Augmented CCL5/CCR5 signaling in brown adipose tissue inhibits adaptive thermogenesis and worsens insulin resistance in obesity

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**Abbreviations list**

BAT, brown adipose tissue; CCL5, Chemokine (C-C motif) ligand 5; DEGs, differentially expressed genes; DM, diabetes mellitus; FFAs, free fatty acids; HFD, high fat diet; HOMA-IR; homeostasis model assessment index; NCD, normal chow diet; RANTES, regulated on activation normal T cell expressed and secreted; SVF, Stromal vascular fraction; TG, triglyceride; WAT, white adipose tissue

**Keywords:** CCL5/CCR5 signaling; adaptive thermogenesis; AMPK

**Clinical perspectives**

Although the CCL5 and its receptor CCR5 have been reported to significantly contribute to the development of obesity-associated adipose tissue inflammation, the link between CCL5/CCR5 signaling and dysregulation of energy metabolism in state of obesity remains unknown. The present study has demonstrated that obesity-induced augmentation of CCL5/CCR5 signaling might suppress adaptive thermogenesis by inhibiting AMPK-mediated lipolysis and oxidative metabolism in BAT to deteriorate the development of obesity. It is implicated that inhibition of the augmented CCL5/CCR5 signaling in BAT might be a promising therapeutic target for the control of obesity and its associated cardiometabolic abnormalities.

**Abstract**

Chemokine (C-C motif) ligand 5 (CCL5) and CCR5, one of its receptors have been reported to be highly expressed in white adipose tissue (WAT) and are associated with the progression of inflammation and the development of insulin resistance in obese humans and mice. However, the role of CCL5/CCR5 signaling in obesity-associated dysregulation of energy metabolism remains unclear. Here, we demonstrate that global CCL5/CCR5 double knockout (DKO) mice have higher cold stress-induced energy expenditure and thermogenic function in BAT than wild-type (WT) mice. DKO mice have higher cold stress-induced energy expenditure and thermogenic function in BAT than wild-type mice. KEGG pathway analysis indicated that deletion of CCL5/CCR5 further facilitated the cold-induced expression of genes related to oxidative phosphorylation and lipid metabolic pathways. In primary brown adipocytes of DKO mice, the augmentation of CL-316243-stimulated thermogenic and lipolysis responses was reversed by co-treatment with AMPKα1 and α2 siRNA. Overexpression of BAT CCL5/CCR5 genes by local lentivirus injection in
WT mice suppressed cold stress-induced lipolytic processes and thermogenic activities. In contrast, knockdown of BAT CCL5/CCR5 signaling further upregulated AMPK phosphorylation as well as thermogenic and lipolysis responses to chronic adrenergic stimuli and subsequently decreased level of body weight gain. Chronic knockdown of BAT CCL5/CCR5 signaling improved HFD-induced insulin resistance in WT mice. It is suggested that obesity-induced augmentation of AT CCL5/CCR5 signaling could, at least in part, suppress energy expenditure and adaptive thermogenesis by inhibiting AMPK-mediated lipolysis and oxidative metabolism in thermogenic AT to exacerbate the development of obesity and insulin resistance.

**Introduction**

Obesity is a major risk factor in the development of type 2 diabetes mellitus (DM). Although the pathologic linkage remains unclear, adipose tissue (AT) dysfunction has been speculated to be crucially involved in the pathogenesis of obesity-associated DM. AT composed of white and brown AT plays a crucial role not only in the maintenance of metabolic homeostasis but also substantially contributes to obesity-associated inflammation and complications (1, 2). The main functions of white adipose tissue (WAT) are the regulation of lipid storage and mobilization, glucose homeostasis, and inflammation. On the other hand, brown adipose tissue (BAT) is rich in mitochondria and specifically expresses uncoupling protein 1 (UCP-1), which is activated by free fatty acids (FFAs) and uncouples oxidative phosphorylation (OxPhos) (3). It has been reported that in both rodents and humans, thermogenic brown adipocytes possess high capacity for fatty acid β-oxidation (4). Many WATs also contain a subset of cells that can express high levels of UCP-1 upon chronic exposure to cold and β-adrenergic stimulation. These cells, called beige adipocytes, and are capable of elevated fuel oxidation and thermogenesis (5). Nevertheless, skeletal muscle is also one of the major sites of adaptive thermogenesis (6,7). Upon cold exposure, the β-adrenergic-cAMP-PKA pathway acts as the major mediator of adaptive thermogenesis in BAT and beige fat by activating UCP-1, PGC-1α and lipolytic mobilization of FFAs, which control mitochondrial biogenesis and thermogenesis (5, 8). On the other hand, FFAs released by lipolysis are the direct activators of UCP-1-mediated leak respiration, and brown fat mitochondria are extremely sensitive to FFAs (9-12). Pharmacological inhibition of ATGL and HSL results in an almost complete blockage of UCP-1-mediated thermogenesis (13). Moreover, the addition of FFAs can directly stimulate thermogenesis in brown adipocytes in the absence of adrenergic
stimulation (14). Global or adipocyte-specific ATGL knockout mice are unable to maintain body temperature upon acute cold and exhibit a lipolytic defect in BAT, leading to severe triglyceride (TG) accumulation in BAT (15, 16).

The chemokine (C-C motif) ligand 5 (CCL5), also known as regulated on activation normal T cell expressed and secreted (RANTES), and its receptor CCR5 are highly expressed in WAT and associated with the progression of inflammation and insulin resistance in obese humans and mice (17-20). For instance, blockade of CCL5/CCR5 axis can reduce body weight of obese mice and prevent hyperlipidemia-induced inflammatory pro-atherosclerotic remodeling (21). High-fat diet (HFD)-induced increases in adipose tissue macrophage recruitment and polarization are not observed in mice with CCR5 gene deletion (19, 21). Interestingly, a previous human study revealed that physical exercise significantly reduces the expression of both CCL5 and CCR5 in the adipose tissue of obese individuals, further combating the deleterious effects associated with obesity through CCL5/CCR5 signaling in adipose tissue (18). However, the role and mechanism underlying CCL5/CCR5-associated weight control and energy metabolism remain ambiguous.

The present study conducted with CCL5/CCR5 double knockout (DKO) mice, primary brown adipocytes, and mice treated with local CCL5/CCR5 gene silencing or overexpression mediated by lentivirus-mediated short hairpin CCL5/CCR5 or CCL5/CCR5 full-length cDNA in BAT, provides important evidence to demonstrate that obesity-induced augmentation of AT CCL5/CCR5 signaling in BAT could suppress adipose tissue energy expenditure by inhibiting AMPK-mediated lipolysis and oxidative metabolism and subsequent adaptive thermogenesis. To unbiasedly gain insight into the comprehensive resource describing the molecular signature of CCL5/CCR5 deletion involved in cold adaptation, the profiling gene expression by RNA-seq was applied to identify differentially expressed genes (DEGs) and perform functional enrichment analysis. It would open a new window for understanding the effect of adipose tissue CCL5/CCR5 signaling on obesity-associated dysregulation of energy metabolism and insulin resistance.

Materials and Methods

Animals and diets

The CCL5/CCR5 double-knockout (DKO) mice were produced by Dr. Yuan-Ji Day laboratory (Hualien Tzu Chi Hospital, Hualien, Taiwan) on the C57BL/6 background. In brief, the DKO mice were generated by the
intercrossing of the single CCR5 KO and CCL5 KO mice that obtained from the Jackson Laboratory (Bar Harbor, ME, USA); the background has been described previously (22). The CCL5/CCR5 DKO mice were obtained by crossing double–heterozygous CCL5+/−; CCR5+/− mice with each other. CCL5 knockout (CCL5−/−;CCR5+/+), CCR5 knockout (CCL5+/+;CCR5−/−), CCL5/CCR5 DKO (CCL5−/−;CCR5−/−), and wild-type (CCL5+/+;CCR5+/+) offspring were identified by PCR genotyping. All animal experiments procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the National Defense Medical Center, Taipei, Taiwan, and the studies were conducted in compliance with the approved protocols IACUC-18-116 in the same institute. The mice were fed either a normal chow diet (NCD) or a 45% HFD (D12451, Research Diets) from 6 to 26 weeks of age. For the cold tolerance test, NCD-fed mice weighing 28-30 g were divided into two subgroups: the room temperature control group, which was exposed to 25°C for 11 days, and the cold exposure group, which was exposed to 4°C for 11 days. At the end of the experiments, mice were killed with overdose inhaled isoflurane and serum samples were collected. An Accu-check glucometer (Roche, Canada) was used to determine blood glucose levels. Insulin was measured with mouse insulin ELISA kits (Mercodia AB, Uppsala, Sweden). The homeostasis model assessment index (HOMA-IR) was calculated as follows: fasting plasma insulin (mU/ml) × fasting plasma glucose (mmol/L)/22.5 (23). Tissue samples were frozen for further analyses.

**Body temperature and indirect calorimetric analysis**

Core body temperature was monitored by measuring the rectal temperature of conscious mice from all experimental groups at noon under room temperature. A Thermalert model TH-5 temperature monitor (Physitemp, Clifton, NJ) was used, and the probe was placed in the rectum at a depth of 1 cm for at least 3 stable measurements. Metabolic performance and activity were measured by using a Comprehensive Lab Animal Monitoring System (CLAMS) with one mouse per chamber at Academia Sinica (Taipei, Taiwan). After a 12h acclimation period, animals were monitored for 24h in order to obtain measurements for the volume of oxygen consumption (VO₂), the volume of carbon dioxide production (VCO₂) and respiratory exchange ratio (RER), which was calculated as the ratio of total VCO₂ produced to total VO₂ consumed and heat production.

**Adipocyte differentiation and cell culture experiments**

Stromal vascular fraction (SVF) cells were isolated from BAT and were differentiated as described
previously (24). To knockdown AMPK α1 and α2 gene expression, On-TargetPlus SmartPool short interfering RNAs (siRNAs) targeting the AMPK α1 (Prkaa1) and α2 (Prkaa2) genes were purchased from Dharmacon (Thermo Scientific, Lafayette, CO, USA), and isolated preadipocytes were differentiated and transiently transfected with the siRNAs by using DharmaFECT1 transfection reagents (Thermo Scientific) according to the manufacturer’s instructions. Experiments were performed 72 h post-transfection in triplicate as stated above. The cells were treated with CL316243 (a selective β3-adrenergic receptor agonist; Tocris Bioscience, Minneapolis, MN, USA) in serum-free DMEM. After incubation for 6 h, the cells were harvested for further analyses.

**RNA-sequencing**

RNA extraction and RNA-seq analysis were performed by Biotools Biotech. Total adipocyte RNA was isolated with TRIzol Reagent (Invitrogen) and purified using the RNeasy mini kit protocol (Qiagen), according to the manufacturer’s instructions. A total amount of 2-μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using VAHTSTM mRNA-seq V2 Library Prep Kit for Illumina®, following the manufacturer’s recommendations and index codes were added to attribute sequences to each sample. The libraries were then quantified and pooled. Paired-end sequencing of the library was performed on the HiSeq XTen sequencers (Illumina, San Diego, CA).

**RNA and qPCR**

cDNA was synthesized from 2 μg RNA. Real-time PCR (TaqMan; Applied Biosystems, Foster City, CA) was performed using specific primers and probes (Supplementary Table 1) as previously described (25). β-actin was used as the reference gene to normalize mRNA levels.

**Mitochondrial respiration assay and seahorse XF24e analysis**

To determine the mitochondrial respiration activities, the O2 concentration in both primary brown adipocytes and BAT were measured using XF24 extracellular flux analyzer and XF24e islet capture microplates separately (Seahorse Bioscience, Billerica, MA) as described previously (26). In brief, SVF cells isolated from BAT of WT mice or DKO mice were seeded in an XFe24 cell culture microplate (Agilent) and differentiated into brown adipocytes. On the other hand, tissue explants were collected and minced in Krebs–Henseleit buffer (KHB). Then, 3-to 5-mg pieces were transferred to Seahorse XF24e islet capture
microplates containing 500 μl of KHB per well. These microplates were then incubated for 1 h at 37°C in the absence of CO₂ and measured by seahorse XF24e Analyzer, and the samples were homogenized in radioimmune precipitation assay buffer.

**Lipolysis Assay**

BAT depots (~100 mg) and primary brown adipocytes were incubated with isoproterenol (1 μM) or vehicle in Krebs-Ringer bicarbonate buffer (KRBBH, Sigma) containing 1% fatty acid-free BSA and glucose (2.5 mM) for the indicated times at 37°C. After incubation, free glycerol contents in the medium were determined by using the free glycerol reagent kit (F6428; Sigma). The measurements were normalized to the protein content of the explants determined with a protein dye reagent (500–0006; Bio-Rad, Hercules, CA).

**Immunoblotting and histological analysis**

For immunoblotting, proteins were extracted, separated by SDS-PAGE, and transferred to nitrocellulose membranes (Bio-Rad, CA). Membranes then were incubated with the corresponding primary antibodies at 4°C overnight. The membranes were washed using 1xPBS/Tween 0.05% and then incubated with an HRP-conjugated secondary antibody (Cell Signaling). The primary antibodies were listed at Supplementary Table 2. β-Actin was used as a loading control (Sigma, MO). Band intensities were quantified using ImageJ (NIH, MD). For immunohistochemistry, adipose tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Tissues were stained with H&E or for UCP1. Quantitation of UCP1 positive adipocytes was carried out according to the previous report (27).

**Transmission electron microscopy (TEM)**

BAT was excised into small pieces (< 1 mm³) and fixed with 2.5% glutaraldehyde (0.1 M phosphate buffer, pH 7.4) for 3 hours. Each specimen was in acetone containing 1% osmium tetroxide, 0.1% uranyl acetate (Electron Microscopy Sciences), and 5% distilled water followed by LX112 resin (Ladd Research Industries). All resin blocks were cut on an Ultracut E ultra-microtome and the ultra-thin sections were placed on formvar and carbon-coated grids. Sections were stained with 2% uranyl acetate and lead citrate (Reynolds) and examined in a JEOL JEM-1400Plus Transmission Electron Microscope. An average of six to seven visual fields was evaluated for mitochondria analysis. The size of mitochondria was analyzed from randomly delineated in five to eight micrographs per group by NIH ImageJ software.

**Local injection of shCCL5/shCCR5 or CCL5/CCR5 lentivirus into BAT**
Recombinant lentiviruses were produced by co-transfection of HEK 293T cells with transfer vector (CCL) and helper plasmids (psPAX2 and pMD2. G) using Lipofectamine 2000 transfection reagent. A total of 5x10^6 293T cells were seeded in 10-cm plates 24 h prior to transfection in DMEM with 10% fetal bovine serum (FBS), penicillin (100 IU/ml), and streptomycin (100 mg/ml) in a 5% CO_2 incubator. The culture medium was changed one hour before transfection. A total of 64 μg of plasmid DNA, including 16 μg of the envelope plasmid Ppax2, 16 μg of the packing plasmid pMD2.G and 32 μg of the transfer vector plasmid, was used per dish for transfection. After the lentiviruses were collected, we used the Lentivirus Purification Kit (ABM, G171) to purify the lentiviruses and increase their titers. The viral titer was determined by the qPCR Lentivirus Titer Kit (ABM, LV900). C57BL/6 mice aged 8-12 weeks were used in experiments.

For BAT injections, control, shCCL5/shCCR5 or CCL5/CCR5 lentivirus (1x10^10 ifu/mouse) was injected directly into the BAT in a total volume of 50 μl. The method used the combination of 0.004% Trypan blue solution (Thermo Fisher Scientific, Gibco™, catalog number: 15250061) with Lentivirus-derived gene or shRNA to make sure that the correct spots were injected (28). For cold challenge experiments, CL316243 (1 mg/kg) was injected intraperitoneally daily for 4 consecutive days. On the other hand, to induce obesity and diabetes, mice were fed a HFD starting at 6 weeks old for 20 weeks, and shCCL5/shCCR5 lentivirus (1x10^10 ifu/mouse) was injected directly into the BAT at weeks 16 of HFD-feeding period, the mice were sacrificed at the end of study.

**Statistical analysis**

Group data were presented as the mean ± SEM. Statistical analyses were performed using SigmaPlot software.

Differences between two groups were analyzed using Student’s t-test (two-tailed), and multiple comparisons were analyzed by ANOVA with Bonferroni’s post hoc test. Correlations were calculated using the Pearson correlation test. CLAMS, and Seahorse data were analyzed by calculating the area under the curve. Functional enrichment analysis of RNA-seq data was performed with HOMER (29) using pathways related to metabolism from the KEGG database (30). KEGG pathways with false discovery rates P < 0.01 were considered as significantly altered.

**Results**

**Protection against HFD-induced obesity in CCL5/CCR5 double-knockout (DKO) mice**
Body weight gain was slightly lower in DKO mice than WT control fed a NCD and more evident during HFD feeding (Fig. 1A). The caloric intake of HFD-fed DKO mice was significantly increased compared to that of HFD-fed WT mice (Fig. 1B). There was a strong positive correlation between the gene expression of CCL5 and CCR5 and body weight in epididymal WAT (eWAT) of WT mice fed NCD and HFD (Fig. 1C). Fat pad weights of inguinal WAT (iWAT) and epididymal WAT (eWAT) in DKO mice were significantly lower than those in WT mice upon HFD feeding (Fig. 1D). H&E staining showed that lipid droplets in BAT, eWAT, and iWAT of DKO mice were smaller than those in WT mice after HFD feeding for 20 weeks (Fig. 1E). HFD-induced ectopic lipid accumulation was not exhibited in the livers of DKO mice (Fig. 1E). As shown in Table 1, the basal plasma NEFA levels were slightly higher in DKO mice than in corresponding WT mice (Table 1). However, fasting insulin level and the value of HOMA-IR in the basal period were slightly lower in DKO mice than those in WT mice. In addition, HFD-fed DKO mice had lower fasting insulin and HOMA-IR levels than HFD-fed WT mice (Table 1). These results demonstrated that DKO mice exhibited protection against HFD-induced obesity and that CCL5/CCR5 signaling might substantially contribute to the regulation of energy utilization in adipose tissue and obesity-induced insulin resistance.

**DKO mice are cold tolerant and exhibit augmented energy expenditure**

Given the morphologic changes in multilocular lipid droplets in liver and fat of DKO mice, we wanted to further examine whether DKO mice are more cold tolerant than WT control. Experimental mice were divided into those in room temperature (RT, ∼25°C) or in cold environment (CE, 4°C) for 11 days, respectively. Cold exposure caused a significant downregulation of CCL5 and CCR5 protein expression in BAT of WT mice (Fig. 2A). A similar finding was also revealed in primary brown adipocytes of WT mice in the presence of CL-316,243, a β3-adrenergic receptor agonist (Fig. 2B). Cold-induced body weight loss was significantly higher in DKO mice than in WT mice during the 11 days of cold exposure (Fig. 2C). However, WT mice suffered a much larger drop in core body temperature during the cold acclimation period than DKO mice (Fig. 2D). Moreover, core body temperature was significantly decreased in WT mice but remained constant in DKO mice during a 4-hr short-term cold exposure (Fig. 2E). Our data suggest that DKO mice were more cold tolerant than WT mice (Fig. 2D and E). We next measured oxygen consumption and carbon dioxide production to evaluate energy expenditure in the experimental groups by using an indirect calorimetric system during 2-day short-term and 11-day long-term cold acclimatation. Cumulative
oxygen consumption (VO$_2$) (Fig. 2F) and carbon dioxide (VCO$_2$) (Fig. 2G) in the dark phase were significantly higher in DKO mice than in WT mice in both the short- and long-term cold stress experiments. Accordingly, cumulative energy expenditure in the dark phase was significantly higher during long-term cold acclimatation in DKO mice than in WT mice (Fig. 2H). However, there were no differences in VO$_2$, VCO$_2$ or energy expenditure between groups during the light period.

**CCL5/CCR5 deletion upregulates the cold-induced expression of genes related to oxidative phosphorylation and lipid metabolism**

To unbiasedly gain insight into the comprehensive molecular signature of CCL5/CCR5 deletion involved in cold adaptation, RNA-seq was applied to identify differentially expressed genes (DEGs) and perform functional enrichment analysis. We assessed whole transcriptome profiling in brown adipocytes from WT and DKO mice as exposed to cold acclimatation (CE) or remained in room temperature (RT). We analyzed the DEGs in brown adipocytes to evaluate its specific response toward cold exposure in WT and DKO mice (WT-CE versus WT-RT and DKO-CE versus DKO-RT) (Fig. 3A). In line with the intrinsic thermogenic response of brown adipocytes toward cold exposure, the transcriptional profiling of WT brown adipocytes were significantly changed with 89 up DEGs and 544 down DEGs under cold stress (orange round figure in Fig. 3A), however, there were 203 up DEGs and 315 down DEGs in DKO brown adipocytes under cold stress (purple round). Furthermore, functional enrichment analysis of these upregulated DEGs identified oxidative phosphorylation as the top KEGG (Kyoto Encyclopedia of Genes and Genomes) pathway in either WT or DKO brown adipocytes under cold exposure (Fig. 3B). Collectively, KEGG pathway analysis indicated that deletion of CCL5/CCR5 facilitated the expression of genes related to oxidative phosphorylation and lipid metabolic pathways after cold exposure. In the aspect of KEGG pathway analysis of downregulated DEGs under cold exposure, ECM receptor interaction was notably influenced in WT brown adipocytes. Some inflammation related pathways, such as TNF signaling pathway, NF-kappa B signaling pathway or MAPK signaling pathway were notably repressed when giving cold exposure on DKO brown adipocytes. Nevertheless, there were no significant changes in inflammatory gene expressions in BAT between WT and DKO mice in room temperature (Supplementary Table 3).

**CCL5/CCR5 deletion upregulates UCP-1 expression and improves metabolic flexibility in BAT**

We next confirmed the RNA-seq results using qRT-PCR and western blot. At room temperature, the mRNA
and protein expression levels of UCP-1 in BAT were barely detectable in WT mice but were significantly enhanced in DKO mice. However, cold stress-induced augmentation of UCP-1 and PGC-1α mRNA or protein levels in BAT between WT and DKO mice was not different (Fig. 4A-D). Accordingly, IHC staining showed that the number of UCP1-positive adipocytes in BAT was increased in DKO mice compared with WT mice at room temperature. Nevertheless, there were similar numbers of UCP-1-positive adipocytes in BAT after cold exposure (Fig. 4E, F). Other thermogenic genes, including Prdm16, Dio2 and ARβ3, were greatly elevated in the BAT of WT and DKO mice under cold stimulation, but were not different between cold-acclimated groups. To investigate the molecular mechanisms of CCL5/CCR5 signaling in non-shivering thermogenesis, we first examined whether deletion of the CCL5/CCR5 axis can activate signaling pathways related to thermogenesis. As expected, cold exposure resulted in stimulation of protein kinase A (PKA) signaling, as previously reported (31, 32), by increasing cAMP response element binding protein (CREB)-S133, activating transcription factor 2 (ATF2) phosphorylation and PPAR-γ expression in BAT (Fig. 4G). However, there were not different between the genotypes under cold exposure (Fig. 4G). This finding indicates that CCL5/CCR5 signaling might play a counterregulatory role in controlling thermogenic programming in BAT, especially UCP-1 activation. Searching for a potential link between the elevated UCP1 expression, we asked the question whether these DKO mice may have altered mitochondrial activity and biogenesis. We investigated whether CCL5/CCR5 gene deletion can impact the expression of genes related to mitochondrial oxidative function in the BAT of DKO mice. The expression of some key OxPhos genes, such as Tfam, Atp5b and Cys, was increased in the BAT of WT and DKO mice exposed to 4°C (Fig. 5A). Notably, the expression of genes involved in mitochondrial oxidative function (Atp5b, and Cys) was elevated in the BAT of DKO mice, even at room temperature (Fig. 5A). TEM micrographs showed that the mitochondria in BAT exhibited a more elongated and connected mitochondrial network and a larger size under cold conditions than at room temperature. The mitochondria from DKO mice were clearly visible and exhibited the tighter cristae organization than those from WT mice under cold exposure (Fig. 5B). To confirm this, mitochondrial oxygen consumption rates (OCRs) were determined in brown adipocytes from WT and DKO mice. The mitochondrial OCR of DKO brown adipocytes (DKO-BRO) remained higher than that of WT brown adipocytes (WT-BRO) (Fig. 5C). Interestingly, DKO brown adipocytes facilitated both basal spared respiration capacity and ATP production (Fig. 5D). We also measured the OCR as an indicator
of mitochondrial respiration and the extracellular acidification rate (ECAR) as an indicator of glycolysis in BAT of WT and DKO mice after cold stress. The ratio of OCR/ECAR can indicate a relative preference of metabolism for either oxidative phosphorylation or glycolysis. The cold exposure-induced decrease in the ratio of OCR/ECAR in BAT indicated that the striking preference of BAT for glycolysis after cold stress was not different between WT and DKO mice (Fig. 5E). These results suggest that deletion of CCL5/CCR5 significantly enhanced the brown-specific thermogenic program in isolated BAT precursors in a cell-autonomous manner.

**CCL5/CCR5 deficiency facilitates lipolysis**

As the enhanced induction of UCP1 and elevated mitochondrial oxidative function were noted in DKO mice, we want to further clarify whether cold-activated BAT in DKO mice could change cold-related lipid metabolism. To confirm the lipolytic capacity of DKO mice, we analyzed the gene expression of factors related to fatty acid breakdown and synthesis, such as HSL, ATGL, and Scd1, and to β-oxidation, such as Cpt1b, Acox1, and the fatty acid elongase Elovl3. Increased levels of Elovl3, ATGL, Cpt1b, Scd1 and Acox1 mRNA were observed in the BAT (Fig. 6A) of all mice under cold stimulation compared to room temperature conditions. We also examined downstream signaling cascades of PKA, which are the key factors that mediate lipolysis. Basal and cold stress-induced phosphorylation levels of HSL (Ser563), ATGL, perilipin-1 and ABHD5 in both BAT (Fig. 6B and C) and inguinal WAT (iWAT) (Supplementary figure 1) were higher in DKO mice than in WT mice. Therefore, these data suggest that CCL5/CCR5 deficiency facilitates lipolysis in BAT and iWAT at room temperature and cold exposure. Next, we measured lipolytic activities of differentiated adipocytes isolated from the BAT of WT and DKO mice. To mimic cold exposure through β-adrenergic activation, the differentiated adipocytes were treated with isoproterenol, a β-adrenergic activator. As shown in Figure 6D, isoproterenol-stimulated lipolysis, measured as glycerol release, was further increased in primary brown adipocytes in mice with CCL5/CCR5 deficiency compared to WT mice. Then, in an *ex vivo* study, we treated brown and inguinal fat pad explants obtained from cold-stressed WT and DKO mice with isoproterenol. Basal and isoproterenol-mediated levels of lipolytic activity (glycerol release) were higher in brown (Fig. 6E) and inguinal fat pads (Fig. 6F) from DKO than those from WT mice at each corresponding time points. Isoproterenol treatment significantly increased fat glycerol release in a time-dependent manner. These findings suggest that the augmented energy expenditure observed in DKO
mice might at least in part, be attributed to the facilitation of adipocyte lipolysis under basal conditions and upon stimulation.

**Importance of AMPK-mediated lipolysis in CCL5/CCR5-mediated energy metabolism**

To determine whether AMPK activation is crucial for the augmentation of adipocyte lipolysis-induced thermogenesis in DKO mice, we measured the phosphorylation of AMPK on Thr172 in BAT obtained from WT and DKO mice exposed to cold for 11 days. As shown in Figure 7A, basal and cold stress-induced phosphorylation levels of AMPK in BAT were higher in DKO mice than in WT mice at the corresponding time point. Consistently, statistical bar chart shows the increase in the ratio of phosphorylated AMPK and total AMPK compared to control in room temperature and cold acclimatation, respectively. Furthermore, the study was conducted with primary brown adipocytes isolated from DKO mice and used siRNA-mediated knockdown of AMPKα1 and α2 to verify the role of AMPK-mediated pathways in regulating CCL5/CCR5-mediated inhibition of adipocyte lipolysis and related thermogenesis. Our results showed that DKO primary brown adipocytes lacking AMPKα1 and α2 completely lost their ability to facilitate UCP-1 and PGC-1α protein expression, even upon CL-316243 stimulation (Fig. 7B). Deletion of AMPKα1 and α2 in DKO brown adipocytes also prevented the enhancement of key protein that regulate lipolysis in adipose tissue, such as p-HSL563 (the PKA regulatory sites), p-HSL565 (the AMPK regulatory site), perilipin-1, ABHD5 (CGI-58) and ATGL (Fig. 7B). Furthermore, an *in vivo* study was performed to clarify whether CCL5/CCR5 signaling in BAT can affect the adipocyte lipolysis-induced thermogenic phenotype. Lentivirus-derived CCL5 and CCR5 full-length cDNA or CCL5 and CCR5 shRNA was injected into BAT. After BAT local lentiviral injection for 5 days, the mice were intraperitoneally injected with saline or CL-316,243 (1 mg/kg) for 4 consecutive days. Increased localized CCL5 and CCR5 expression was observed only in BAT with CCL5 and CCR5-expressing lentivirus compared to those injected with control vector (Fig. 7C). Overexpression of CCL5/CCR5 in BAT did not result in significant body weight changes in response to CL-316,243 administration for 4 days (Fig. 7D). Compared to the control mice, mice overexpressing CCL5/CCR5 in BAT exhibited significantly decreased UCP-1 and PGC-1α expression and phosphorylation of AMPK, HSL p563 and ABHD5 in BAT upon treatment with saline and CL-326243, respectively (Fig. 7E). The expression of both TNF-α, Nos2 and IL-6 genes was about 1.3~1.9 folds higher in mice overexpressing CCL5/CCR5 in BAT than control mice (Supplementary Figure 2), implicating that CCL5/CCR5...
overexpression-induced inflammation might at least in part, be involved in the suppression of UCP-1 and related thermogenic gene and protein. In another experiment, a lentivirus expressing CCL5 and CCR5 shRNA or control shRNA was injected into BAT. Decreased localized CCL5/CCR5 expression was only observed at BAT injected with shRNA CCL5 and CCR5 compared to those injected with control shRNA (Fig. 7F). Mice with local injection of shCCL5/CCR5 into the BAT caused significant body weight loss during the CL-316,243 treatment period (Fig. 7G). Notably, knockdown of the gene expression of CCL5 and CCR5 in BAT increased UCP-1 and PGC-1α protein expression and the phosphorylation of AMPK, HSL p563 and ABHD5 in BAT both upon saline and CL-316,243 treatment (Fig. 7H).

Improved HOMA-IR and insulin sensitivity in HFD-fed mice with global deletion and local BAT knockdown of the CCL5 and CCR5 genes

An increase in adipose tissue thermogenesis can be accompanied by increases in whole-body energy expenditure and improved glucose homeostasis in vivo (33). Our data showed that global CCL5/CCR5 deletion (DKO) mice exhibited a decrease in fasting insulin and HOMA-IR levels after HFD feeding for 20 weeks (Table 1). Moreover, we found the significant increases in CCL5 and CCR5 protein expressions in BAT of HFD-fed mice compared to those on regular diet (Fig. 7I). To test whether local knockdown of BAT CCL5/CCR5 could significantly attenuates the development of HFD-induced insulin resistance, we first injected a lentivirus carrying shCCL5 and shCCR5 directly into the BAT of mice with HFD feeding for 16 weeks and measured the changes in HOMA-IR and insulin tolerance test (ITT) after additional 4 weeks of diet intervention. The HFD-induced increase in the value of HOMA-IR was significantly suppressed in mice that received local injection of shCCL5/CCR5 into BAT (Fig. 7J). Accordingly, the ITT showed that the elevated area under the curve of glucose in HFD mice was significantly decreased while combined with lentivirus-mediated BAT CCL5/CCR5 knockdown treatment (Fig. 7K,L).

Discussion

Although chemokine-associated proteins and their receptors, such as CCL5 and CCR5, have been reported to substantially contribute to the development of obesity-associated inflammation, the effect of obesity-induced augmented CCL5/CCR5 signaling in BAT on the regulation of energy metabolism and adaptive thermogenesis remains unknown. Our study suggested for the first time that obesity-induced augmentation of AT CCL5/CCR5 signaling not only triggers inflammation in WAT but also suppresses
adaptive thermogenesis through AMPK-mediated lipolysis and oxidative metabolism in BAT to exacerbate the development of obesity and associated insulin resistance (Fig. 8).

Previous studies have reported that CCL5 and CCR5 are upregulated in the WAT of obese mice and human subjects (18-20, 34). In the present study, protection against obesity and HFD-induced insulin resistance in mice with global CCL5/CCR5 deletion was indicated by increased calorie consumption, decreased adipocyte size and fat pad weight in WAT. It is implicated that energy expenditure is augmented in DKO mice. In addition, manipulation of CCL5/CCR5 gene expression in BAT significantly altered the regulation of tissue energy expenditure including adaptive thermogenesis. Moreover, local injection of a lentivirus expressing shCCL5/CCR5 into BAT improved the metabolic phenotypes of HFD-fed mice, as assessed by HOMA-IR and ITT results. This study provides compelling evidence that CCL5/CCR5-mediated signaling in BAT, acts as a negative regulator of energy expenditure and adaptive thermogenesis under physiological and pathological conditions. On the other hand, augmented CCL5/CCR5 expression in BAT during the development of obesity could further exacerbate the progression of obesity at least in part, by inhibiting energy expenditure and adaptive thermogenesis.

Accordingly, our data suggest that CCL5/CCR5-mediated signaling negatively regulates lipolysis and oxidative metabolism, as indicated by increased expression levels of ATGL, ABHD5 and pHSL in BAT and enhanced basal and isoproterenol-induced lipolysis in isolated brown adipocytes and cold-exposed BAT. It has been reported that stimulation with β3-adrenergic receptor agonists or low temperature promotes lipolysis and oxidative metabolism in adipose tissue and thereby boosts thermoregulatory responses (35, 36). In BAT, lipolysis is essential for the production of FAs, which act as primary fuel substrates for the activation of UCP-1-mediated non-shivering thermogenesis (5, 14, 3, 37). Therefore, CCL5/CCR5-mediated signaling in BAT might affect energy expenditure, at least in part, through the regulation of lipolysis.

In vivo gain- and loss-of-function studies revealed that knockdown of BAT CCL5/CCR5 gene expression significantly upregulated AMPK phosphorylation, which can facilitate thermogenic programming in BAT through AMPK-induced Ser563-HSL phosphorylation and ABHD5 expression. Moreover, AMPK α1- and α2-silencing significant suppressed the augmentation of UCP-1 and PGC-1α expression in isolated DKO brown adipocytes indicated the importance of AMPK activation in counter-regulation of CCL5/CCR5 signaling in adaptive thermogenesis. AMPK is considered a cellular...
sensor of energy homeostasis and is activated upon β-adrenergic stimulation to enhance FA oxidation for ATP production in human brown adipocytes and mouse BAT (15, 38-40). In addition, the other reports have demonstrated that AMPK may increase adipocytes HSL activation through PKA-mediated phosphorylation at Ser660/563 and concomitantly increased perilipin and ATGL activation in lipolysis (41-43). Deletion of AMPK in adipocytes leads to the defects in BAT mitochondrial structure and function and reduces oxidative metabolism in response to cold exposure (44). Moreover, obesity and insulin resistance are associated with reduced AMPK activity in BAT (36, 3). In this regard, our results further demonstrate that the augmentation in lipolysis and mitochondrial FA oxidation in BAT of mice with CCL5/CCR5 deletion are AMPK dependent upon cold exposure and even in the basal state, might result in elevated thermogenic responses. These results suggest that the obesity-induced the enhancement of CCL5/CCR5-mediated signaling could suppress AT lipolysis and oxidative metabolism via the inhibition of AMPK activation in BAT to further deteriorate the development of obesity.

RNA-seq was used in this study to determine the molecular signature of mouse BAT during long-term (11-day) cold challenge (4°C) and to provide a comprehensive view of changes in the gene expression profile. The significantly enriched metabolic (p < 0.01) pathways extracted from the KEGG database are shown genes that were affected by cold exposure. Consistent with previous studies in mice and human subjects (45-48), we showed the most highly enriched pathways of upregulated genes in WT brown adipocytes in response to cold, which were further enhanced in CCL5/CCR5-deficient mice, were oxidative phosphorylation and glycolysis metabolic reactions. Notably, KEGG pathway analysis revealed that the PPAR signaling pathway and fatty acid metabolism were enriched by cold exposure in DKO brown adipocytes, which was not significantly altered in WT brown adipocytes. These data implied that the enhanced thermogenesis observed in DKO mice not only facilitated the utilization of thermogenic fuels but also upregulated transcriptional programs in brown adipocytes during cold exposure. In addition, the upregulated genes associated with carbon metabolism, fatty acid metabolism and the PPAR signaling pathway were also markedly increased in DKO mice. These results further suggest that CCL5/CCR5-mediated signaling plays counterregulatory roles in AT energy metabolism and adaptive thermogenesis in BAT under conditions that require energy (cold exposure).

The slight increase in basal plasma NEFA levels and decrease in the HOMA-IR value might attribute to
the augmented energy expenditure and lipolysis exhibited in DKO mice. Nevertheless, CCL5 is also found in spleen, lungs and small intestine other than BAT and inflammatory cells (49). Thereby, the study was further conducted with WT mice with local knockdown or overexpression of CCL5/CCR5 in BAT to rule out the collateral effects of global CCL5/CCR5 deletion on data interpretation. On the other hand, BAT and skeletal muscle have emerged as the two major sites of adaptive thermogenesis. Under cold-induced adaptive thermogenesis, the skeletal muscles and BAT could exhibit functional interplay (6, 7). For instance, in murine models of increased BAT thermogenesis, fatty acid oxidation in skeletal muscle is increased simultaneously (50,51). Consequently, some defects in muscle would influence the heat generation and physical activity at thermoneutrality. Therefore, it is possible that the muscle-mediated thermogenic response might contribute at least in part, to the CCL5/CCR5-mediated effects on adaptive thermogenesis in BAT. Nevertheless, there were some gene expressions in the inflammatory pathways such as TNF and NFκB signaling repressed under cold acclimation in DKO mice. Due to the limitation of this study, the potential impact on the data interpretation of CCL5/CCR5-mediated inflammatory effect on energy expenditure and adaptive thermogenesis is still needed to be further clarified.

In summary, our study suggests that obesity-associated enhancement of the AT CCL5/CCR5 signaling pathway plays a pivotal role in both the pathogenesis of AT inflammation and the development of obesity-associated energy imbalance. In addition, inhibition of augmented CCL5/CCR5-mediated signaling in BAT, protects against obesity and diet-induced insulin resistance and at least in part, exerts beneficial metabolic effects via AMPK-mediated lipolysis and oxidative metabolism and subsequent adaptive thermogenesis. CCL5/CCR5 signaling in BAT could be a promising therapeutic target for the treatment of obesity and its associated cardiometabolic abnormalities.

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**Author Contributions**

PSH wrote the proposal, designed the experiments and wrote the manuscript. PCC performed and organized
experiments and wrote the manuscript under the guide of PSH. LMH, JPH, CJY and YJD helped to perform some of the animal experiments. FCK and CHL help to analyze and interpret the NGS data. YFT help to discuss and paper writing.

**Conflict of interest**

The authors declare no competing interests.

**Data Availability**

All supporting data are included within the main article and its supplementary files. Derived data supporting the findings of this study are available from the corresponding author PSH on request. The complete RNA-Seq dataset is available from the [https://github.com/peggyndmc/RNA-seq.git](https://github.com/peggyndmc/RNA-seq.git).

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Table 1. Metabolic characteristics in wild-type mice fed with normal chow diet (WT-NCD) or high fat diet (WT-HFD) and CCR5 and CCL5 double knockout mice fed with normal chow diet (DKO-NCD) or high fat diet (DKO-HFD) for 20 weeks.

| Parameter       | WT-NCD (n=10) | DKO-NCD (n=8) | WT-HFD (n=8) | DKO-HFD (n=8) |
|-----------------|---------------|---------------|--------------|---------------|
| Cholesterol (mg/dl) | 64.01 ± 2.27 | 70.41 ± 4.32 | 198.07 ± 7.72 * | 194.79 ± 7.14 # |
| Triglyceride (mg/dl) | 70.64 ± 5.58 | 77.50 ± 4.62 | 75.52 ± 2.77      | 79.17 ± 5.10   |
| NEFA (mmol/L)    | 1.25 ± 0.05   | 1.51 ± 0.11 * | 0.63 ± 0.06 *     | 0.77 ± 0.03 #  |
| Glucose (mg/dL)  | 89.2 ± 3.92   | 83.6 ± 2.3    | 103.7 ± 5.99 *    | 109.59 ± 6.72 # |
| Insulin (ug/L)   | 1.28 ± 0.2    | 0.79 ± 0.2*   | 1.86 ± 0.19 *     | 0.98 ± 0.24 # † |
| HOMA-IR          | 8.24 ± 1.65   | 5.18 ± 1.37 * | 13.73 ± 1.8 *     | 9.01 ± 2.63 # † |

Data are presented as mean ± SEM in each group, *, p<0.05 vs. WT-NCD; #, p<0.05 vs. DKO-NCD; †, p<0.05, vs. WT-HFD.
**Figure Legends**

**Figure 1. Protection against HFD-induced obesity in CCL5/CCR5 double-knockout (DKO) mice.** WT and DKO mice were fed HFD (n=9-12 for each experimental group) for 20 weeks from 6 weeks of age. (A) Body weights of WT and DKO mice on high fat diet. *P < 0.05 vs. DKO-NCD; # P < 0.05 vs. DKO-HFD. (B) Calorie intake of WT and DKO mice on high fat diet. * P < 0.05. (C) Relation of CCL5 and CCR5 gene expression levels in eWAT to body weight. (D) Tissue weights in WT and DKO mice after 20 weeks of HFD. *P < 0.05. (E) Representative images of H&E staining of adipose tissues and liver of WT and DKO mice after 20 weeks of HFD. Bars represent 50 µm (n = 3 mice/group). All data presented are mean values ± SEM.

**Figure 2. DKO mice are cold-tolerant with augmented energy expenditure.** (A) Western blot analysis of CCL5 and CCR5 in BAT of WT mice after cold exposure for 11 days. (B) Western blot analysis of CCL5 and CCR5 in brown adipocytes of WT mice after CL-316243 treatment. Differentiated adipocytes were treated with or without CL316243 (10 µM) for 6 h. (C) Body weight of WT and DKO mice during experimental period of 25°C or 4°C for 11 days. *P < 0.05 vs. WT-RT; # P < 0.05 vs. DKO-RT. (D) Core body temperature of WT and DKO mice during experimental period of 25°C or 4°C for 11 days. (E) Core body temperature of WT and DKO mice during experimental period of 4°C for 4 hrs. (F) VO₂ of WT and DKO mice were measured during indirect calorimetry tests using a Comprehensive Lab Animal Monitoring System (CLAMS) during room temperature (RT), 2-day (2D) short-term cold exposure (CE) and 11-day (11D) long-term CE. (G) VO₂ of WT and DKO mice analyzed by TSE Systems GmbH during RT, 2D short-term CE and 11D long-term CE. (H) Energy expenditure (EE) of WT and DKO mice analyzed by CLAMS. Values are calculated as the ratio of VCO₂ to VO₂ produced and consumed by the mice, respectively. Each dot represents a mouse. (n=8/group) Data represent the mean ± SEM. *P < 0.05.

**Figure 3. CCL5/CCR5 deletion upregulated cold stress-induced gene programs involved in oxidative phosphorylation and lipid metabolism** (A) RNA-sequencing gene expression signatures of brown adipocytes isolated from WT and DKO mice after cold exposure. (B) Genes that were among the top 250 induced in response to cold in brown adipocytes were subjected to functional enrichment analyses using HOMER. Significantly enriched (p < 0.01) metabolic pathways extracted from the KEGG database are shown for genes with cold-induced expression.

**Figure 4. CCL5/CCR5 deletion up-regulated UCP-1 expression in BAT** (A) Total mRNA was extracted from BAT and used for qPCR analysis of thermogenesis genes after cold exposure for 11 days. (n = 8/group). (B to D) Western blot analysis of protein expressions UCP-1 and PGC-1α in BAT (n = 6/group, each lane represents a mix of 3 mice). * P < 0.05. vs. WT-RT; # P < 0.05 vs. DKO-RT. (E) Immunohistochemical staining of UCP1 in BAT of WT and DKO mice exposed to RT or CE for 11 days (n = 3 mice/group). Bars represent 100 µm. (F) Quantitation of UCP1 positive adipocytes in (E). UCP1 levels are relative to WT at RT. (G) Western blot analysis of cold-induced thermogenesis related signaling pathway in BAT of mice after cold exposure for 11 days (n = 6/group, each lane represents a mix of 3 mice). All data presented are mean values ± SEM.

**Figure 5. CCL5/CCR5 deletion promotes mitochondrial activity and biogenesis upon cold exposure.** (A) mRNA expression of mitochondrial biogenesis and oxidative function genes in BAT of mice after cold exposure for 11 days. (n = 8/group). (B) Mitochondrial morphology and quantification of mitochondrial area in BAT from WT and DKO mice using electronic microscopy after cold exposure for 11 days (n = 3 mice/group). Data presented are representative micrographs with 0.5-µm scale bars and quantification of mitochondrial area in based on ~30 mitochondria per condition. (C to D) OCR of isolated primary brown adipocytes from BAT of WT and DKO mice at room temperature (n = 12 mice/group). (E) OCR/ECAR ratio of in BAT of mice exposed to 25°C or 4°C (n = 8 mice/group). All data presented are mean values ± SEM. * P < 0.05.

**Figure 6. CCL5/CCR5 deficiency promotes lipolysis upon cold exposure.** (A) mRNA expression of lipolysis genes in BAT of mice after cold exposure for 11 days (n = 8/group). (B to C) Western blot analysis of lipolytic activity in BAT (n = 6/group, each lane represents a mix of 3 mice). (D) Primary pre-adipocytes were isolated from BAT of WT and DKO mice and were fully differentiated into...
adipocytes. For basal and stimulated lipolysis, differentiated adipocytes were treated with or without isoproterenol (1 µM) for 3 h. The levels of glycerol were measured from conditional media (n = 3–5 mice/group). (E) Glycerol levels in BAT and (F) Glycerol levels in iWAT isolated from cold challenged WT and DKO mice were treated with or without isoproterenol (1 µM) for indicated time (n = 3–5 /group). All data presented are mean values ± SEM.; *P < 0.05; **P < 0.01 vs. control group.

**Figure 7. CCL5/CCR5 deficiency promotes AT lipolysis through AMPK activation.**

(A) Western blot analysis for phosphorylation levels of AMPK in BAT of WT and DKO mice exposed to 25°C or 4°C for 11 days (n = 8 /group). Actin was used as a loading control. (B) AMPK phosphorylation and lipolysis markers measured by immunoblotting in differentiated DKO brown adipocytes treated with non-targeting control (non-silencing shRNA) and AMPKα1 & α2-targeting shRNA (AMPKα1 & α2 shRNA) in the presence or absence of CL-316243 (CL, 10 µM) for 6h. (C-E): BAT of C57BL/6 mice was injected with either control or CCL5 & CCR5 lentivirus for increase in CCL5 & CCR5 expression (LacZ vs. L5R5 ov, n = 6/group) at day 5 post-injection and were injected intraperitoneally with CL-316,243 at a dose of 1mg/kg. (C) Western blot analysis of BAT and iWAT CCL5 and CCR5 protein level of C57BL/6 mice which injected with LacZ or lentivirus-derived CCL5 & CCR5 full length cDNA in BAT. (D) Body weight change in mice. (E) Western blot analysis of PGC1-α, UCP-1, p-AMPK, p-HSL563 and ABHD5 in BAT. (F-H): BAT of C57BL/6 mice was injected with either control or scrambled or shCCL5 & shCCR5 lentivirus for knockdown in CCL5 & CCR5 expression (scrambled vs. shL5R5, n = 6/group) at day 5 post-injection and were injected intraperitoneally with CL-316,243 at a dose of 1mg/kg. (F) Western blot analysis of BAT and iWAT CCL5 and CCR5 protein level of C57BL/6 mice which injected with scrambled or lentivirus-derived shCCL5 & shCCR5 in BAT. (G) Body weight change in mice. (H) Western blot analysis of PGC1-α, UCP-1, p-AMPK, p-HSL563 and ABHD5 in BAT. (I) Western blot analysis of CCL5 and CCR5 protein levels in BAT of 20-weeks HFD fed C57BL/6 mice. (J) C57BL/6 mice injected a lentivirus carrying shCCL5 and shCCR5 directly into the BAT of mice with HFD feeding for 16 weeks and measured the changes in HOMA-IR and (K, L) insulin tolerance test (ITT) after additional 4 weeks of diet intervention (n = 6 /group). Data represent the mean ± SEM; * P < 0.05

**Figure 8. Schematic proposed model.**

The vicious cycle initiating by obesity-induced augmentation in AT CCL5/CCR5 signaling not only trigger AT inflammation but also suppress BAT energy expenditure via inhibiting AMPK-mediated lipolysis and oxidative metabolism and subsequent adaptive thermogenesis to deteriorate the development of AT inflammation.
### WT-RT vs WT-CE (Brown adipocytes) - induced by cold

### KEGG pathway (p<0.01) associated with top 9 genes induced by cold

| Term ID | Term                                | P-value | Genes in Term | Target Genes in Term | Gene Symbols                  |
|---------|-------------------------------------|---------|---------------|----------------------|-------------------------------|
| mmu00190 | Oxidative phosphorylation           | 4.58E-15 | 135           | 15                   | mt-Cytb, mt-Nd4, Uqcrx, mt-ND3, Cox8b, mt-Coa3, mt-ND1, mt-ND5, Cox7a1, mt-Coa1, mt-ND2, mt-Atp6, mt-Atp8, mt-Co2, mt-Atp6 |
| mmu00010 | Glycolysis / Gluconeogenesis        | 2.77E-05 | 65            | 7                    | Pdk1, Eno1, Pdk4, AldoA, Gpd1, Gapdh, Pkm |
| mmu00120 | Carbon metabolism                   | 1.07E-04 | 111           | 8                    | Pdk1, Eno1, Gapdh, Pdk4, AldoA, Gpd1, Mel, Pkm |
| mmu00123 | Biosynthesis of amino acids         | 3.46E-03 | 78            | 5                    | Gapdh, Pdk4, Eno1, Pkm, AldoA |

### DKO-RT vs DKO-CE (Brown adipocytes) - induced by cold

### KEGG pathway (p<0.01) associated with top 203 genes induced by cold

| Term ID | Term                                | P-value | Genes in Term | Target Genes in Term | Gene Symbols                  |
|---------|-------------------------------------|---------|---------------|----------------------|-------------------------------|
| mmu00190 | Oxidative phosphorylation           | 7.63E-12 | 135           | 18                   | Ndufb4, Ndufa5, Ndufa12, mt-ND3, Atp6e6e, mt-Atp8, Ndufa11, Ndufa5, Ndufa4, Ndufa12, Atp6e6e, Cox7a1, Uqcrx, Ndufa11, mt-ND3, Atp6e6e, Uqcrx, mt-ND1, Hko1, Cog51, Mec, Pdk4, Got1, Eno1, Pdk4, Gapdh, Acs2, Pkp4, Pdhb, AldoA, Gpd1, Mel, Pkm |
| mmu00120 | Carbon metabolism                   | 1.32E-10 | 111           | 18                   | Hko1, Cog51, Mec, Pdk4, Got1, Eno1, Pdk4, Gapdh, Acs2, Pkp4, Pdhb, AldoA, Gpd1, Gapdh, Pgm1, Tik, Pkm |
| mmu00010 | Glycolysis / Gluconeogenesis        | 5.29E-09 | 65            | 13                   | Hko1, Pbk1, Eno1, Pdgk1, AldoA, Acs2, Pkp4, Pdhb, AldoA, Gpd1, Gapdh, Pgm1, Tik, Pkm |
| mmu00104 | Biosynthesis of unsaturated fatty acids | 2.88E-06 | 25            | 7                    | Hsd17b12, Aca11b, Elov5, Scd2, Elov16, Scd1, Acox2 |
| mmu01230 | Biosynthesis of amino acids         | 1.15E-05 | 78            | 10                   | Pdk1, Got1, Eno1, Pdk4, AldoA, Pkp4, Gapdh, Pgm1, Tik, Pkm |
| mmu03220 | PPPA signaling pathway              | 9.49E-05 | 82            | 9                    | Acs1b, Glk, Scd2, Scd1, Acs5, Mel, Fabp3, Fabp5, Ucp1 |
| mmu01211 | Fatty acid metabolism               | 6.00E-04 | 56            | 7                    | Hsd17b12, Aca11b, Elov5, Scd2, Elov16, Scd1, Acox2 |
| mmu00062 | Fatty acid elongation               | 6.00E-04 | 24            | 5                    | Acox2, Hsd17b12, Elov16, Elov5 |
| mmu00055 | Fructose and mannose metabolism     | 0.001215 | 35            | 5                    | Pdk1, Pkp4, Pmn1, AldoA, Hko1 |
| mmu00625 | Pyruvate metabolism                | 0.00177 | 39            | 5                    | Mel, AldoA, Acs2, Pdp4, Pkm |
| mmu00052 | Galactose metabolism               | 0.00643 | 33            | 4                    | Pdk1, Ugp2, Pkp4, Hko1 |
| Parameter     | WT-NCD (n=10) | DKO-NCD (n=8) | WT-HFD (n=8) | DKO-HFD (n=8) |
|--------------|--------------|--------------|--------------|--------------|
| Cholesterol  | 64.01 ± 2.27 | 70.41 ± 4.32 | 198.07 ± 7.72 * | 194.79 ± 7.14 # |
| Triglyceride | 70.64 ± 5.58 | 77.50 ± 4.62 | 75.52 ± 2.77 | 79.17 ± 5.10 |
| NEFA (nmol/L)| 1.25 ± 0.05  | 1.51 ± 0.11 * | 0.63 ± 0.06 * | 0.77 ± 0.03 # |
| Glucose      | 89.2 ± 3.92  | 83.6 ± 2.3   | 103.7 ± 5.99 * | 109.59 ± 6.72 # |
| Insulin      | 1.28 ± 0.2   | 0.79 ± 0.2 * | 1.86 ± 0.19 * | 0.98 ± 0.24 # \|
| HOMA-IR      | 8.24 ± 1.65  | 5.18 ± 1.37 * | 13.73 ± 1.8 * | 9.01 ± 2.63 \# |