% agarose-formaldehyde gel. A Csf1r probe was constructed using the mouse Csf1r gene digested with KpnI enzyme. The relative intensity of the Csf1r/β-actin bands was normalized against that in the brain. Complementary DNA (cDNA) was prepared from total RNA using M-MLV Reverse Transcriptase and oligo-dT primers (Invitrogen). Real-time PCR was performed using cDNA, QuantiTect SYBR Green PCR Master Mix (QIAGEN), and specific primers. The primers used are described in Table S2. Relative expressions were calculated normalized with 18s ribosomal RNA, using Rotor-Gene 6000 real-time rotary analyzer Software (Version 1.7, Corbett Life Science).

Complementary DNA microarray analysis

Total RNA was prepared from fully-differentiated 3T3-L1 adipocytes transfected with control or Csf1r siRNA. RNA amplification and labeling were performed with the Low RNA Input Linear Amplification kit PLUS (Agilent Technologies). Array hybridization and scanning were performed with a DNA microarray Chip and scanner (Agilent Technologies). Array data was analyzed using the Feature Extraction and GeneSpring Software (Agilent Technologies).

ROS staining

Dihydroethidium (DHE) or MitoSOX were used to detect intracellular superoxide. Fully-differentiated 3T3-L1 cells were incubated with 10 μM DHE or 5 μM MitoSOX at 37°C for 15 min. Fully-differentiated 3T3-L1 cells were washed with Krebs-HEPES buffer (pH 7.4) or HBSS. Images of cells stained with DHE or MitoSOX were obtained by fluorescence microscopy (Olympus, Japan). Cells were trypsinized and analyzed using a FACScan flow cytometer (BD Bioscience) and data analysis was performed using BD FACSDiva software (BD Bioscience).

Blue native-polyacrylamide gel electrophoresis (BN-PAGE)

Before BN-PAGE, mitochondrial isolation was performed as previously described [56] with modifications. Pellets of ADSCs or tissues from mice were resuspended in buffer B (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 5 mM HEPES, pH 7.2) and incubated for 5 min at 4°C. After centrifugation at 600×g for 10 min, the supernatant was re-centrifuged at 17,000×g for 10 min. The pellet containing the mitochondrial fraction was used in the Native PAGE. Novex Bis-Tris Gel system (Invitrogen) to determine the content of the OXPHOS complex. A total of 20 μg of the mitochondrial fraction in Native PAGE sample buffer supplemented with 0.5% n-dodecyl-β-maltoside was loaded onto a Native PAGE Novex 3–12% Bis-Tris gel. The mitochondrial fraction was mixed with Native PAGE sample buffer containing 1% of digitonin to detect the supercomplexes. After electrophoresis, the separated proteins in the gel were transferred to a PVDF membrane, which was then incubated with an anti-OXPHOS antibody mixture (Invitrogen).

Oxygen consumption rate (OCR)

OCR was measured using a Seahorse XF-24 analyzer (Seahorse Bioscience). Control Csf1r+/+ and Csf1r+/− ADSCs were prepared from the eWAT of Csf1r+/+Fabp4+/+. Csf1r−/−Fabp4−/− mice. After seeding ADSCs on an XF-24 plate, cells were incubated in differentiation M199 media contained with FBS, IBMX, dexamethasone, insulin and rosiglitazone. After 2 days later, Csf1r+/+ and Csf1r+/− ADSCs maintained M199 media with insulin for 8 days. The day before OCR measurement, the sensor cartridge was calibrated with calibration buffer (Seahorse Bioscience) at 37°C. Fully-differentiated ADSCs were washed and incubated with
M199 (Gibco BRL) without sodium bicarbonate at 37°C in an incubator. Three readings were taken after each addition of mitochondrial inhibitor before injection of the subsequent inhibitors. The mitochondrial inhibitors used were oligomycin (2 μg/ml), carbonyl cyanide m-chlorophenyl hydrazine (CCCP, 10 μM), and rotenone (1 μM). OCR was automatically calculated and recorded by the sensor cartridge and Seahorse XF-24 software. The plates were saved and the protein concentration was calculated to confirm that there were an approximately equal number of cells in each well.

Mice

Floxed GypI (GypIPm/fox) mice were generated as previously described [18]. Fabp4-Cre, Albumin-Cre transgenic mice (C57BL/6) were purchased from the Jackson Laboratory. Adiponectin-Cre transgenic mice were kindly provided by Dr. Evan Rosen. Dr. Steven J Burden provided the MLC-Cre mouse strain. The HFD, which contained 60% fat, was purchased from Research Diets Inc. (D12492). Mice were maintained in a controlled environment (12 h light/12 h dark cycle; humidity 50–60%; ambient temperature 25°C±1°C) and fed ad libitum. For the cold challenge experiments, mice were individually housed in cages pre-chilled to 4°C. Body temperature was monitored using a rectal probe attached to a digital thermometer (TD-300, Shibaura Denshi, Japan) with/without cold stress. For the thermoneutrality experiments, 2-week-old mice were housed with their mothers at a temperature of 30°C±1°C. All mouse experiments were performed in the animal facility according to institutional guidelines, and the experimental protocols were approved by the institutional review board of Korean Research Institute of Biotechnology and Bioscience, and Chungnam National University.

Genomic PCR

To measure the activity of Cre recombinase, PCR was performed as previously reported [36]. Briefly, after isolation of genomic DNA from WAT, BAT, and diaphragm-induced peritoneal macrophages, PCR was performed with a combination of three primers: forward primer 1, GGGCTGGTGA AATGTGTGGT; reverse primer 2, TCAAGCTAGGG TGGGACAGA; and reverse primer 3, TATCAGTTCAAGACACTGT. To ensure product specificity from PCR, the extension time was limited to 30 sec.

Histological and morphometric analysis

WAT was fixed in 10% neutralized formalin for 16 h, washed, and then embedded in paraffin. Tissue sections of 5 μm thickness were deparaffinized, rehydrated, and heated in a microwave for 10 min in citrate buffer. The tissue sections were then incubated with primary antibodies (anti-F4/80 (diluted 1:100; Abcam)) for 16 h at 4°C. Immunohistochemistry was performed using a Polink-1 HRP Rat-NM DAB Detection System (GIBI Inc).

Transmission electron microscopy (TEM)

WAT and BAT were fixed in 1% glutaraldehyde at 4°C and then washed with 0.1 M cacodylate buffer at 4°C. After washing five times, the tissue was post-fixed with 1% OsO4 in an 0.1 M cacodylate buffer (pH 7.2) containing 0.1% CaCl2 for 1 h at 4°C. Samples were dehydrated by serial ethanol and propylene oxide treatment and embedded in Embed-812 (EMS). The resin was then polymerized at 60°C for 36 h. Tissue was sectioned using an EM UC6 ultramicrotome (LEICA) and stained with 4% uranyl acetate and citrate. Observation was performed using a Tecnai G2 Spirit Twin transmission electron microscope (FEI Company, USA) and a JEM ARM 1300S high-voltage electron microscope (JEOL, Japan).

Intraperitoneal glucose tolerance (IPGTT) and insulin tolerance tests (ITT)

For IPGTT, mice were fasted for 16 h and then 2 g/kg or 1 g/kg glucose was injected into the intraperitoneal cavity of each mouse. Blood glucose levels were measured at 0, 15, 30, 60, and 90 min using a glucometer (Bayer breeze). ITT was performed by measuring blood glucose after 6 h of fasting followed by intraperitoneal insulin injection (0.75 U/kg; Humalog).

Hyperinsulinemic euglycemic clamping

Hyperinsulinemic euglycemic clamping was performed as previously described [57]. Briefly, after an overnight fast, a 2 h hyperinsulinemic euglycemic clamping was performed in GypIPm/fox and control littermates (n = 8). The insulin clamp began with a primed-continuous infusion of insulin (0.3 U/kg bolus followed by 2.5 mU/kg/min). Blood samples [20 μl] were collected at 10 to 20 min intervals for immediate measurement of plasma glucose concentrations, and 20% glucose was infused at variable rates to maintain glucose at basal concentrations (~120 mg/dl). Basal and insulin-stimulated whole-body glucose uptake was estimated with a continuous infusion of 1H glucose (Perkin Elmer Life and Analytical Sciences) for 2 h before clamping (0.05 μCi/min) and throughout the clamping (0.1 μCi/ min), respectively. At 75 min after the start of the clamp, 2-deoxy-d-1-14C glucose (PerkinElmer Life and Analytical Sciences) was injected with a Hamilton syringe to measure insulin-stimulated glucose transport activity and metabolism in skeletal muscle. Blood samples were taken before, during, and at the end of the clamps for measurement of plasma 1H glucose and 2-deoxy-d-1-14C glucose concentrations, and/or insulin concentrations. At the end of the clamps, tissue samples (gastrocnemius, eWAT, and liver) were rapidly taken and stored at −70°C prior to biochemical and molecular analysis.

Flow cytometry

To quantified M1 macrophages, M2 macrophages, and CD4+ and CD8+ T cell populations, the stromal vascular fractions (SVF) was isolated from mouse eWAT. The SVF was prepared by the lysis of eWAT with type 1 collagenase (Gibco BRL) in collagenase buffer at 37°C in a shaking water bath for 40 min, followed by centrifuging at 2000 rpm for 5 min. The suspended solid matter comprised adipocytes and the cell pellet comprised T cells, B cells and macrophages. The cell pellet was then incubated with RBC lysis buffer and the remaining cells were stained with specific antibodies. Anti-CD3 (BD bioscience), anti-CD4 (BD Bioscience) and anti-CD8 (eBioscience) were used to stain the T cell population [58], and F4/80 (eBioscience), CD206 (eBioscience) and CD11c (eBioscience) were used to stain the M1/M2 macrophages. The stained SVF cells were analyzed using a FACScan flow cytometer (BD Bioscience) and data analysis was performed using BD FACSDiva software (BD Bioscience).

Thiobarbituric acid reactive substance (TBAR) assay

The TBAR assay kit (Cayman Chemicals) was used to measure lipid peroxidation in the WAT and plasma of mice. WAT (23 mg) suspended in RIPA buffer was sonicated, centrifuged at 1,600 g for 10 min at 4°C, and the supernatant was collected. The SDS solution was added to the supernatant, which was then mixed with the Color reagent according to the manufacturer’s instructions.
The sample was boiled for 1 h, centrifuged, and the supernatant was collected. Fluorescence at the excitation wavelength of 530 nm and emission wavelength of 550 nm was measured.

Macrophage depletion by clodronate

The generation of liposome-encapsulated clodronate was performed as previously described [38]. Cholesterol (10 mg/ml; Sigma-Aldrich) was dissolved in 100% ethanol, and 100 mg/ml phosphatidylcholine in 100% ethanol (Sigma-Aldrich) was made into a phospho-lipid film by drying with a low-vacuum rotary. Clodronate (0.6 M; Sigma-Aldrich) was dissolved in purified water and incubated with the phospho-lipid film by gentle rotation at room temperature and sonication in a water bath for 3 min at 55 kHz. After removing the non-encapsulated clodronate, liposome-encapsulated clodronate was resuspended in 1X PBS. Two intraperitoneal injections (3 days apart) of clodronate were administered to mice fed a HFD for 8 weeks. IPGTT and ITT were performed 6 days after the first injection.

Analysis of triglyceride, ceramide, and saturated fatty acids in tissues

Measurement of hepatic triglycerides: Liver triglycerides were extracted with chloroform and methanol, dissolved in 1x PBS, and measured in a Hitachi 7150 chemistry analyzer (Hitachi, Japan).

Measurement of ceramides in WAT, liver, and muscle: Prior to extraction of total lipids, C17 ceramide was added as an internal standard. Ceramides were measured as previously described [59]. All liquid chromatography-mass spectrometry (LC-MS/MS) experiments were performed using an Agilent 1200 HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled to a Thermo LTQ linear ion trap mass spectrometer. GC interfaced with an HP5973N MSD. A DB-5 column was used. Lipid molecules separated by LC were detected by the mass spectrometer in Positive ESI mode using selected reaction monitoring (ESI) source. Briefly, LC separation was achieved using a LunaC18 RP column (150 mm×2 mm I.D., 5 μm 100 Å particles; Phenomenex, Torrance, CA) with gradient elution. Lipid molecules separated by LC were detected by the mass spectrometer in Positive ESI mode using selected reaction monitoring (SRM). The SRM channels were arranged as follows: 533→264 for C16, 552→264 for C17, 566→264 for C18, 594→264 for C20, 648→264 for C24:1, and 650→264 for C24. The peak area was normalized according to the internal standard and tissue weight. All values are presented as relative differences in the ratio of the extracted lipids to the internal standard.

To measure the level of saturated fatty acids, tissues were homogenized in ice-cold methanol, and 1 μg of pentadecanoic acid (C15:0) was added as an internal standard. Samples were incubated at 45°C overnight, then cooled to room temperature. Hexane and 1 mL of H2O were added, samples were vortexed and incubated at 45°C for 30 min, then cooled to room temperature. The sample was boiled for 1 h, centrifuged, and the supernatant was collected. The peak area was normalized according to the internal standard.

Serum analysis

Whole cardiac blood from the mice was collected at room temperature for 2 h. The blood was centrifuged at 2,500 rpm for 5 min, and the supernatant was collected. TNFα and IL-4 were measured using a mouse cytokine/chemokine multiplex panel (Millipore). MCP1 and IP10 were measured using an ELISA kit (R&D Systems). Serum triglycerides and FFAs were measured with a Hitachi 7150 chemistry analyzer (Hitachi, Japan).

Statistical analyses

Data are presented as means ± or + standard deviation (SD). Statistical significance for comparisons was determined using the Student’s two-tailed T-test. A p value less than 0.05 was considered statistically significant.

Supporting Information

Figure S1 Expression of Crif1 mRNA in the various tissues in control C57BL/6 mouse, ob/ob and db/db mice. (A) Measurement of Crif1 mRNA in multiple tissues in 8 weeks-of-aged C57BL/6 male mouse by northern blot analysis. The bars represent the relative density of Crif1 mRNA compared with the value of brain in northern blots. BAT, brown adipose tissue; eWAT, epididymal white adipose tissue; EDL, extensor digitorum longus; GA, gastrocnemius. (B) Crif1 expression was measured in 7 weeks old db/db (left) or ob/ob (right) mice and control heterozygous mice by real-time PCR, normalized with 18s ribosomal RNA. (n = 6). Values are means ± SD. *p<0.05 versus control mice. (C) Crif1 expression in mice fed a high fat diet (HFD) for 8 weeks starting from 6 weeks of age (n = 10). Values are means ± SD. *p<0.05 versus control mice. NCD, normal chow diet; HFD, high-fat diet. (PDF)

Figure S2 Impaired non-shivering thermogenesis in Crif1+/Fabp4 mice. All analysis of these mice fed with a NCD was performed at 3 weeks-of-age. (A) Hematoxylin and eosin (H&E) staining of BAT. Scale: 100 μm. (B) Transmission electron microscopy (TEM) of BAT revealed that the mitochondria of Crif1+/Fabp4 mice developed severe swollen cristae (red arrows). L, lipid droplet; N, nucleus. Scale: 6,000 nm. (C) Number of mitochondria per area and relative mitochondria size in BAT (n = 20). Values are means ± SD. *p<0.05 versus control mice. (D) Body temperature was measured rectally with a digital thermometer at an ambient temperature (23°C) and after emersion in cold water (4°C) for 5 min (n = 5). Values are means ± SD. *p<0.05 versus control mice. (E) Survival rate of Crif1+/Fabp4 and Crif1−/−Fabp4 mice housed in ambient (23°C) and thermoneutrality (30°C) conditions (n = 20). (PDF)

Figure S3 Levels of mitochondrial OXPHOS complexes in non-adipose tissues of Crif1−/−Fabp4 mice. (A) Western blotting of Crif1 and the ND1 subunit of OXPHOS complex I in liver and heart. ND1, subunit of OXPHOS complex I. (B) Blue native-PAGE analysis of OXPHOS complexes I, II, III, IV and V in mitochondria isolated from liver of Crif1−/−Fabp4 and Crif1+/−Fabp4 mice. (C) Level of ATP in the heart of Crif1−/−Fabp4 and Crif1+/−Fabp4 mice (n = 8). Values are means ± SD. n.s., not significant. (PDF)

Figure S4 Adipose development and lipid metabolites in Crif1−/−Fabp4 mice. Crif1−/−Fabp4 and Crif1+/−Fabp4 mice were generated from floxed Crif1 mice with Fabp4-Cre recombinase mice. NCD or a HFD were begun at 6-weeks-of-age. (A) Body weight changes in mice fed a NCD or 60% HFD for 8 weeks or 14 weeks, starting at 6 weeks-of-age (n = 8). Values are means ± SD. wks, weeks; n.s, not significant. (B) Daily food intake of Crif1−/−Fabp4 and Crif1+/−Fabp4 mice (n = 8). (Crif1+/−Fabp4, 3.24±0.42 g/day vs Crif1−/−Fabp4, 2.94±0.14 g/day) Values are
means + SD, n.s. not significant. (C) MR images in Cref1+/+;Fabp4−/− and Cref1+/+;Fabp4−/− mice fed with NCD and HFD for 14 weeks. (D) and (E) Serum TG and FFA levels in control (Cref1+/+;Fabp4−/−) and adipose tissue-specific Cref1 heterozygous mice (Cref1+/+;Fabp4−/−) (n = 8). Values are means + SD, *p<0.05, n.s. not significant. (F) TG levels in the liver. The peak area was normalized according to a liver weight (n = 8). Values are means + SD, *p<0.05 versus control mice. (G) Combined level of saturated fatty acids such as C16, C18, C18:1, C18:3, and C20:4 in WAT, gastrocnemius muscle and liver quantified by gas chromatography–mass spectrometry (GC-MS). The data was normalized according to an internal standard and tissue wet weight (n = 4). Values are means + SD, n.s. not significant. (H) Combined level of ceramides, hydrosilated C18, C18:1, C18:2, C18:3, C16:0, C18:0, C16:1 and C18:1, C18:2, C18:3, C20:4 in WAT, gastrocnemius muscle, and liver of mice fed a HFD for 14 weeks quantified by liquid chromatography-mass spectrometry (LC-MS/MS). The peak area was normalized according to an internal standard (C17 ceramide) and tissue weight (n = 4). Values are means + SD, n.s. not significant.

(PDF)

**Figure S5** Gene expression profiles in 3T3-L1 adipocytes following silencing of Cref1 determined by a complementary DNA microarray. A microarray was performed using 3T3-L1 adipocytes treated with control or Cref1 siRNA with Agilent’s DNA microarray Chip. Data were analyzed using the Feature Extraction and GeneSpring Software (Agilent Technologies).

(PDF)

**Figure S6** Activation of p38 MAPK and expression of chemokines in MEFs. (A) p-p38 MAPK and t-p38 MAPK levels in control (+/Δ) and Cref1-null (−/−Δ) MEFs. p38 MAPK, p38 mitogen-activated protein kinase. (B) Real-time PCR with Mqpl and Ip10 primers in MEF null cells (−/−Δ) (n = 6). Values are means + SD, n.s. not significant.

(PDF)

**Figure S7** ROS and chemokine secretion in mice. (A) Measurement of lipid peroxidation with the TBAR assay in the WAT and plasma of Cref1+/+;Fabp4−/− and Cref1+/+;Fabp4−/− mice fed a NCD or HFD for 8 weeks (n = 8). Values are means + SD, *p<0.05, n.s. not significant. (B) Secreted MCP1 and IP10 levels in the serum of Cref1+/+;Fabp4−/− and Cref1+/+;Fabp4−/− mice fed a NCD or HFD for 8 weeks (n = 8). Values are means + SD, *p<0.05, n.s. not significant.

(PDF)

**Figure S8** Phenotypic analysis and detection of Cre recombinase activity in peritoneal macrophages from Cref1+/+;Fabp4−/− and Cref1+/+;Fabp4−/− mice. (A) Peritoneal macrophages were collected following intraperitoneal injection of thioglycollate into 6-week-old Cref1+/+;Fabp4−/− and Cref1+/+;Fabp4−/− mice. Expression of Cref1 and phenotypic markers for M1 and M2 macrophages were measured by real-time PCR using specific primers (n = 10). Values are means + SD, *p<0.05, n.s. not significant. (B) Schematic showing the lox site in the Cref1 gene and the location of primers 1, 2, and 3. (C) PCR analysis of homologous recombination in genomic DNA extracted from WAT, BAT, and peritoneal macrophages isolated from 6-week-old control and Cref1+/+;Fabp4−/− mice. Genomic DNA for Cre recombinase was detected in WAT, BAT, and peritoneal macrophages (upper panel), but the recombination product was only present in WAT and BAT (lower panel).

(PDF)

**Figure S9** Generation of adipocyte specific Cref1 knockout mouse with Adipoq-Cre mice. (A) All analysis of these mice fed with a NCD was performed at 8 weeks-of-age. Cref1 mRNA levels in eWAT and BAT of control (Cref1+/+;Adipoq), adipose-specific Cref1 heterozygous (Cref1+/+;Adipoq−/−), and homozygous (Cref1+/+;Adipoq−/−) knock-out mice (n = 6). Values are means + SD, *p<0.05 versus the control mice. (B) Western blot analysis of Cref1, subunit of OXPHOX complex I (ND1 and NDUF9), OXPHOX complex III (UQCRC2), OXPHOX complex IV (COX4) in eWAT, BAT, heart and liver from the three strains of mice. (C) The body temperature of 3-week-old mice was measured when exposed to an ambient temperature (23°C) and after emersion in cold water (4°C) for 5 min (n = 5). Values are means + SD, *p<0.05 versus control mice, n.s. not significant.

(PDF)

**Figure S10** Metabolic phenotypes of liver specific- or skeletal muscle specific-Cref1 KO mice. (A and B) Body weight and daily food intake of control (Cref1+/+;Ab) and liver specific-Cref1 homozygous KO mice (Cref1+/+;Ab) fed a HFD diet for 8 weeks (n = 8). Values are means + SD, n.s. not significant. (C) IPGTT experiment with Cref1+/+;Ab and Cref1+/+;Ab mice injected with 1 g/kg glucose after 16 h of fasting (n = 8). Values are means ± SD. (D) Real time PCR with specific primers to measure gluconeogenic gene expression in the livers of fasted mice. (E) Body weight of control (Cref1+/+;MEC) and skeletal muscle-specific Cref1 haploinsufficient mice (Cref1+/+;MEC) fed a NCD diet. Values are means + SD, n.s. not significant. (F) IPGTT experiment with Cref1+/+;MEC mice injected with 2 g/kg glucose after 16 h of fasting (n = 8). Values are means ± SD.

(PDF)

**Table S1** Summary of the phenotypes of adipocyte-specific Cref1 mutant mice under the control of the Fabp4-Cre or Adipoq-Cre promoters. The percentages represent residual levels in comparison to control mice. **The band intensities of Crif1 protein were determined by Western blot analysis using 6 male mice per group.** Adipose mass was evaluated by totaling the weights of eWAT of 6 male mice per group. n.d. not determined.

(OCX)

**Table S2** Sequence of primers used in real-time PCR.

(OCX)

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**Author Contributions**

Conceived and designed the experiments: MJR, JC, MS. Performed the experiments: MJR, SJ, TK, MJ, CM, SH, SY, J, MSK. Contributed reagents/materials/analysis tools: YYK, C-HL. Wrote the paper: M-JR, JC, KCP, JUL. Analyzed the data: M-JR, SBJ, H-JK, YSJ, KSK. Contributed

References


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