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Constitutively active receptor ADGRA3 signaling induces adipose thermogenesis

Zewei Zhao, Longyun Hu, Bigui Song, Tao Jiang, Qian Wu, Jiejing Lin, Xiaoxiao Li, Yi Cai, Jin Li, Bingxiu Qian, Siqi Liu, Jilu Lang ✉, Zhonghan Yang ✉

Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China • Department of Cardiovascular Surgery, Peking University Shenzhen Hospital, Shenzhen, China

eLife Assessment

The study identifies the adhesion G-protein-coupled receptor A3 (ADGRA3) as a potential target for activating adaptive thermogenesis in white and brown adipose tissue, providing **valuable** information for scientists in the field of adipose tissue biology and metabolism. Although the authors have addressed some concerns raised by reviewers, the interpretations remain somewhat limited, and the work is deemed **incomplete**. The evidence supporting ADGRA3's role in thermogenesis is insufficient, necessitating more rigorous experiments to validate the receptor's relevance in adipose tissue. Additionally, the lack of experiments using primary cultures, despite feedback from multiple reviewers, highlights significant shortcomings.

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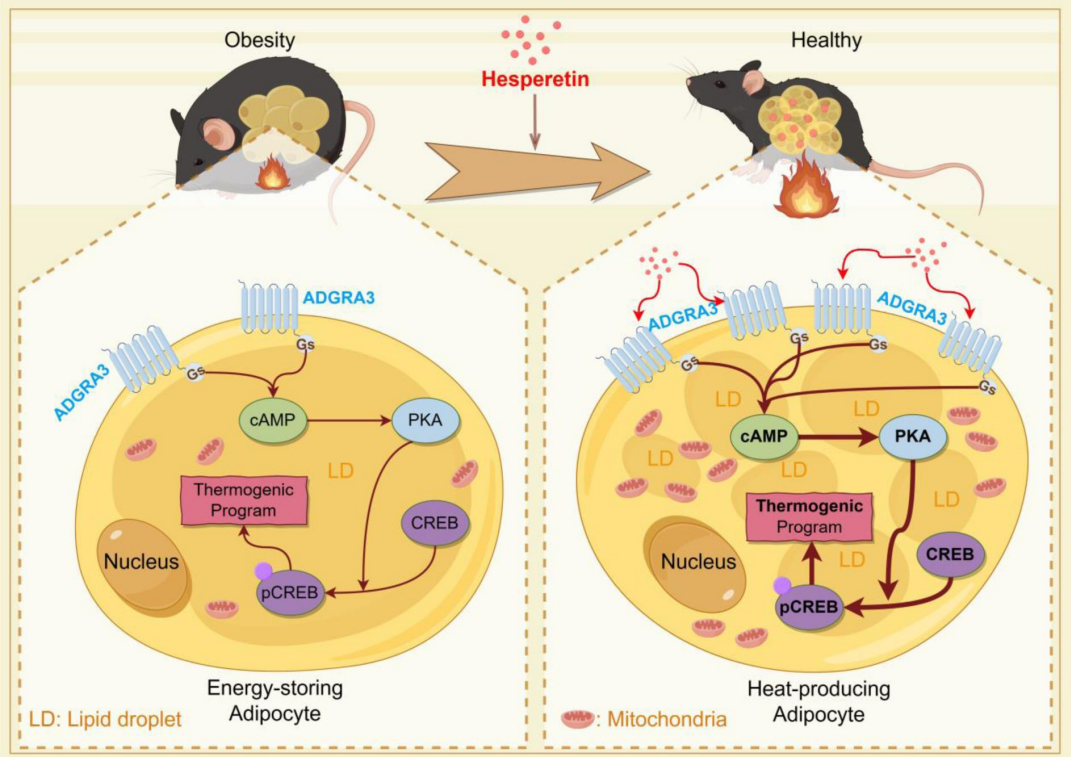
Abstract

The induction of adipose thermogenesis plays a critical role in maintaining body temperature and improving metabolic homeostasis to combat obesity. β3-adrenoceptor (β3-AR) is widely recognized as a canonical β-adrenergic G protein-coupled receptor (GPCR) that plays a crucial role in mediating adipose thermogenesis in mice. Nonetheless, the limited expression of β3-AR in human adipocytes restricts its clinical application. The objective of this study was to identify a GPCR that is highly expressed in human adipocytes and to explore its potential involvement in adipose thermogenesis. Our research findings have demonstrated that the adhesion G protein-coupled receptor A3 (ADGRA3), an orphan GPCR, plays a significant role in adipose thermogenesis through its constitutively active effects. ADGRA3 exhibited high expression levels in human adipocytes and mouse brown fat. Furthermore, the knockdown of *Adgra3* resulted in an exacerbated obese phenotype and a reduction in the expression of thermogenic markers. Conversely, *Adgra3* overexpression activated the adipose thermogenic program and improved metabolic homeostasis without an exogenous ligand supplementation. We found that ADGRA3 facilitates the biogenesis of beige adipocytes through the Gs-PKA-CREB axis. Moreover, hesperetin was identified as a potential agonist of ADGRA3, capable of inducing adipocyte browning and ameliorating insulin resistance. In

conclusion, our study demonstrated that the overexpression of constitutively active ADGRA3 or the activation of ADGRA3 by hesperetin can induce adipocyte browning by Gs-PKA-CREB axis. These findings indicate that the utilization of hesperetin and the selectively overexpression of ADGRA3 in adipose tissue could serve as promising therapeutic strategies in the fight against obesity.

Graphic abstract



This schema summarizes the main roles and functions of ADGRA3 in lipid metabolism. ADGRA3 promotes the biogenesis of beige adipocytes via the Gs-PKA-CREB axis. This figure was drawn by Figdraw (Copyright ID: IWAIA0d9f9).

1. Introduction

Since 1975, there has been a substantial increase in the global prevalence of obesity, with the magnitude nearly tripling. The World Health Organization projects that the prevalence of obesity among adults will exceed 20% by the year 2025 [1]. Currently, the management of excessive adiposity poses a paramount economic burden and healthcare predicament [2, 3]. In addition to the detrimental social and psychological implications, a multitude of studies have consistently demonstrated a significant association between obesity and an increased vulnerability to a range of health conditions, such as type 2 diabetes, cardiovascular diseases, and cancer [4–7].

Activating and maintaining the thermogenesis of beige/brown fat has been shown to be effective in treating obesity and related metabolic disorders in humans [8, 9]. As a well-established β-adrenergic GPCR, the β3-AR has been identified as a prominent target for stimulating adipose thermogenesis in mice. Regrettably, the clinical application of β3-AR has been impeded due to its low expression in human adipocytes and the cardiovascular risks associated with other

adrenergic receptors [10, 11]. G protein-coupled receptors (GPCRs) are the most prevalent class of drug targets among all drugs approved by the U.S. Food and Drug Administration (FDA). They also play a crucial role in the clinical treatment of obesity [12–14]. Therefore, it is of clinical significance to identify novel GPCR targets that induce adipose thermogenesis.

ADGRA3 is classified as an orphan adhesion G protein-coupled receptor (aGPCR) and exhibits the typical domains found in aGPCRs within its N-terminal extracellular region (ECR), including a leucine-rich repeat (LRR), an immunoglobulin-like domain (Ig), a hormone-binding domain (HBD), and a GAIN domain [15]. ADGRA3 was initially discovered as a distinctive indicator of various spermatogonial progenitor cells [16, 17]. Recent studies have shown that the orphan status of receptors has posed challenges to the study of aGPCRs. However, these studies have also uncovered a conservative mechanism of aGPCR activation, which involves the use of tethered ligands in the GAIN domain [18, 19]. ADGRA3 has been previously identified as a receptor capable of auto-cleavage [20]. However, the functional activity of ADGRA3 in a constructive manner is still uncertain. A genome-wide association study (GWAS) demonstrated a significant correlation between single nucleotide polymorphisms (SNPs) of ADGRA3 and body weight in chickens [21].

Nevertheless, the precise role of ADGRA3 in the progression of obesity and adipose thermogenesis remains uncertain. This study aimed to investigate three main aspects: (1) the impact of ADGRA3 on browning of white adipose tissue (WAT) and brown adipose tissue (BAT), (2) the effects of ADGRA3 on metabolic homeostasis, and (3) the underlying mechanisms by which ADGRA3 induces adipose thermogenesis.

2. Material and methods

2.1. Mice

Wild-type (WT) C57BL/6J mice were obtained from the Center of Laboratory Animal at Sun Yat-sen University. All mice were housed in the Sun Yat-sen University Laboratory Animal Center, where they were subjected to a 12-hour light-dark cycle and maintained at a controlled environmental temperature of 21±1℃. Eight-week-old male C57BL/6J mice were fed with a normal chow diet (NCD) or a high fat diet (HFD, 60% kcal) for 12 weeks to render mice obese. With the exception of mice fed with a HFD, male mice at the age of eight weeks were utilized in all experimental procedures.

For the knockdown and over-expression experiments of *Adgra3* in mice fed with a NCD, the following procedures were conducted: sh*Adgra3* (pLKO.1-U6-sh*Adgra3*-2 plasmid encapsulated in nanomaterials) and shNC (pLKO.1-U6-shNC plasmid encapsulated in nanomaterials) were injected intraperitoneally (i.p.) for knockdown experiments, while *Adgra3* OE (pLV3-CMV-*Adgra3* (mouse)-3×FLAG plasmid encapsulated in nanomaterials) and CON (pLV3-CMV-MCS-3×FLAG plasmid encapsulated in nanomaterials) were injected i.p. for over-expression experiments. The frequency of the sessions was twice a week over a period of four weeks. For the knockdown and over-expression experiments of *Adgra3* in mice fed with a HFD, the following procedures were conducted: sh*Adgra3* and shNC were injected intraperitoneally (i.p.) for knockdown experiments, while *Adgra3* OE and CON were injected i.p. for over-expression experiments. The frequency of the sessions was twice a week over a period of 12 weeks. For the local over-expression of *Adgra3* in BAT of mice fed with a NCD, *Adgra3* OE and CON were injected locally into BAT once.

For the treatment with a selective β3-adrenoceptor agonist, CL-316,243 (hereafter referred to as CL), mice fed a HFD were injected intraperitoneally (i.p.) with CL (1 mg/kg daily) for 7 days. For the treatment with hesperetin (Hes), hesperetin is dissolved in drinking water (200mg/L) and water were available ad libitum.

Intraperitoneal injections of glucose (2g/kg for mice fed with a NCD and 1g/kg for mice fed with a HFD) or insulin (0.5U/kg for mice fed with a NCD and 1U/kg for mice fed with a HFD) were administered. At the designated time points of 0 minutes, intraperitoneal glucose or insulin tolerance tests were conducted on mice that had been fasted for six hours. After administration, the blood glucose concentration was assessed at specific time intervals using a OneTouch Ultra Glucometer. Finally, the animals were euthanized, followed by the collection of tissue samples. Cohorts of ≥3 mice per genotype or treatment were assembled for all in vivo studies. All in vivo studies were repeated 2-3 independent times. All procedures related to animal feeding, treatment and welfare were conducted at Sun Yat-sen University Laboratory Animal Center.

2.2. Stromal Vascular Fraction (SVF) and mature adipocytes isolation

SVF from inguinal white adipose tissue (iWAT) and BAT of WT male mice at 4 weeks of age were washed with PBS, minced and digested with 0.1% type Ⅱ collagenase in Dulbecco's modified eagle medium (DMEM) containing 3% BSA and 25μg/ml DNase I for 30 min at 37℃. During the digestion, the mixed solution was shaken by a hand every 5 min. The mixed solution was filtered through a 70 μm cell strainer and then centrifuged at 500 g for 5 min at 4℃. The floating mature adipocytes were collected for subsequent analysis, and the pellets containing SVF were resuspended in red blood cell lysis buffer for 5 min at 37℃. Cells were centrifuged at 500 g for 10 min at 4℃ and the SVF pellets were collected for subsequent analysis.

2.3. Cell culture

3T3-L1 and 293T cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences in Shanghai. Human adipose-derived mesenchymal stem cells (hADSCs) were purchased from the National Stem Cell Translational Resource Center. 3T3-L1 cells were culture and grown to confluence in high-glucose DMEM supplemented with 10% newborn calf serum (NCS). 293T cells were culture and grown to confluence in high-glucose DMEM supplemented with 10% FBS. Confluent 3T3-L1 pre-adipocytes were induced into mature beige-like adipocytes with 0.5 mM isobutyl methylxanthine (IBMX), 1 μM dexamethasone, 5 μg/ml insulin, 1 nM 3, 3', 5-Triiodo-L-thyronine (T3), 125 μM indomethacin and 1 μM rosiglitazone in high-glucose DMEM containing 10% FBS for 2 days, then treated with high-glucose DMEM containing 5 μg/ml insulin, 1 nM T3, 1 μM rosiglitazone and 10% FBS for 6 days and cultured with high-glucose DMEM containing 10% FBS for 2 days. hADSCs were seeded on plates coated with 0.1% gelatin and culture and grown to confluence in human mesenchymal stem cells (hMSCs) specialized culture medium (ZQ-1320). Confluent hADSCs were induced into mature human adipocytes with adipogenic induction medium (PCM-I-004) according to the manufacturer's instructions.

The sh*Adgra3*, sh*Gnas* (pLKO.1-U6-sh*Gnas* plasmid encapsulated in nanomaterials) and shNC were added to 3T3-L1 mature beige-like adipocytes for 72 hours. The sh*ADGRA3* (pLKO.1-U6-sh*ADGRA3*-(1/2/3) plasmid encapsulated in nanomaterials) and shNC were added to human adipocytes for 72 hours. The pcDNA3.1(+)-m*Gnas*-6×His (mixture of pcDNA3.1(+)-*Gnas*(mouse)-6×His plasmid and transfection reagent), pcDNA3.1(+)-m*Gnai1*-6×His (mixture of pcDNA3.1(+)-*Gnai1*(mouse)-6×His plasmid and transfection reagent), pcDNA3.1(+)-m*Gnaq*-6×His (mixture of pcDNA3.1(+)-*Gnaq*(mouse)-6×His plasmid and transfection reagent), pcDNA3.1(+)-m*Gna12*-6×His (mixture of pcDNA3.1(+)-*Gna12*(mouse)-6×His plasmid and transfection reagent), *Adgra3* OE and CON were added to 3T3-L1 mature beige-like adipocytes or 293T for 48 hours. The *ADGRA3* OE (pLV3-CMV-*ADGRA3*(human)-3×FLAG plasmid encapsulated in nanomaterials) and CON were added to human adipocytes for 48 hours. Hesperetin (10 μM) and PKAi (protein kinase A inhibitor, 20 μM H-89) was added to 3T3-L1 mature beige-like adipocytes for 48 hours. All in vitro studies were repeated 2-3 independent times.

2.4 Construction of plasmid

The pLV3-CMV-*Adgra3*(mouse)-3×FLAG, pLV3-CMV-*ADGRA3*(human)-3×FLAG, pLKO.1-U6-sh*ADGRA3*-(1/2/3), pLV3-CMV-MCS-3×FLAG, pcDNA3.1(+)-*Gnas*(mouse)-6×His, pcDNA3.1(+)-*Gnai1*(mouse)-6×His, pcDNA3.1(+)-*Gnaq*(mouse)-6×His and pcDNA3.1(+)-*Gna12*(mouse)-6×His plasmids were purchased from Shenzhen Yanming Biotechnology Co., LTD. The pLKO.1-U6-sh*Adgra3*-(1/2/3) and pLKO.1-U6-shNC plasmids were purchased from Guangzhou Hanyi Biotechnology Co., LTD.

2.5. Temperature measurements

The body temperature was measured at 9:00 using a rectal probe connected to a digital thermometer.

2.6. Real-time Polymerase Chain Reaction (PCR)

Total RNA from tissue or cells was extracted with Trizol reagent. RNA concentration was measured by a NanoDrop spectrometer. 1000ng total RNA was reverse transcribed into cDNA by All-in-One RT SuperMix (G3337). Real-time PCR analysis using SYBR-Green fluorescent dye was performed with Step One Plus RT PCR System. Primers used for real-time PCR were listed in **Table S1**.

2.7. Histology and immunohistochemistry

Subcutaneous, epididymal white adipose tissue, interscapular brown adipose tissue and liver were fixed in 4% paraformaldehyde. Tissues were embedded with paraffin and sectioned by microtome. The slides were stained with hematoxylin and eosin (HE) using a standard protocol. For UCP1 and ADGRA3 immunohistochemistry, slides of various tissue were blocked with goat serum for 1h. Subsequently, the slides were incubated with anti-UCP1 (1:1000; ab10983) or anti-ADGRA3 (1:200; 11912-1-AP) overnight at 4℃ followed by detection with the EnVision Detection Systems. Hematoxylin was used as counterstain.

2.8. Western-blot

Tissues and cells were lysed in RIPA buffer supplemented with 1 mM PMSF and protease inhibitor cocktail (K1007). The protein concentration was measured by the BCA protein assay kit (BL521), and total cellular protein (25 μg) was subject to Western-blot analysis. The protein transferred to the PVDF membrane was probed with primary antibodies specific for HSP90 (1:1000; C45G), α-tubulin (1:10000; 666031-1-Ig), ADGRA3 (1:1000; 11912-1-AP), UCP1 (1:1000 for iWAT and cells or 1:10000 for brown adipose tissue (BAT); ab10983), FLAG-tag (HRP Conjugated, 1:2000; AF2855), HIS-tag (HRP Conjugated, 1:2000; AF2879), p-CREB (1:1000; AF5785) and CREB (1:1000; AF6566) overnight at 4 °C. Except FLAG-tag protein and HIS-tag protein, after being incubated with HRP conjugated secondary antibody, proteins were detected with chemiluminescence using Immobilon Western HRP Substrate on ChemiDoc MP Imaging System. The ImageJ software was used for gray scanning. For all Western-blots, each lane represented an independent sample and all experiments were replicated 2 to 3 times.

2.9. IP assay

HEK293T cells were transfected using PEI 40K transfection reagent (G1802) with indicated cDNAs and cultured using the manufacture's protocol. Cells were lysed with IP lysis buffer (G2038) containing protease inhibitor cocktail (K1007). The lysates were precipitated with the FLAG-tag antibody (GB15938) or HIS-tag antibody (GB151251) in the presence of protein A+G agarose (P2055). The precipitants were washed five times with the IP lysis buffer and analyzed by immunoblot with the indicated antibodies.

2.10. Enzyme-linked immunosorbent assay (ELISA)

Mouse cAMP level was detected using a sensitive ELISA kit (MM-0544M2) purchased from Jiangsu Meimian Industrial Co., Ltd. Mouse IP1 level was detected using a sensitive ELISA kit (MM-0790M2) purchased from Jiangsu Meimian Industrial Co., Ltd. Mouse insulin level was detected using a sensitive ELISA kit (MM-0579M1) purchased from Jiangsu Meimian Industrial Co., Ltd. Mouse free tetraiodothyronine (fT4) level was detected using a sensitive ELISA kit (RXJ202449M) purchased from Quanzhou Ruixin Biological Technology Co., Ltd. All measurements were performed using the manufacture's protocol.

2.11. Bodipy staining

For the lipid staining, the differentiated adipocytes were washed twice with PBS. The cells were then stained with 2 μM BODIPY staining solution (GC42959) for 15 min at 37℃, then washed three times with PBS according to manufacturer's instructions. The stained cells were observed using a fluorescence microscope.

2.12. Mito-Tracker staining

The differentiated adipocytes were incubated with 100nM Mito-Tracker Red CMXRos (C1049) for 30 min according to manufacturer's instructions. Then cells were washed with PBS and visualized under the confocal microscope.

2.13. Determination of 2-deoxy-D-glucose (2-NBDG) uptake

The differentiated adipocytes were washed twice with PBS. The cells were then incubated with 100 μM 2-NBDG staining solution (HY-116215) for 30 min at 37℃, then washed three times with PBS. The stained cells were observed using a fluorescence microscope.

2.14. Measurement of Triacylglycerol (TG)

The triacylglycerol in adipocytes, tissues and plasma was measured by using Triglyceride Assay Kit (A110-1-1) according to the manufacturer's instructions.

2.15. Transmission electron microscopy

BAT sections were fixed in 2% (vol/vol) glutaraldehyde in 100mM phosphate buffer, pH 7.2 for 12 h at 4℃. The sections were then post-fixed in 1% osmium tetroxide, dehydrated in ascending gradations of ethanol and embedded in fresh epoxy resin 618. Ultra-thin sections (60-80 nm) were cut and stained with lead citrate before being examined on the FEI-Tecnai G2 Spirit Twin transmission electron microscope.

2.16. Differential expression analysis

The R package Linear Models for Microarray Data (limma) was used to analyze differential RNA-Sequencing expression. For screening high-expressed G-protein-coupled receptors in mouse BAT, limma was applied in the GSE118849 dataset to screen out BAT-elevated genes. For screening ADGRA3 high-expressed gene sets in human subcutaneous adipose, limma was applied in the human subcutaneous adipose dataset from GTEx Portal to screen out ADGRA3 high-expressed gene sets. Genes highly expressed in human adipocytes were obtained from the human protein atlas database. Genes with the cutoff criteria of $|\log FC| \geq 1.0$ and $P < 0.05$ were regarded as differentially expressed genes (DEGs). The DEGs of the GSE118849 dataset and the human subcutaneous adipose dataset were visualized as volcano plots by using the R package ggplot2.

2.17. Functional annotation for genes of interest

To explore DisGeNET, Gene Ontology (GO), WikiPathwas, Kyoto Encyclopedia of Genes and Genomes (KEGG) and Reactome of selected genes, Metascape was used to explore the functions among DEGs, with a cutoff criterion of $p < 0.05$. GO annotation that contains the biological process (BP) subontology, which can identify the biological properties of genes and gene sets for all organisms.

2.18. Gene set enrichment analysis (GSEA)

GSEA was performed to detect a significant difference in the set of genes expressed between the *ADGRA3* high-expressed and *ADGRA3* low-expressed groups in the enrichment of the KEGG collection.

2.19. Oxygen consumption rate (OCR)

The basal oxygen consumption rate of cells was measured using a BBoxiProbe R01 kit (BB-48211) according to the manufacturers' instructions. The maximum oxygen consumption rate of cells was measured with the addition of FCCP (Trifluoromethoxy carbonylcyanide phenylhydrazone) with a final concentration of 1μM.

2.20. Infrared Thermography

BAT temperature was measured at room temperature by infrared thermography according to previous publications [22, 23]. The same batch of representative infrared images of mice were all captured using a thermal imaging camera (FLIR ONE PRO), measured at the same distance perpendicular to the plane on which the mice were located. To quantify interscapular region temperature, the average surface temperature from a region of the interscapular BAT was taken with FLIR Tools software.

2.21. Availability of Data and Materials

The transcriptomic dataset analyzed in this study can be accessed on the GTEx Portal database (*https://gtexportal.org/home/multiGeneQueryPage*), human protein atlas database (*https://www.proteinatlas.org/*) and GEO repository under accession number GSE118849. The PRESTO-Salsa dataset of ADGRA3 in this study can be accessed on the PRESTO-Salsa database (*https://palmlab.shinyapps.io/presto-salsa/*)[24]. All other data associated with this paper can be found in the main text or the Supplementary Materials.

2.22. Statistical Analysis

All data are presented as mean ± *SEM*. In this study, outliers that met the three-sigma rule were excluded from analysis, with the exception of those presented in **Figure S1E**. Given the possibility that the outliers in **Figure S1E** represent extreme expressions of the inherent variability within the population sample, we have chosen to retain these specific outliers for further analysis. Student's t-test was used to compare two groups. One-way analysis of variance (ANOVA) or Two-way ANOVA was applied to compare more than two different groups on GraphPad Prism 9.0 software. For each parameter of all data presented, NS (No Significance), *$p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ and **** $p < 0.0001$. $p < 0.05$ is considered significant.

3. Results

3.1. ADGRA3 is identified as a potential GPCR inducing the development of beige fat

We conducted a comprehensive analysis of three datasets to identify ADGRA3 as a potential GPCR target that promotes the development of beige fat (**Figure 1A**). To identify novel GPCRs that induce the biogenesis of beige fat, we conducted differential gene expression analysis (**Figure 1B**) and Venn diagram analysis (**Figure 1C**) using the GSE118849 dataset obtained from the Gene Expression Omnibus (GEO) database. Additionally, we utilized the human subcutaneous adipocytes dataset (**Figure 1C**, red) and human visceral adipocytes dataset (**Figure 1C**, purple) from the human protein atlas database to obtain genes that are highly expressed in human white adipocytes. The GSE118849 dataset comprises samples of brown adipose tissue (BAT) and inguinal white adipose tissue (iWAT) obtained from mice subjected to a 72-hour cold exposure at a temperature of 4℃.

A total of 1134 differentially expressed genes (DEGs) that exhibited up-regulation in BAT compared to iWAT under cold stimulation were identified in the analysis, which might play a role in adipose thermogenesis. These DEGs were further screened to identify highly expressed GPCRs in BAT relative to WAT (**Figure 1B**, red). We conducted additional annotation on 1134 DEGs and identified that 27 of these genes were associated with the encoding of GPCRs (**Table S2**). Among the set of 27 genes, it was found that 24 genes were not present in the group of genes that exhibited high expression levels in human adipocytes, as determined by the human protein atlas database. Consequently, these 24 genes were excluded from further analysis. We conducted a comprehensive literature review and discovered that out of the three remaining GPCRs namely ADGRA3, ADRA1A, and ADRB1, only ADGRA3 has not been documented to have any association with brown fat. Therefore, our research subsequently shifted towards investigating the potential regulatory role of ADGRA3 in obesity and brown fat.

The findings indicated that the level of *Adgra3* expression in mouse adipose tissue (**Figure 1D**) was comparatively lower than that of *Adrb3*, the coding gene for β3-AR. Conversely, in human adipose tissue, *ADGRA3* expression was observed to be higher than that of *ADRB3* (**Figures 1E** and **S1E**). We conducted an investigation to examine the regulatory effects of a high-fat diet on the transcription of *Adgra3* and *Ucp1* (Uncoupling protein 1, a functional protein and marker of beige/brown fat). The findings of the study demonstrated that a HFD had a significant inhibitory effect on the expression of ADGRA3 and UCP1 in iWAT and BAT, while CL robustly increased the expression of ADGRA3 and UCP1 in iWAT and BAT (**Figures 1F-G** and **Figures S1A-D**). Interestingly, in human subcutaneous fat, there was a moderate positive correlation between the expression level of *ADGRA3* and the expression level of *UCP1* (R=0.5, **Figure 1H**). On the other hand, the expression level of *ADRB3* showed a weak positive correlation with the expression level of *UCP1* (R=0.21, **Figure S1F**). The data presented in this study indicate that ADGRA3 is a GPCR that exhibits high expression levels in BAT and may participate in inducing adipose thermogenesis.

3.2. Adgra3 overexpression induces the biogenesis of beige adipocytes in vitro

To ascertain the predominant expression of ADGRA3, the isolation of stromal Vascular Fraction (SVF) and mature adipocytes from WAT and BAT was conducted for subsequent validation. The results showed that ADGRA3 is predominantly expressed in adipocytes. Furthermore, the expression level of ADGRA3 in BAT adipocytes was found to be higher compared to WAT adipocytes (**Figure 1I**). However, no significant difference was observed in the expression level

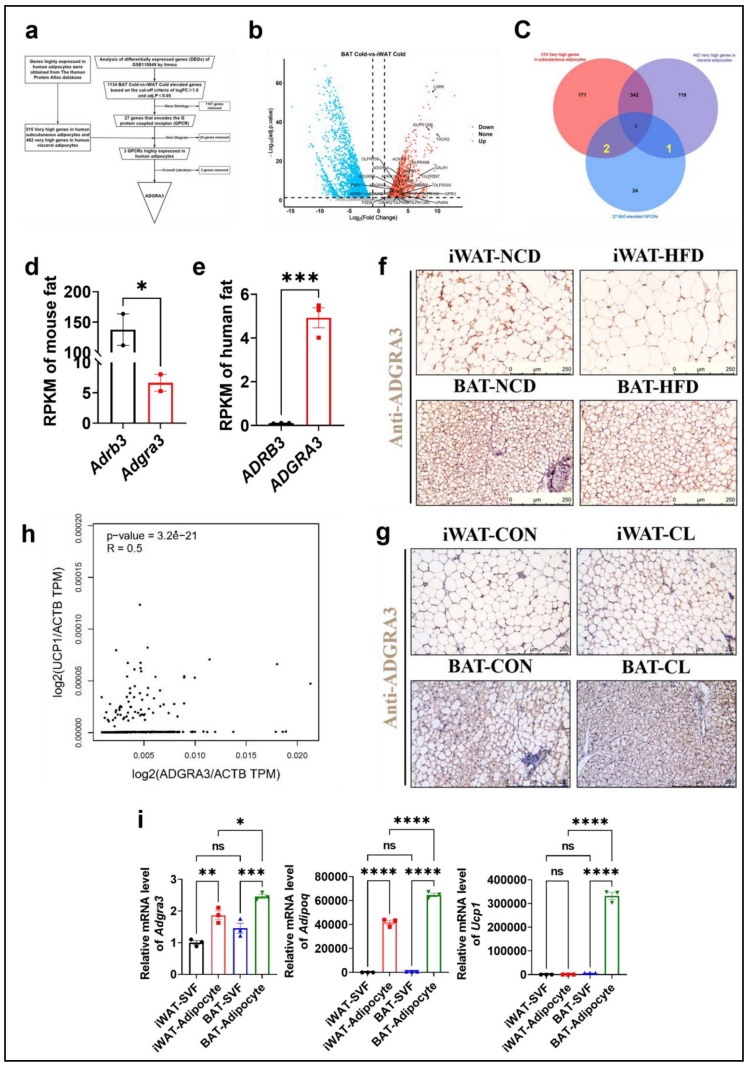


Figure 1.

ADGRA3 is a high-expressed GPCR in human adipocytes and mouse brown fat.

(A-F) ADGRA3 screening as a high-expressed GPCR in human adipocytes and mouse brown fat via comprehensive analysis. Brown adipose tissue and subcutaneous WAT were dissected from mice that were treated in cold (4℃) temperature for 72 hours. A total of six samples with three replicates for each adipose tissue were evaluated. The datasets of human subcutaneous adipocytes and human visceral adipocytes were acquired from the human protein atlas database. (A) Flowchart of screening. (B) Volcano plot summarizing the differentially expressed genes (DEGs) between cold temperature BAT group and cold temperature iWAT group. Blue and red shading are used to indicate down-regulation and up-regulation, respectively. (C) 27 BAT-elevated GPCRs from transcriptome, 515 very high genes in subcutaneous adipocytes and 462 very high genes in visceral adipocytes from the human protein atlas database were analyzed by using a Venn diagram. (D-E) The RPKM of *ADRB3* and *ADGRA3* genes in mouse fat (D) from Mouse ENCODE transcriptome data (PRJNA66167, N = 2) and human fat (E) from HPA RNA-seq normal tissues (PRJEB4337, N = 3). (F) C57BL/6J mice fed with a NCD or a HFD for 12 weeks. Representative images of iWAT and BAT stained with ADGRA3. Scale bars, 250μm. (G) C57BL/6J mice fed with a HFD for 12 weeks were injected with vehicle or CL (1 mg/kg daily) over 7 days. Representative images of iWAT and BAT stained with ADGRA3. Scale bars, 250μm. (H) Correlation between *UCP1* expression level normalized by *ACTB* gene and *ADGRA3* expression level normalized by *ACTB* gene in human subcutaneous fat dataset from GTEx Portal database (N = 663). (I) qPCR analysis of *Adgra3*, *Adipoq* and *Ucp1* genes in Stromal Vascular Fraction (SVF) and mature adipocyte isolated from iWAT and BAT (N = 3 for each group). iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; RPKM, Reads Per Kilobase per Million mapped reads; TPM, Transcripts Per Kilobase Million; GPCR, G-protein-coupled receptor; NCD, normal chow diet; HFD, high-fat diet; CL, CL-316,243; SVF, Stromal Vascular Fraction. All data are presented as mean ±*SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (D-E), simple linear regression (H) and one-way ANOVA (I).

of ADGRA3 in the SVF of WAT and BAT (**Figure 1I**). Moreover, it was observed that the modulation of the expression levels of *Adgra3/ADGRA3* and *Ucp1/UCP1* exhibited a similar pattern during the differentiation process between mouse and human adipocytes (**Figure S1G-H**).

To investigate the role of ADGRA3 in the biogenesis of beige adipocytes, we conducted an experiment where we transformed pre-adipocytes 3T3-L1 into mature beige-like adipocytes with a knockdown of *Adgra3*. Our findings indicate that the knockdown of *Adgra3* resulted in a decrease in the expression of genes related to thermogenesis and lipolysis (**Figure 2A**). Western blot analysis and Mito-Tracker staining revealed a decrease in the expression of UCP1 (**Figure 2B**) and a reduction in the number of mitochondria (**Figure 2C**) following *Adgra3* knockdown. Lipid droplet fluorescence staining and intracellular triglyceride assay were performed on adipocytes to assess the impact of *Adgra3* knockdown. The results revealed a significant increase in the number of lipid droplets and intracellular triglyceride levels (**Figures 2C-D**) following *Adgra3* knockdown. Moreover, the uptake of 2-deoxy-D-glucose (2-NBDG), a fluorescently-labeled deoxyglucose analog, by adipocytes was significantly inhibited following the knockdown of *Adgra3* (**Figure 2E**). Furthermore, oxygen consumption rate (OCR) was detected to verify the effect of ADGRA3 on the oxygen consumption of adipocytes. The results indicated that the loss of ADGRA3 decreased the both basal and max OCR of adipocytes (**Figures 2F-G**).

Following the overexpression of *Adgra3*, there was an observed up-regulation in the expression of UCP1 in 3T3-L1 mature beige-like adipocytes (**Figures 2H-I**). Additionally, Mito-Tracker staining revealed an increase in the quantity of mitochondria (**Figure 2J**). There was a notable reduction observed in the lipid droplets and intracellular triglyceride levels (**Figures 2J-K**) subsequent to the overexpression of *Adgra3*. Moreover, the findings indicated that the overexpression of *Adgra3* resulted in an increased uptake of 2-NBDG by adipocytes (**Figure 2L**) and increased basal and maximum OCR (**Figures 2M-N**). The presented data suggest that ADGRA3 has the ability to stimulate the formation of beige adipocytes in vitro.

3.3. Adgra3 knockdown suppresses adipose thermogenic program and impairs metabolic homeostasis in vivo

To evaluate the role of ADGRA3 in the biogenesis of beige fat in vivo, mice fed with a NCD were injected with shNC or sh*Adgra3* for 28 days (**Figure 3A**). After knocking down *Adgra3* in mice (**Figures S2A-B**), there was a significant increase in the weight of sh*Adgra3* mice (mice with *Adgra3* knockdown) (**Figure 3B**). Furthermore, the food intake of sh*Adgra3* mice was elevated slightly (**Figure S2C**). Serum triacylglycerol (TG) levels (**Figure S2D**), weight of iWAT, epididymal white adipose tissue (eWAT) and BAT (**Figure 3C**) were significantly higher in sh*Adgra3* mice. Liver weight (**Figure 3C**) and TG levels in the liver (**Figure S2E**) did not show a significant difference between shNC mice and sh*Adgra3* mice. Meanwhile, hematoxylin-eosin staining showed that *Adgra3* knockdown induced adipose expansion in iWAT (**Figure S2F**), eWAT (**Figure S2G**) and BAT (**Figure S2F**) but not lead to hepatic steatosis (**Figure S2G**).

Remarkably, the knockdown of *Adgra3* resulted in a significant reduction in both body temperature (**Figure 3D**) and BAT temperature (**Figure 3E**). Given the crucial influence of thyroid activity on thermogenesis, we measured the levels of serum free tetraiodothyronine (fT4) to evaluate the consequences of *Adgra3* knockdown on thyroid activity, which indicated that the nanoparticle-mediated *Adgra3* knockdown does not exert an inhibitory effect on thyroid activity (**Figure S2H**). The knockdown of *Adgra3* resulted in a significant decrease in the expression of genes related to thermogenesis and lipolysis in both iWAT (**Figure 3F**) and BAT (**Figure 3G**). Moreover, the Western blot analysis (**Figures 3H-I**) and Immunohistochemical staining (**Figure 3J**) of UCP1 revealed comparable outcomes. Additionally, it was observed that the knockdown of *Adgra3* resulted in an increase in the size of lipid droplets and a decrease in the number of mitochondria in BAT (**Figure 3K**). These findings indicate that ADGRA3 plays a crucial role as a receptor in the biogenesis of beige fat and the activation of BAT.

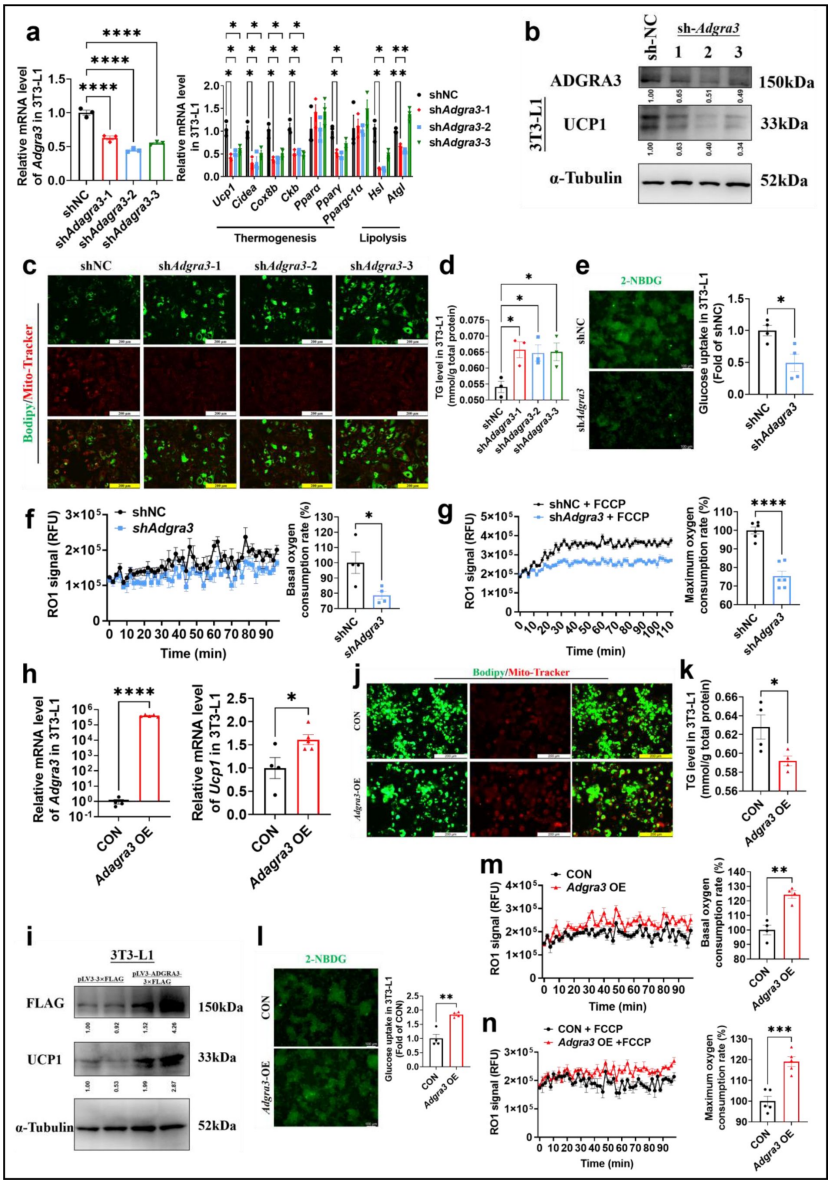


Figure 2.

Adgra3 overexpression promotes the biogenesis of beige adipocytes.

(A, H) qPCR analysis of *Adgra3*, thermogenesis and lipolysis genes in 3T3-L1 mature beige-like adipocytes (A: N = 3 for each group; H: N = 4 for CON, N = 5 for *Adgra3* OE). (B, I) Western blot analysis for level of ADGRA3, UCP1 and ADGRA3-3×FLAG protein in 3T3-L1 mature beige-like adipocytes treated with sh*Adgra3* (pLKO.1-U6-sh*Adgra3*-(1/2/3) plasmid encapsulated in nanomaterials), shNC (pLKO.1-U6-shNC plasmid encapsulated in nanomaterials), *Adgra3* OE (pLV3-CMV-*Adgra3*(mouse)-3×FLAG plasmid encapsulated in nanomaterials) or CON (pLV3-CMV-MCS-3×FLAG plasmid encapsulated in nanomaterials). The ImageJ software was used for gray scanning. (C, J) Bodipy green staining for lipid droplet and Mito-Tracker red staining for mitochondria in 3T3-L1 mature beige-like adipocytes. Scale bars, 200 μm. (D, K) The level of intracellular triglyceride in 3T3-L1 mature beige-like adipocytes (D: N = 3 for each group; K: N = 4 for each group). (E, I) Glucose uptake assay in 3T3-L1 mature beige-like adipocytes and staining intensity analysis diagram (right, N = 4 for each group). (F, M) When 3T3-L1 mature beige-like adipocytes were treated with shNC, sh*Adgra3*, CON or *Adgra3* OE, fluorescence of the oxygen probe (RO1) in the cells was monitored and the rate of basal oxygen consumption was analyzed (N = 4 for each group). (G, N) When FCCP-treaded 3T3-L1 mature beige-like adipocytes were treated with shNC, sh*Adgra3*, CON or *Adgra3* OE, fluorescence of the oxygen probe (RO1) in the cells was monitored and the rate of maximum oxygen consumption was analyzed (G: N = 6 for each group; N: N = 5 for each group). All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (E-H and K-N) and one-way ANOVA (A and D).

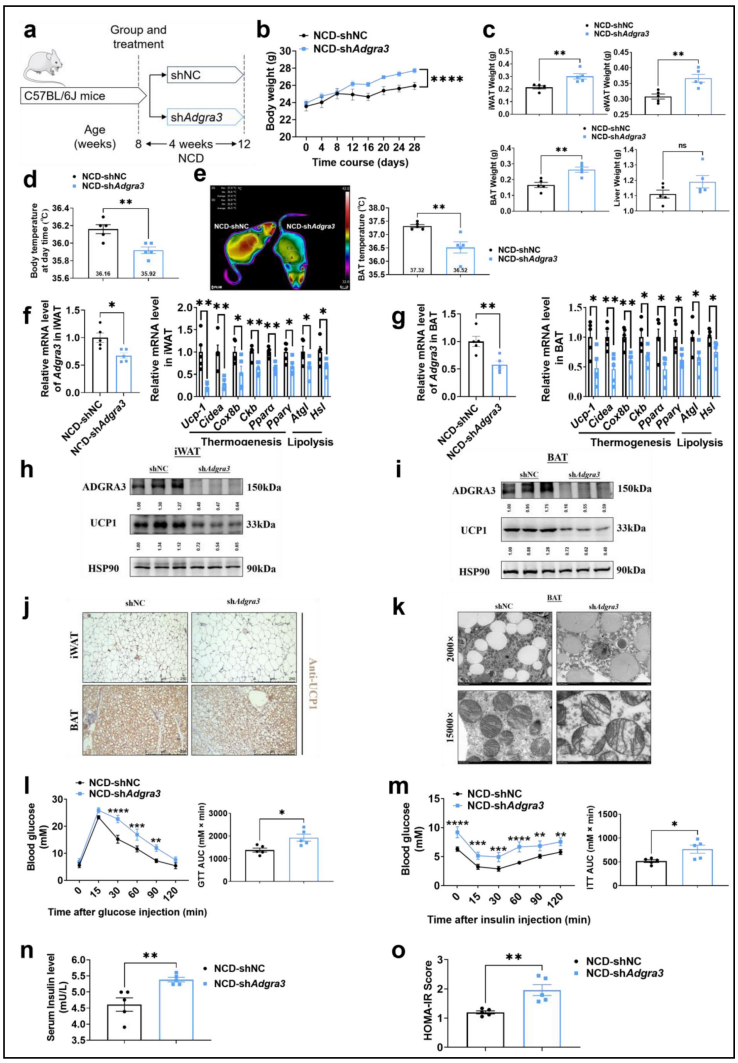


Figure 3.

Knockdown of *Adgra3* suppressed the adipose thermogenic program and impaired metabolic homeostasis in mice.

(A) Experimental schematic. C57BL/6J mice fed with a NCD for eight weeks were injected with sh*Adgra3* (pLKO.1-U6-sh*Adgra3*-2 plasmid encapsulated in nanomaterials) or shNC (pLKO.1-U6-shNC plasmid encapsulated in nanomaterials) twice a week for four weeks. (B-D) Changes in body mass (B), tissue weight (C) and body temperature (D) in mice injected with shNC or sh*Adgra3* for 28 days (N = 5 for each group). (E) Thermal image and BAT temperature of mice injected with shNC or sh*Adgra3* for 28 days (N = 5 for each group). (F-G) qPCR analysis of *Adgra3*, genes associated with thermogenesis and lipolysis in iWAT (F) and BAT (G) from different treatment mice (N = 5 for each group). (H-I) Western-blot analysis for the level of ADGRA3 and UCP1 protein in iWAT (H) and BAT (I) from differently treated mice. (J) Representative images of iWAT (top) and BAT (bottom) stained with UCP1. Scale bars, 250 μm. (K) Transmission electron microscope photograph of BAT treated with shNC or sh*Adgra3*. (L) Glucose tolerance test (GTT) was conducted by intraperitoneal injection of glucose (2g/kg) and measurement of blood glucose concentration with a OneTouch Ultra Glucometer at designed time points in six hours fasted mice (N = 5 for each group). (M) Insulin tolerance test (ITT) was done by intraperitoneal injection of insulin (0.5U/kg) and measurement of blood glucose concentration by a OneTouch Ultra Glucometer at designed time points in six hours fasted mice (N = 5 for each group). (N-O) The fasting serum insulin (N) and HOMA-IR (O) in mice injected with either shNC or sh*Adgra3* for 28 days (N = 5 for each group). HOMA-IR=Fasting glucose level (mmol/L) * Fasting insulin level (mIU/L) /22.5. NCD, normal chow diet; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; GTT, Glucose tolerance test; ITT, Insulin tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance. All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (C-G and N-O) and two-way ANOVA (B and L-M).

Moreover, the genes that were highly expressed in ADGRA3 high-expressed human subcutaneous adipose tissue (**Figure S3A**, red) exhibited enrichment in various biological processes. These processes included hyperinsulinism, obesity (**Figure S3B**), metabolic processes (**Figure S3C**), adipogenesis (**Figure S3D**), regulation of lipolysis in adipocytes (**Figure S3E**), and lipid metabolism (**Figure S3F**). GSEA was conducted to search the enriched KEGG pathways based on the expression level of ADGRA3 in human subcutaneous adipose dataset and human visceral adipose dataset from GTEx portal database. For ADGRA3 high-expressed group, both subcutaneous adipose dataset (**Figure S3G**) and visceral adipose dataset (**Figure S3H**) enriched in insulin signaling pathway, which indicates that ADGRA3 may be involved in the regulation of glucose metabolism in addition to its influence on lipid metabolism. Furthermore, it was observed that sh*Adgra3* mice exhibited significant disruptions in overall glycemic homeostasis (**Figure 3L**) and insulin sensitivity (**Figure 3M**). Moreover, the fasting serum insulin level was increased and the homeostasis model assessment of insulin resistance (HOMA-IR) showed an increase in sh*Adgra3* mice (**Figures 3N-O**). Hence, the findings of this study provide evidence that the knockdown of *Adgra3* hampers adipose thermogenesis and disrupts metabolic homeostasis in vivo.

3.4. ADGRA3 activates the adipose thermogenic program and counteracts metabolic disease in vivo

To identify whether *Adgra3* overexpression induces adipose thermogenesis and improves the metabolic homeostasis against obesity, *Adgra3* OE and CON were injected i.p. into mice fed with a NCD (**Figure S4A**) and a HFD (**Figure 4A**), thereby establishing models of *Adgra3*-overexpressed mice (**Figures 4F-G** and **Figures S4B-C**). The growth of body weight of *Adgra3* OE mice was alleviated (**Figure 4B**) during the HFD feeding accompanied with a slight decrease of food intake (**Figure S5A**). Furthermore, the weight of iWAT, eWAT, BAT and liver (**Figure 4C**) were significantly decreased in *Adgra3* OE mice. The *Adgra3* OE mice exhibited an elevation in both body temperature (**Figures 4D** and **S4D**) and BAT temperature (**Figures 4E** and **S4E**), while there was no difference in serum fT4 levels (**Figures S4F** and **S5B**). Meanwhile, *Adgra3* overexpression decreased the TG level in serum and liver (**Figures S5C-D**) as well as the area of adipocytes in iWAT, eWAT, BAT and liver (**Figures S4G** and **S5E-F**).

Moreover, the expression levels of thermogenic and lipolysis-related genes were elevated in iWAT (**Figures 4F** and **S4H**) and BAT (**Figures 4G** and **S4I**). Western-blot (**Figures 4H-I** and **S4J-K**) and immunohistochemical staining of UCP1 (**Figures 4J** and **S4L**) showed that the expression of UCP1 was increased dramatically in iWAT and BAT after *Adgra3* overexpression. In addition, we found that after *Adgra3* overexpression, BAT presented multiple thermogenesis fat features (**Figure 4K**). Furthermore, nanomaterials carrying *Adgra3* OE were directly injected into BAT (**Figure S5G**), resulting in overexpression of *Adgra3* and upregulation of thermogenic and lipolysis-related genes, as compared to BAT injected with CON (**Figure S5H**). These findings indicate that the overexpression of *Adgra3* is capable of inducing the hallmarks of thermogenesis in mice, independently of other organs.

We then investigated the metabolic impact of ADGRA3. The glucose tolerance test (GTT) presented that *Adgra3* overexpression improved the glucose homeostasis of HFD mice (**Figure 4L**). The insulin tolerance test (ITT) showed that *Adgra3* overexpression alleviated the insulin resistance of HFD mice (**Figure 4M**). Moreover, the fasting serum insulin level was reduced after *Adgra3* overexpression (**Figure 4N**) and the HOMA-IR also showed a robust improvement (**Figure 4O**) in *Adgra3*OE mice. Taken together, *Adgra3* overexpression activates the adipose thermogenic program and improves the metabolic homeostasis in diet-induced obese mice against obesity and insulin resistance in vivo.

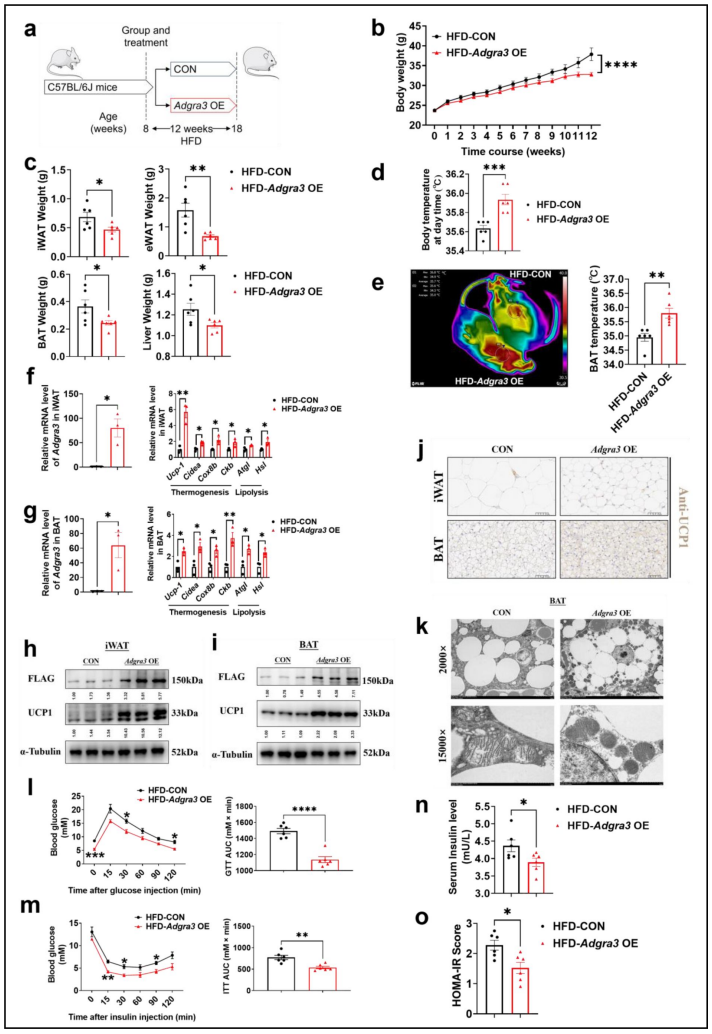


Figure 4.

Adgra3 overexpression activated the adipose thermogenic program and facilitated metabolic homeostasis in mice with diet-induced obesity (DIO).

(A) Experimental schematic. C57BL/6J mice were fed with a HFD and injected with *Adgra3* OE (pLV3-CMV-*Adgra3* (mouse)-3×FLAG plasmid encapsulated in nanomaterials) or CON (pLV3-CMV-MCS-3×FLAG plasmid encapsulated in nanomaterials) once a week for 12 weeks. (B-D) Changes in body mass (B), tissue weight (C) and body temperature (D) of mice injected with CON or *Adgra3* OE (N = 6 for each group). (E) Thermal image and BAT temperature in mice injected with CON or *Adgra3* OE (N = 6 for each group). (F-G) qPCR analysis of *Adgra3*, genes associated with thermogenesis and lipolysis in iWAT (F) and BAT (G) from different treatment mice (N = 3 for each group). (H-I) Western-blot analysis for the level of ADGRA3-3×FLAG and UCP1 protein in iWAT (H) and BAT (I) from differently treated mice. (J) Representative images of iWAT (top; Scale bars, 50 μm.) and BAT (bottom; Scale bars, 50 μm.) stained with UCP1. (K) Transmission electron microscope photograph of BAT treated with CON or *Adgra3* OE. (L) Glucose tolerance test (GTT) was conducted by intraperitoneal injection of glucose (1g/kg) and measurement of blood glucose concentration with a OneTouch Ultra Glucometer at designed time points in six hours fasted mice (N = 6 for each group). (M) Insulin tolerance test (ITT) was done by intraperitoneal injection of insulin (1U/kg) and measurement of blood glucose concentration by a OneTouch Ultra Glucometer at designed time points in six hours fasted mice (N = 6 for each group). (N-O) The fasting serum insulin (N) and HOMA-IR (O) in mice injected with CON or *Adgra3* OE (N = 6 for each group). HOMA-IR=Fasting glucose level (mmol/L) * Fasting insulin level (mIU/L) /22.5. HFD, high-fat diet; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; GTT, Glucose tolerance test; ITT, Insulin tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance. All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (C-G and N-O) and two-way ANOVA (B and L-M).

3.5. ADGRA3 activates the adipose thermogenic program via the G_s-PKA-CREB axis

To ascertain the ADGRA3-conjugated Gα protein, we conducted an overexpression of FLAG-labeled mouse ADGRA3 and four different types of His-labeled G_α proteins (G_s, G_i, G_q and G_{12}) in 293T cells. The lysate obtained from the 293T cells was then utilized for the subsequent co-immunoprecipitation (co-IP) analysis. The results of the co-IP experiment demonstrated that mouse ADGRA3 coupled to the G_s protein (**Figures 5A-B**), while no interaction was observed with the other three Gα proteins (G_i, G_q and G_{12}; **Figures S6A-C**) ADGRA3 exhibits intrinsic and auto-cleavable receptor activity, allowing it to signal even in the absence of an exogenous ligand [16, 20]. Hence, the overexpression of *Adgra3* is capable of inducing cAMP production (**Figure 5C**), which serves as a second messenger indicating the activation of downstream signals mediated by G_s protein. This response is comparable to the effect of a ligand. However, there is no production of IP1, which is a metabolite of the downstream second messenger IP3 associated with G_q protein (**Figure S6D**). Additionally, our findings indicate that the effect of *Adgra3* overexpression on cAMP production is dependent on G_s protein (**Figures 5D** and **S6E**). These results suggest that ADGRA3 is involved in the coupling of G_s protein, leading to the stimulation of downstream cAMP production.

Hence, it was hypothesized that the overexpression of *Adgra3* could potentially stimulate adipocyte thermogenesis by activating the PKA signaling pathway. As expected, the Western-blot analysis revealed that the overexpression of *Adgra3* leads to an elevation in the phosphorylated form of CREB (p-CREB), indicating an increase in PKA-CREB signaling activity. This effect was observed in 3T3-L1 (**Figure 5E**), as well as in the iWAT and BAT (**Figure 5F**). Consistently, the knockdown of *Adgra3* resulted in a decrease in the level of p-CREB in 3T3-L1 (**Figure 5G**), as well as in iWAT and BAT (**Figure 5H**). To investigate the potential role of *Adgra3* overexpression in promoting the biogenesis of beige adipocytes and activating the PKA-CREB signaling pathway via G_s protein, we conducted an experiment using 3T3-L1 cells. The cells were treated with *Adgra3* OE and sh*Gnas*, respectively. *Adgra3* overexpression was found to be adequate in inducing the expression of UCP1 in 3T3-L1 cells. However, this effect was observed to be eliminated when *Gnas* was knocked down (**Figure 5I** and **S6E**). Furthermore, the utilization of PKAi (protein kinase A inhibitor, H-89) was employed to ascertain the dependence of the browning effect of Adgra3 overexpression on the PKA-CREB signal. The results showed that PKAi effectively inhibited the activation of PKA-CREB signaling and UCP1 expression induced by *Adgra3* overexpression (**Figures 5J** and **S6F**). These results suggest that the observed browning effect resulting from *Adgra3* overexpression is mediated through the PKA-CREB signaling pathway. Collectively, these findings indicate that ADGRA3 facilitates the biogenesis of beige adipocytes through the G_s-PKA-CREB signaling pathway.

3.6. Hesperetin: a screened ADGRA3 agonist that induces the biogenesis of beige adipocytes

Considering the difficulty of overexpressing ADGRA3 in clinical application, hesperetin was screened as a potential agonist of ADGRA3 by PRESTO-Salsa database (**Figure 6A**). The results showed that hesperetin treatment stimulates cAMP production (**Figure 6B**) and increases the expression level of UCP1 and p-CREB (**Figures 6C-D**). To verify whether hesperetin induces the biogenesis of beige adipocyte and activates PKA-CREB signal via ADGRA3, we treated 3T3-L1 with hesperetin and sh*Adgra3*, respectively. We found that the induction effect of hesperetin on UCP1 and p-CREB is eliminated when *Adgra3* is knocked down (**Figures 6E-F**). In addition, OCR was detected to verify the effect of hesperetin on the oxygen consumption of adipocytes. The results indicated that hesperetin increased the both basal and max OCR of adipocytes, which was ADGRA3-dependent (**Figures 6G-H**).

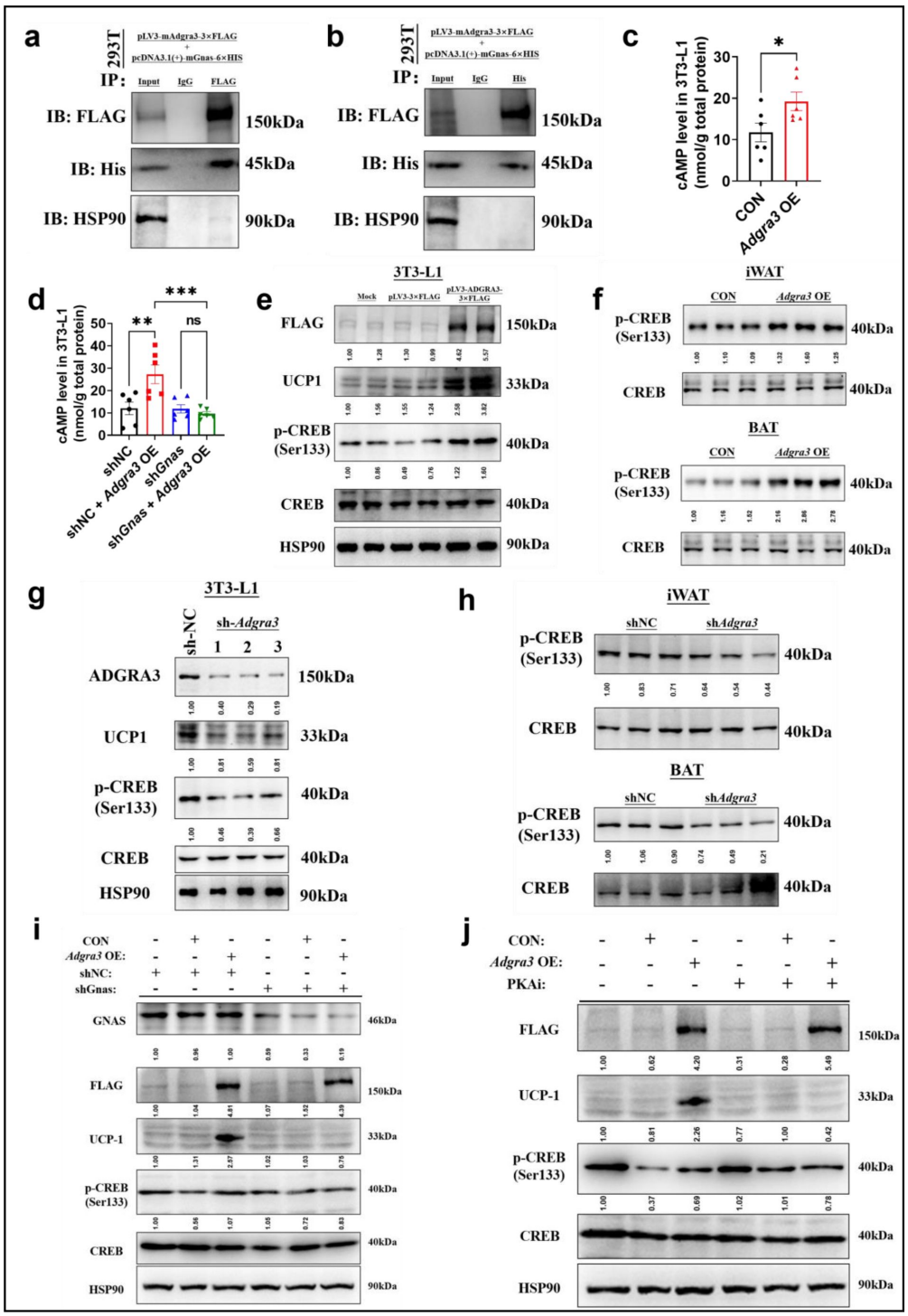


Figure 5.

ADGRA3 promotes the biogenesis of beige adipocytes via the Gs-PKA-CREB axis.

(A-B) Western-blot analysis for level of ADGRA3-3×FLAG, GNAS-6×HIS and HSP90 proteins in 293T transfected with different plasmids. (C-D) The level of cAMP in 3T3-L1. An ELISA kit was used to measure the level of cAMP (N = 6 for each group). (E, G and I-J) Western-blot analysis for level of ADGRA3, ADGRA3-3×FLAG, UCP1, p-CREB and CREB protein in 3T3-L1 mature beige-like adipocytes. (F and H) Western-blot analysis for level of p-CREB and CREB proteins in iWAT and BAT from differently treated mice. PKAi, protein kinase A inhibitor, 20 μM H-89. All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (C) and one-way ANOVA (D).

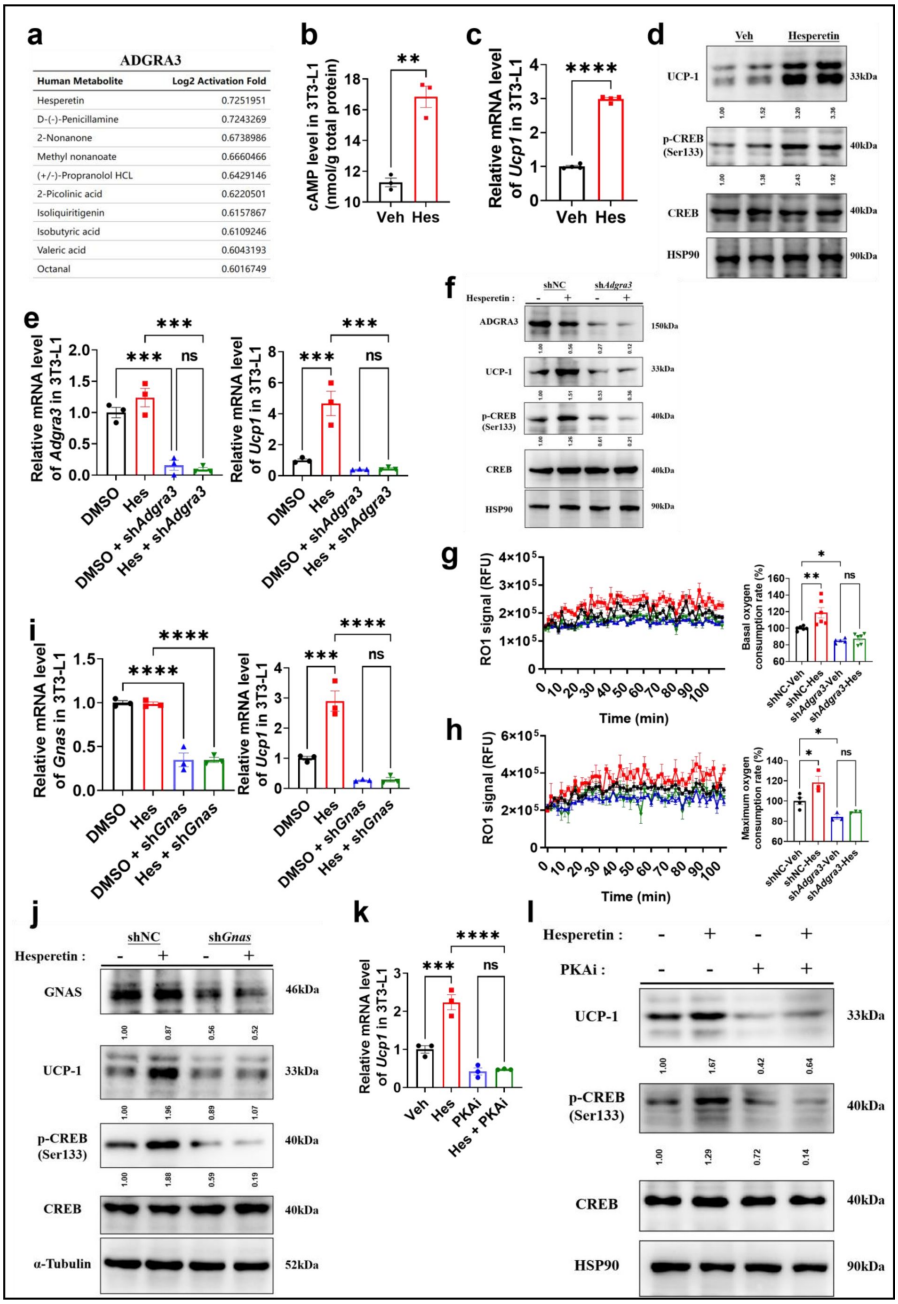


Figure 6.

Hesperetin promotes the biogenesis of beige adipocytes via ADGRA3-G_s-PKA-CREB axis.

(A) Table of human metabolites with the ability to activate ADGRA3, from the PRESTO-Salsa database. (B) The level of cAMP in 3T3-L1. ELISA kit was used to measure the level of cAMP (N = 3 for each group). (C, E, I and K) qPCR analysis of *Adgra3*, *Gnas* and *Ucp1* in 3T3-L1 mature beige-like adipocytes (N = 3 for each group). (D, F, J and L) Western-blot analysis for level of ADGRA3, GNAS, UCP1, p-CREB and CREB protein in 3T3-L1 mature beige-like adipocytes. (G) When 3T3-L1 mature beige-like adipocytes were treated with shNC, sh*Adgra3*, or Hesperetin, fluorescence of the oxygen probe (RO1) in the cells was monitored and the rate of basal oxygen consumption was analyzed (N = 5 for sh*Adgra3*-Veh; N = 6 for each other group). (H) When FCCP-treaded 3T3-L1 mature beige-like adipocytes were treated with shNC, sh*Adgra3*, or Hesperetin, fluorescence of the oxygen probe (RO1) in the cells was monitored and the rate of maximum oxygen consumption was analyzed (N = 4 for shNC-Veh; N = 3 for each other group). Hes, 10 μM Hesperetin; PKAi, protein kinase A inhibitor, 20 μM H-89. All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (B-C) and one-way ANOVA (E, G-I and K).

Moreover, the results showed that the induction effect of hesperetin on UCP1 and p-CREB is attenuated after *Gnas* knocked down (**Figures 6I-J**), suggesting that hesperetin up-regulates UCP1 and activates PKA-CREB axis dependent on G_s. Furthermore, PKAi was used to verify whether the browning effect of hesperetin was dependent on PKA-CREB signal. The results revealed that hesperetin treatment resulted in the upregulation of UCP1 and p-CREB. However, this effect was found to be eliminated when PKAi was applied (**Figures 6K-L**), suggesting that the induction of UCP1 and p-CREB by hesperetin is dependent on PKA. These findings suggest that hesperetin exerts an induction effect on biogenesis of beige adipocytes via ADGRA3-G_s-PKA-CREB axis.

3.7. Hesperetin: a potential ADGRA3 agonist that activates the adipose thermogenic program and counteracts metabolic disease dependent on ADGRA3

To identify whether hesperetin induces adipose thermogenesis and improves the metabolic homeostasis against obesity via ADGRA3, shNC mice or sh*Adgra3* mice were treated with hesperetin and fed with a HFD (**Figure 7A**). Hesperetin was found to alleviate the growth of body weight (**Figure 7B**) during the HFD feeding and the weight of iWAT, eWAT, BAT and liver weight ratio (**Figure 7C**), which was dependent on ADGRA3. It is noteworthy that the food consumption of sh*Adgra3* mice slightly surpassed that of shNC mice, while the administration of hesperetin remained uninfluential on their dietary intake (**Figure S7A**). Hesperetin increased body temperature (**Figure 7D**) and BAT temperature (**Figure 7E**) in shNC mice, which were significantly blunted in sh*Adgra3* mice. The levels of serum fT4 were measured to evaluate the consequences of hesperetin treatment on thyroid activity, which indicated that hesperetin treatment does not activate thyroid activity (**Figure S7B**).

Concurrently, hesperetin induced a decline in TG level in both serum (**Figure S7C**) and liver (**Figure S7D**), and also reduced the area of adipocytes in iWAT (**Figure S7E**) and BAT (**Figure S7F**). However, these effects were absent in sh*Adgra3* mice. Moreover, the expression level of UCP1 were elevated in both iWAT (**Figures 7F** and **7H**) and BAT (**Figures 7G** and **7I**) after hesperetin treatment in shNC mice but not in sh*Adgra3* mice. The results indicated that hesperetin treatment resulted in a substantial decrease in lipid droplet size and a significant increase in mitochondria quantity in BAT (**Figure 7J**). However, this browning effect was weakened in sh*Adgra3* mice. These findings suggest that hesperetin is sufficient to orchestrate the hallmarks of thermogenesis in mice, which is dependent on ADGRA3.

We then investigated the metabolic impact of hesperetin treatment. The GTT presented that hesperetin improved the glucose resistance of HFD mice which showed no effect in sh*Adgra3* mice (**Figure 7K**). The ITT showed that hesperetin alleviated the insulin resistance of HFD mice which showed no significance in sh*Adgra3* mice (**Figure 7L**). Moreover, the fasting serum insulin level was reduced after hesperetin treatment (**Figure 7M**) and the HOMA-IR also showed a moderate improvement (**Figure 7N**), which were dependent on ADGRA3. Taken together, hesperetin activates the adipose thermogenic program and improves the metabolic homeostasis in diet-induced obese mice against obesity and insulin resistance in vivo, which is ADGRA3 dependent.

3.8. ADGRA3 overexpression induces the biogenesis of human beige adipocytes in vitro

Given the elevated expression level of *ADGRA3* compared to *ADRB3* in human adipose tissue (**Figures 1E** and **S1E**), we induced human adipose-derived mesenchymal stem cells (hADSCs) into adipocytes to evaluate the effect of ADGRA3 on human adipocytes. The results showed that *ADGRA3* knockdown led to a diminished expression of *UCP1* (**Figure 8A**), whereas its overexpression elicited an enhancement in *UCP1* expression (**Figure 8B**). Furthermore, Mito-Tracker and lipid droplet fluorescence staining illuminated a notable increase in lipid droplet

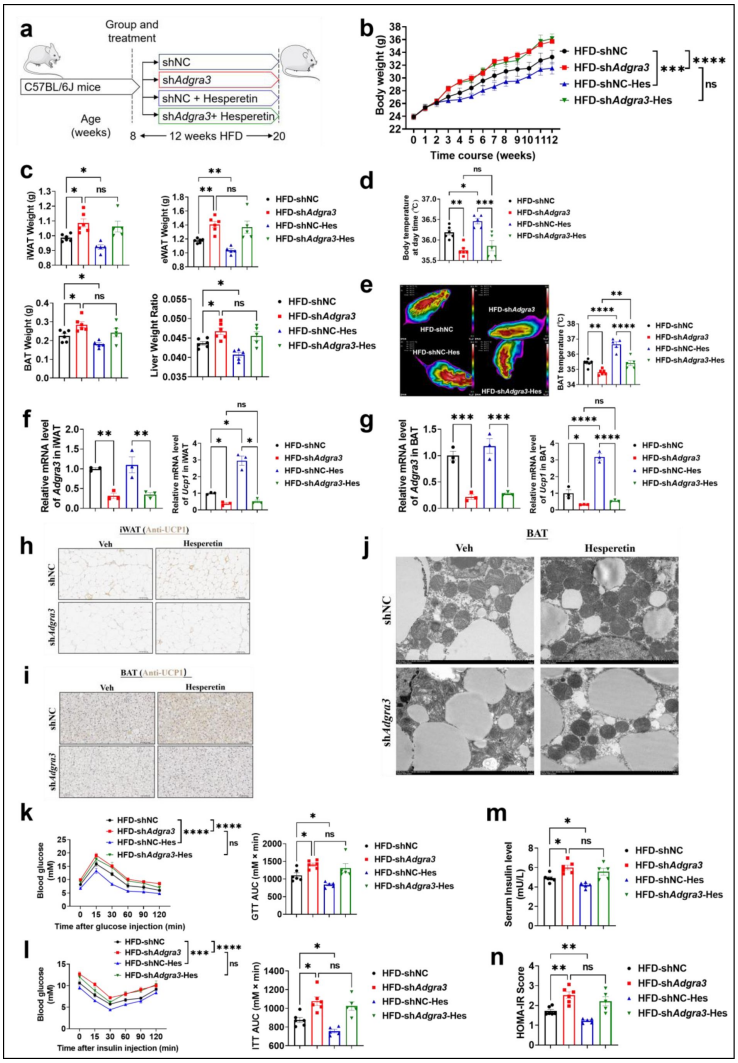


Figure 7.

Hesperetin activated the adipose thermogenic program and facilitated metabolic homeostasis in mice with diet-induced obesity (DIO) dependent on ADGRA3.

(A) Experimental schematic. Different treated C57BL/6J mice were fed with a HFD for 12 weeks. (B-D) Changes in body mass (B), tissue weight (C) and body temperature (D) of different treated mice (N = 6 for HFD-shNC and HFD-sh*Adgra3*; N = 5 for HFD-shNC-Hes and HFD-sh*Adgra3*-Hes). (E) Thermal image and BAT temperature of different treated mice (N = 6 for HFD-shNC and HFD-sh*Adgra3*; N = 5 for HFD-shNC-Hes and HFD-sh*Adgra3*-Hes). (F-G) qPCR analysis of *Adgra3* and *Ucp1* in iWAT (F) and BAT (G) from different treated mice (N = 3 for each group). (H-I) Representative images of iWAT (H; Scale bars, 50 μm.) and BAT (I; Scale bars, 50 μm.) stained with UCP1. (J) Transmission electron microscope photograph of BAT from different treated mice (Scale bars, 2 μm.). (K) Glucose tolerance test (GTT) was conducted by intraperitoneal injection of glucose (1g/kg) and measurement of blood glucose concentration with a OneTouch Ultra Glucometer at designed time points in six hours fasted mice (N = 6 for HFD-shNC and HFD-sh*Adgra3*; N = 5 for HFD-shNC-Hes and HFD-sh*Adgra3*-Hes). (L) Insulin tolerance test (ITT) was done by intraperitoneal injection of insulin (1U/kg) and measurement of blood glucose concentration by a OneTouch Ultra Glucometer at designed time points in six hours fasted mice (N = 6 for HFD-shNC and HFD-sh*Adgra3*; N = 5 for HFD-shNC-Hes and HFD-sh*Adgra3*-Hes). (M-N) The fasting serum insulin (M) and HOMA-IR (N) in different treated mice (N = 6 for HFD-shNC and HFD-sh*Adgra3*; N = 5 for HFD-shNC-Hes and HFD-sh*Adgra3*-Hes). HOMA-IR=Fasting glucose level (mmol/L) * Fasting insulin level (mIU/L) /22.5. HFD, high-fat diet; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; GTT, Glucose tolerance test; ITT, Insulin tolerance test; HOMA-IR, homeostasis model assessment of insulin resistance; Hes, Hesperetin. All data are presented as mean ± *SEM*. Statistical significance was determined by one-way ANOVA (C-G and M-N) and two-way ANOVA (B and K-L).

count accompanied by a decrease in mitochondrial number following *ADGRA3* knockdown (**Figure 8C**). Conversely, *ADGRA3* overexpression resulted in a visible surge in mitochondrial quantity and a marked reduction in lipid droplet presence (**Figure 8D**).

4. Discussion

In the present study, we have elucidated a novel role of ADGRA3 and hesperetin in inducing the development of beige adipocytes through the activation of the G_s-PKA-CREB signaling pathway. ADGRA3 is responsible for the activation of the adipose thermogenic program and plays a significant role in maintaining systemic glucose homeostasis. Additionally, the development of beige adipocytes induced by hesperetin is contingent upon the presence of ADGRA3. The novelty of this study is the discovery that ADGRA3 plays a role in the advancement of beige fat and the regulation of metabolic homeostasis. This suggests that targeting the ADGRA3-G_s-PKA-CREB signaling pathway could potentially be a therapeutic approach for obesity and related metabolic disorders.

The induction of beige fat has been investigated as a potentially effective therapeutic approach in combating obesity [25]. A clinical trial revealed that treatment with the chronic β3-AR agonist mirabegron leads to an increase in human brown fat, HDL cholesterol, and insulin sensitivity [26]. Subsequently, Blondin et al discovered that oral administration of mirabegron only elicits an increase in BAT thermogenesis when administered at the maximal allowable dose, indicating that human brown adipocyte thermogenesis is primarily driven by β2-adrenoceptor (β2-AR) stimulation [11]. Consistent with this finding, we found much higher levels of ADRB2 expression in human white adipose tissue than ADRB3 (**Figure S1E**). Furthermore, a recent study has demonstrated that simultaneous activation of β2-AR and β3-AR enhances whole-body metabolism through beneficial effects on skeletal muscle and BAT [27].

While the promotion of thermogenesis in brown and beige adipocytes in rodents was effectively achieved by the β3-adrenoceptor agonist, the clinical implications of this finding appear to be unfeasible in humans due to the low efficacy of β3-adrenoceptor agonists [28, 29]. It is of utmost importance to investigate alternative therapeutic targets that can effectively and selectively enhance beige adipogenesis in order to combat obesity and its related metabolic disorders. In this study, we have identified ADGRA3 as a novel GPCR therapeutic target that exhibits high expression in human adipocytes. In human adipose tissue, ADGRA3 is expressed at a lower level than ADRB2 (**Figure S1E**), which has been shown to be the main receptor mediating adrenergic activation of thermogenesis in human brown adipocytes [11]. Nevertheless, given ADRB2's pivotal role in bronchodilation and vasodilation [30, 31], we believe that ADGRA3 has the potential to be an alternative target for inducing adipose thermogenesis. Overall, these findings suggest that ADGRA3, when overexpressed or stimulated by its potential agonist, hesperetin, can induce the biogenesis of beige fat.

Hesperetin has been reported to attenuate the age-related metabolic decline, reduce fat and improve glucose homeostasis in naturally aged mice [32]. Previous studies showed that hesperetin improved glycemic control [32, 33] and was involved in adipocyte differentiation [34], but whether hesperetin induces the biogenesis of beige adipocyte was uncertain. Previously, the influence of hesperetin on ADGRA3 has remained unreported. In this study, we screened hesperetin as a potential agonist for ADGRA3 by using the PRESTO-Salsa tool as well as discovered that hesperetin has an agonist effect on ADGRA3 through a series of experiments. This study focuses on the regulatory effect of hesperetin on adipose thermogenesis and explores whether this effect is dependent upon ADGRA3. As such, we refrained from conducting further investigations into other potential effects of hesperidin, including its potential role in antioxidant and in apoptosis.

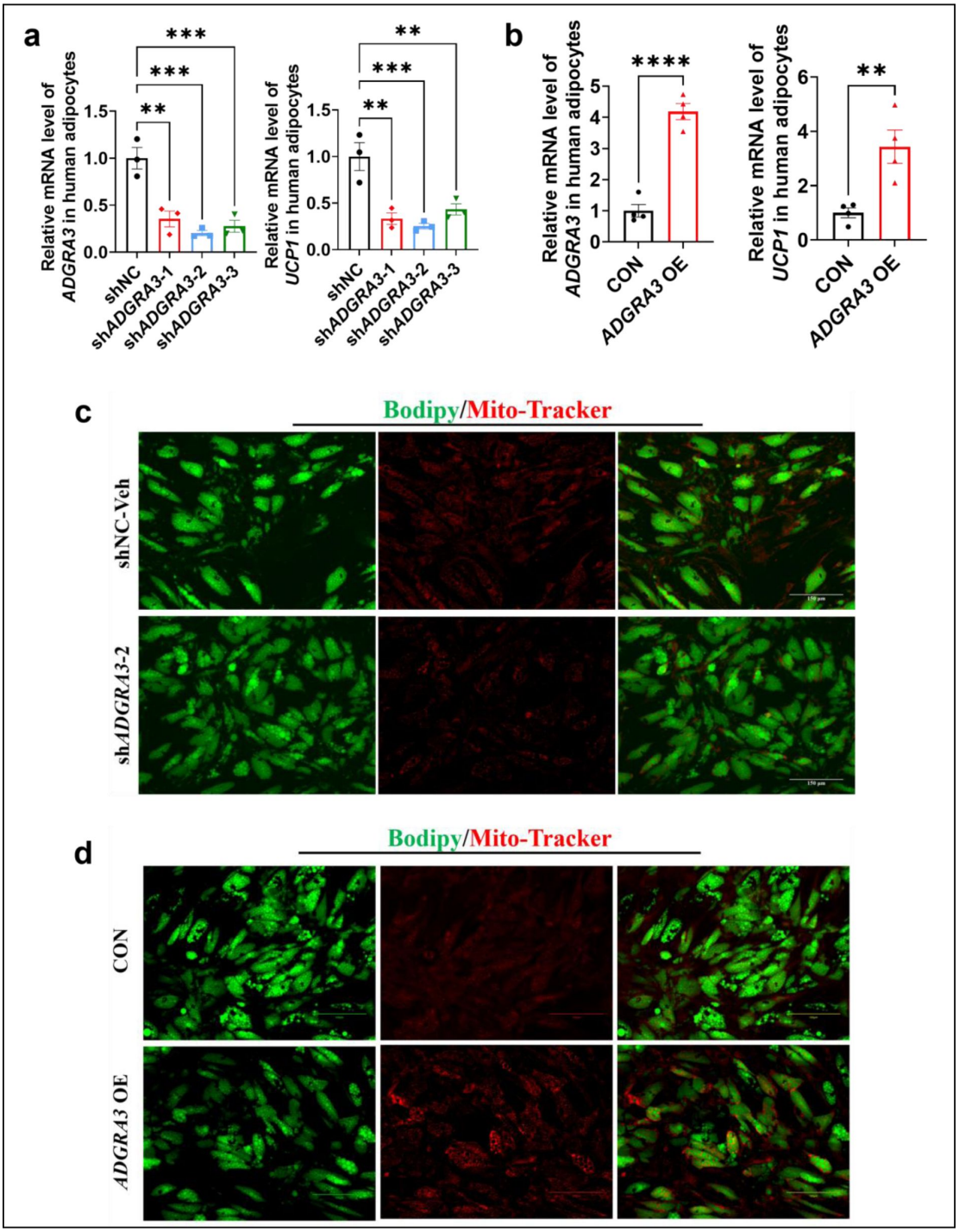


Figure 8.

***ADGRA3* overexpression induces the beiging of human adipocytes.**

(A-B) qPCR analysis of *ADGRA3* and *UCP1* genes in adipocytes induced from human adipose-derived mesenchymal stem cells (hADSCs) (A: N = 3 for each group; B: N = 4 for each group). (C-D) Bodipy green staining for lipid droplet and Mito-Tracker red staining for mitochondria in adipocytes induced from hADSCs. Scale bars, 150 μm. sh*ADGRA3* (pLKO.1-U6-sh*ADGRA3*-(1/2/3) plasmid encapsulated in nanomaterials), shNC (pLKO.1-U6-shNC plasmid encapsulated in nanomaterials), *ADGRA3* OE (pLV3-CMV-*ADGRA3*(human)-3×FLAG plasmid encapsulated in nanomaterials) or CON (pLV3-CMV-MCS-3×FLAG plasmid encapsulated in nanomaterials). All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (B) and one-way ANOVA (A).

In previous reports, male mice deficient in ADGRA3 showed obstructive azoospermia with high penetrance [15]. Moreover, a genome-wide association study (GWAS) identified a single nucleotide polymorphism (SNP) located in the downstream region of *ADGRA3* as a genomic locus associated with body weight in chickens, suggesting that the ADGRA3 is a potential regulator of body weight [21]. Nevertheless, the agonist and the downstream signal axis of ADGRA3 remain unclear as well as the effects of ADGRA3 on adipose thermogenesis and glucose homeostasis have not been explored. Consequently, our study has confirmed that the knockdown of *Adgra3* exacerbates obesity and disrupts glucose homeostasis. Additionally, both the overexpression of *Adgra3* and the administration of hesperetin have been found to stimulate the biogenesis of beige adipocytes through the ADGRA3-G_s-PKA-CREB signaling pathway and improve glucose homeostasis.

Given the consideration that the non-targeted nanoparticle approach utilized in this study for modulating *Adgra3* expression levels in vivo alter *Adgra3* expression in tissues beyond adipose tissue (**Figures S2A-B** and **S4B-C**), notably the liver and skeletal muscle, the construction of *Adgra3* adipose tissue-specific knockout/overexpression mouse models is imperative for a more nuanced understanding of the precise mechanisms underlying the influence of on adipose thermogenesis. We will employ more sophisticated models in subsequent studies to further elucidate the effects of ADGRA3 on adipose thermogenesis and metabolic homeostasis. Nevertheless, our findings underlie a potential therapeutic feature of ADGRA3 and hesperetin in obesity and the associated metabolic diseases from the thermogenic viewpoint of beige fat.

5. Conclusion

In conclusion, the activation of the G_s-PKA-CREB axis by ADGRA3 has been found to induce adipose thermogenesis, promote lipid metabolism, and alleviate lipid accumulation in adipose tissues. Furthermore, the induction of beige adipocyte biogenesis by hesperetin occurs through the ADGRA3-G_s-PKA-CREB axis. Given the importance of identifying signaling pathways that induce beige fat and alleviate obesity-related dysfunction in adipose tissue, our research findings suggest that hesperetin and activation of the intracellular signaling of ADGRA3 could serve as a promising and innovative therapeutic approach.

Acknowledgements

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Additional information

Compliance with Ethics Requirements

All the animal experiments were conducted with the approval of the Animal Care and Use Committee of Sun Yat-sen University (Approval ID: SYSU-IACUC-MED-2023-B082). This study was conducted in accordance with the ethical principles derived from the Declaration of Helsinki and Belmont Report and was approved by the review board of Sun Yat-sen University (Guangzhou, China).

CRediT authorship contribution statement

Zewei Zhao: Investigation, Conceptualization, Data curation, Formal analysis, Writing original draft, Writing-review & editing. **Longyun Hu:** Data curation, Formal analysis. **Bigui Song:** Data curation, Formal analysis. **Tao Jiang:** Data curation, Formal analysis. **Qian Wu:** Data curation, Formal analysis. **Jiejing Lin:** Data curation, Formal analysis. **Xiaoxiao Li:** Data curation, Formal analysis. **Yi Cai:** Data curation, Formal analysis. **Jin Li:** Data curation, Formal analysis. **Bingxiu Qian:** Data curation, Formal analysis. **Siqi Liu:** Data curation, Formal analysis. **Jilu Lang:** Conceptualization, Supervision, Writing-review & editing. **Zhonghan Yang:** Funding acquisition, Conceptualization, Supervision, Writing-review & editing.

Declaration of competing interests

The authors have declared no conflict of interest.

Abbreviations

- ANOVA: analysis of variance
- BAT: brown adipose tissue
- β2-AR: β2-adrenoceptor
- β3-AR: β3-adrenoceptor
- BMI: body mass index
- ADGRA3: adhesion G protein-coupled receptor A3
- CL: CL-316,243
- DEGs: differentially expressed genes
- edgeR: Empirical Analysis of Digital Gene Expression Data in R
- eWAT: epididymal white adipose tissue
- FDA: Food and Drug Administration
- GEO: Gene Expression Omnibus
- GPCRs: G protein-coupled receptors
- GSEA: Gene set enrichment analysis
- NCD: normal-chow diet
- HFD: high-fat diet
- GTT: glucose tolerance test
- HOMA-IR: homeostasis model assessment of insulin resistance
- ITT: insulin tolerance test
- iWAT: inguinal white adipose tissue
- Limma: Linear Models for Microarray Data
- Hes: Hesperetin
- PKAi: protein kinase A inhibitor
- NS: No Significance
- RPKM: Reads Per Kilobase per Million mapped reads
- TPM: Transcripts Per Kilobase Million
- WT: Wild-type
- GWAS: genome-wide association study
- SNP: single nucleotide polymorphism
- OCR: oxygen consumption rate
- SVF: Stromal Vascular Fraction
- hADSCs: human adipose-derived mesenchymal stem cells

Supplementary materials and methods

Table S1.

Primer sequences used for qPCR.

Gene	Forward Primer	Reverse Primer
Ppargc1α	TATGGAGTGACATAGAGTGTGCT	CCACTTCAATCCACCCAGAAAG
Pparα	AGGCTGTAAGGGCTTCTTTCG	GGCATTTGTTCCGGTTCTTC
Ppargγ	TGTGGGGATAAAGCATCAGGC	CCGGCAGTTAAGATCACACCTAT
Adipoq	TGTTCCTCTTAATCCTGCCCA	CCAACCTGCACAAGTTCCCTT
Ucp1	AGGCTTCCAGTACCATTAGGT	CTGAGTGAGGCAAAGCTGATTT
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG
Cox8b	TGTGGGGATCTCAGCCATAGT	AGTGGGCTAAGACCCATCCTG
Adgra3	CACGGTCACCCTGATTTTAAGC	GCACCTGGGGCTATCCTACTAA
Gnas	TGCCTCGGCAACAGTAAGAC	GCCGCCCTCTCCGTTAAAC
Atgl	GGAGGAATGGCCTACTGAACC	ATCCTCTTCCTGGGGGACAA
Hsl	CCAGCCTGAGGGCTTACTG	CTCCATTGACTGTGACATCTCG
Actb	TGTCCACCTTCCAGCAGATGT	AGCTCAGTAACAGTCCGCCTAGA
Hexo	GCCAGCCTCTCCTGATTTTAGTGT	GGGAACACAAAAGACCTCTTCTGG
mito-16s	CCGCAAGGGAAAGATGAAAGAC	TCGTTTGGTTTCGGGGTTTC
ACTB	CATGTACGTTGCTATCCAGGC	CTCCTTAATGTCACGCACGAT
ADGRA3	GCGTCATTACGGTCTTTGGAA	ACGGCAATTCAAGCGGAGG
UCP1	AGGTCCAAGGTGAATGCCC	TTACCACAGCGGTGATTGTTC

Gene ID	Gene Name	GO Annotation
14748	***Gpr3***	GO0004930: G-protein coupled receptor activity
70693	***Adgra3***	GO0004930: G-protein coupled receptor activity
224792	***Adgrf5***	GO0004930: G-protein coupled receptor activity
170757	***Adgrl4***	GO0004930: G-protein coupled receptor activity
11549	***Adra1a***	GO0004930: G-protein coupled receptor activity
11554	***Adrb1***	GO0004930: G-protein coupled receptor activity
12778	***Ackr3***	GO0004930: G-protein coupled receptor activity
12922	***Crhr2***	GO0004930: G-protein coupled receptor activity
13618	***Ednrb***	GO0004930: G-protein coupled receptor activity
14368	***Fzd6***	GO0004930: G-protein coupled receptor activity
14427	***Galr1***	GO0004930: G-protein coupled receptor activity
242425	***Gabbr2***	GO0004930: G-protein coupled receptor activity
329252	***Lgr6***	GO0004930: G-protein coupled receptor activity
78134	***Lpar4***	GO0004930: G-protein coupled receptor activity
258218	***Olfr102***	GO0004930: G-protein coupled receptor activity
258027	***Olfr1385***	GO0004930: G-protein coupled receptor activity
258334	***Olfr1396***	GO0004930: G-protein coupled receptor activity
634104	***Olfr287***	GO0004930: G-protein coupled receptor activity
259010	***Olfr393***	GO0004930: G-protein coupled receptor activity
258816	***Olfr466***	GO0004930: G-protein coupled receptor activity

Table S2.

Gene annotation for screened genes.

The 1134 screened genes were annotated by David database and 27 of these genes were identified as G-protein coupled receptor encoding gene.

259097	***Olfr558***	GO0004930: G-protein coupled receptor activity
259062	***Olfr667***	GO0004930: G-protein coupled receptor activity
258469	***Olfr90***	GO0004930: G-protein coupled receptor activity
258825	***Olfr975***	GO0004930: G-protein coupled receptor activity
18441	***P2ry1***	GO0004930: G-protein coupled receptor activity
94226	***S1pr5***	GO0004930: G-protein coupled receptor activity
21337	***Tacr2***	GO0004930: G-protein coupled receptor activity

Table S2. (continued)

Supplementary figures

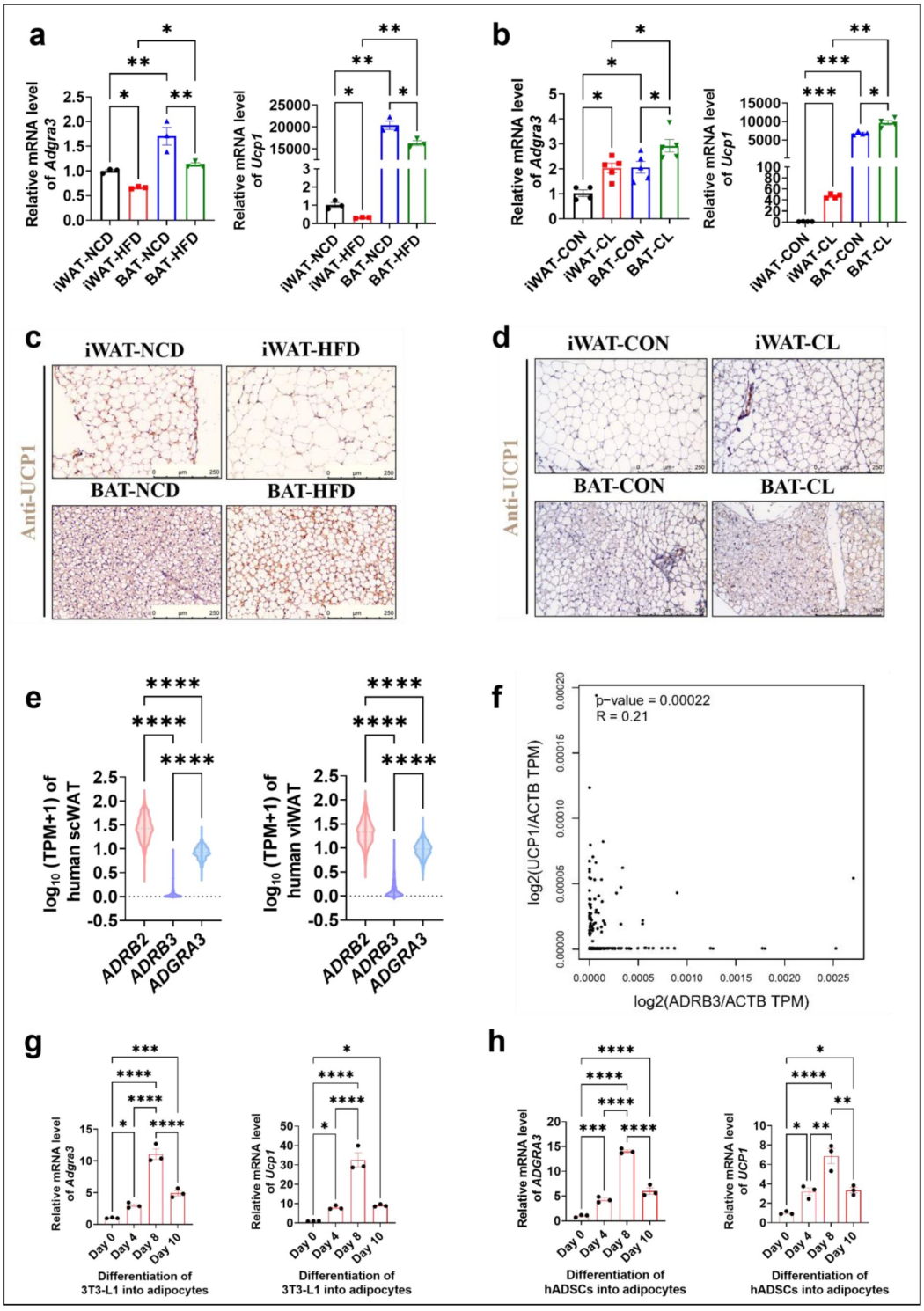

Figure S1.

ADGRA3 positively correlated with beige fat.

(A-B) qPCR analysis of *Adgra3* and *Ucp1* in iWAT and BAT from different treatment mice (A: N = 3 for each group; B: N = 4 for iWAT-CON, N = 5 for each other group). (C) C57BL/6J mice fed with a NCD or a HFD for 12 weeks. Representative images of iWAT and BAT stained with UCP1. Scale bars, 250μm. (D) C57BL/6J mice fed with a HFD for 12 weeks were injected with vehicle or CL (1 mg/kg daily) over 7 days. Representative images of iWAT and BAT stained with UCP1. Scale bars, 250μm. (E) The TPM of *ADRB2*, *ADRB3* and *ADGRA3* genes in human scWAT (left, N = 663) and viWAT (right, N = 541) from the GTEx database. (F) Correlation between *UCP1* expression level normalized by *ACTB* gene and *ADRB3* expression level normalized by *ACTB* gene in human subcutaneous fat dataset from GTEx Portal database (N = 663). (G) qPCR analysis of *Adgra3* and *Ucp1* during the differentiation of 3T3-L1 into adipocytes (N = 3 for each group). (H) qPCR analysis of *ADGRA3* and *UCP1* during the differentiation of hADSCs into adipocytes (N = 3 for each group). iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; scWAT, subcutaneous white adipose tissue; viWAT, visceral white adipose tissue; NCD, normal chow diet; HFD, high-fat diet; CL: CL-316,243; qPCR, quantitative real-time PCR; hADSCs, human adipose-derived mesenchymal stem cells. All data are presented as mean ±*SEM*. Statistical significance was determined by one-way ANOVA (A-B, E and G-H) and simple linear regression (F).

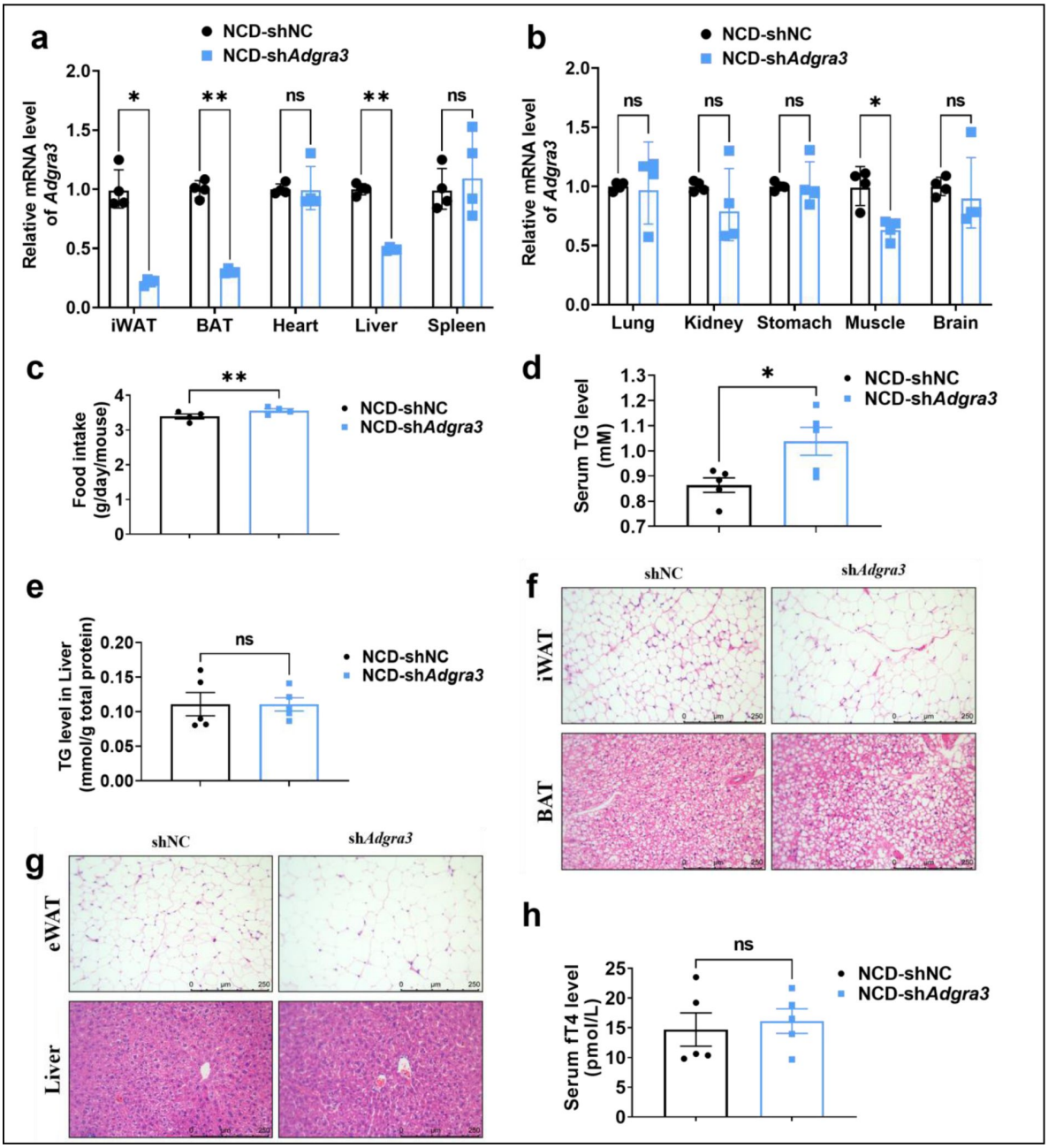

Figure S2.

Characterization of wild-type and *Adgra3*-knockdown mice.

(A-H) C57BL/6J mice fed with a NCD for eight weeks were injected with sh*Adgra3* (pLKO.1-U6-sh*Adgra3*-2 plasmid encapsulated in nanomaterials) or shNC (pLKO.1-U6-shNC plasmid encapsulated in nanomaterials) twice a week for four weeks. (A) qPCR analysis of *Adgra3* gene in iWAT, BAT, heart, liver and spleen from different treatment mice (N = 4 for each group). (B) qPCR analysis of *Adgra3* gene in lung, kidney, stomach, skeletal muscle and brain from different treatment mice (N = 4 for each group). (C) Food intake of different treated mice (N = 4 for each group). (D-E) The TG level of serum (D) and liver (E) from different treated mice (N = 5 for each group). (F) Representative images of iWAT (top) and BAT (bottom) stained with hematoxylin and eosin. Scale bars, 250 μm. (G) Representative images of eWAT (top) and Liver (bottom) stained with hematoxylin and eosin. Scale bars, 250 μm. (H) The fT4 level of serum from different treated mice (N = 5 for each group). shNC, pLKO.1-U6-shNC plasmid encapsulated in nanomaterials; sh*Adgra3*, pLKO.1-U6-sh*Adgra3*-2 plasmid encapsulated in nanomaterials; iWAT, inguinal white adipose tissue; eWAT, epididymal white adipose tissue; BAT, brown adipose tissue; NCD, normal chow diet; fT4, free tetraiodothyronine. All data are presented as mean ± *SEM*. Statistical significance was determined by paired two-tailed student's t-test (C) and unpaired two-tailed student's t-test (A-B, D-E and H).

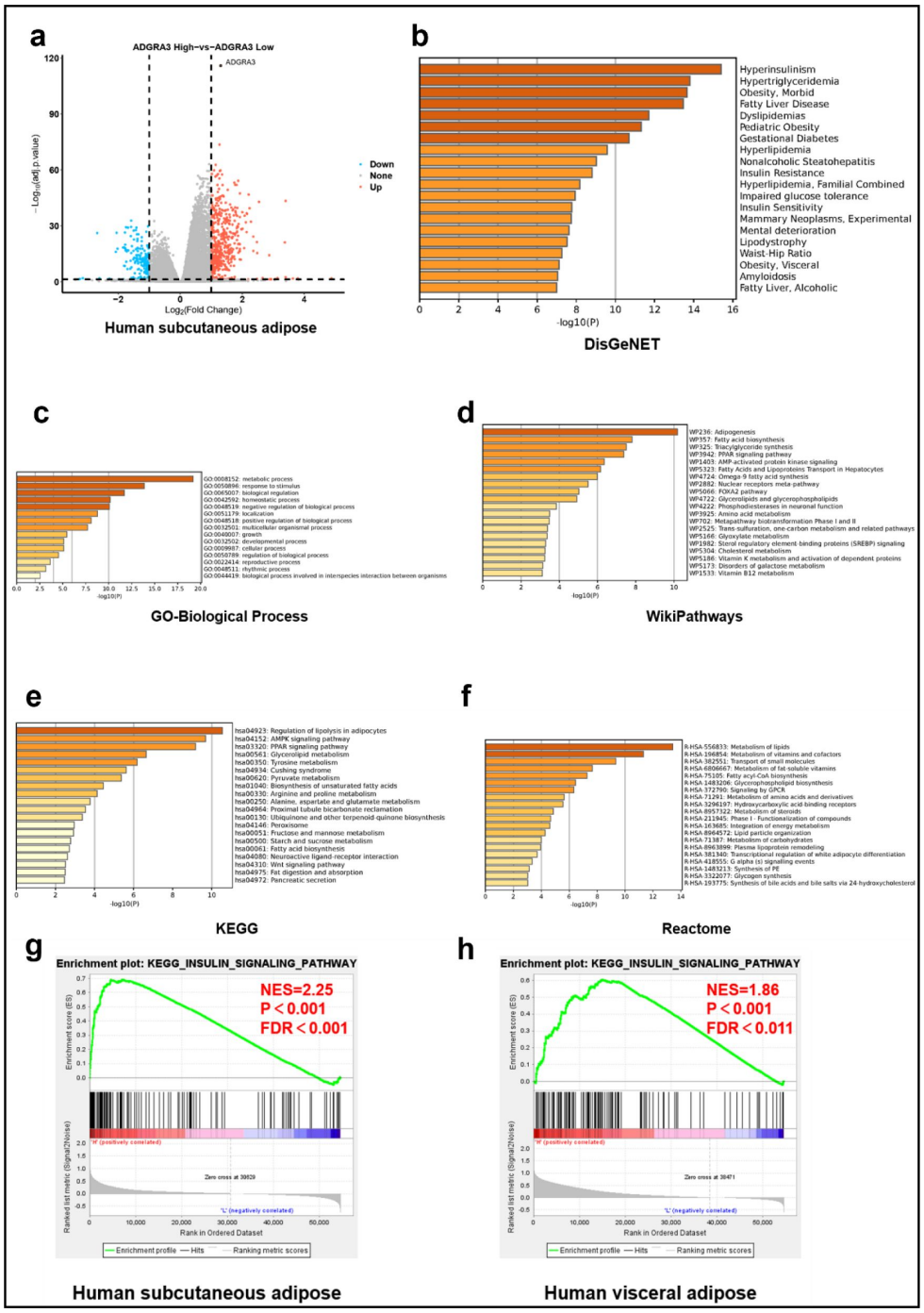

Figure S3.

ADGRA3 high expressed gene sets in human subcutaneous fat are enriched to lipid metabolism and adipocyte differentiation.

(A) Volcano plot summarizing the differentially expressed genes (DEGs) between *ADGRA3* high-expressed human subcutaneous adipose group and *ADGRA3* low-expressed human subcutaneous adipose group. Blue and red shading indicates down-regulation and up-regulation, respectively. (B-F) Enrichment analysis for the high-expressed genes in *ADGRA3* high-expressed human subcutaneous adipose in DisGeNET (B), GO-Biological Process (C), WikiPathways (D), KEGG (E) and Reactome (F) databases. (G-H) Gene set enrichment analysis (GSEA) analysis for gene signatures of insulin signaling pathway in human subcutaneous adipose (G) and human visceral adipose (H) from *ADGRA3* high-expressed group compared with *ADGRA3* low-expressed group. NES, normalized enrichment score. FDR, false discovery rate.

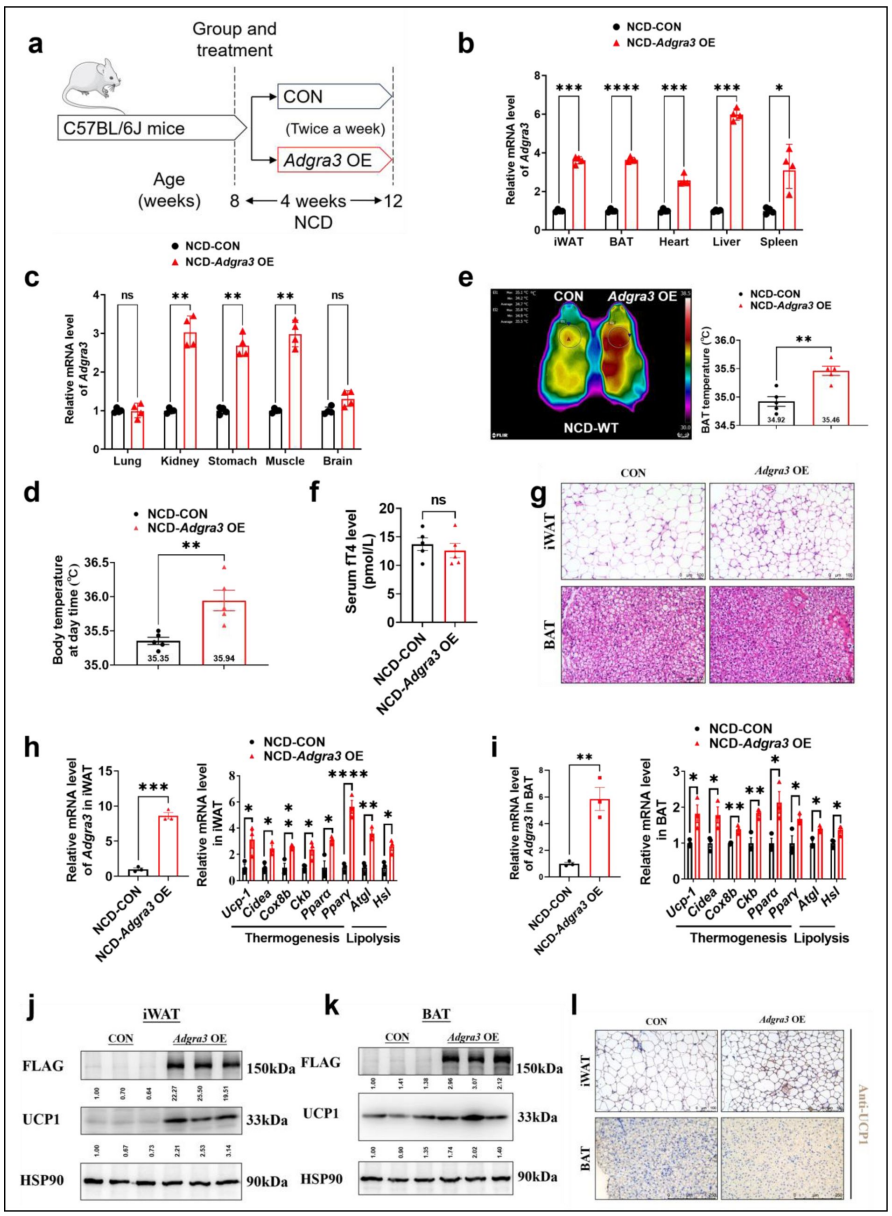

Figure S4.

Adgra3 overexpression activated the adipose thermogenic program in mice.

(A) Experimental schematic. C57BL/6J mice fed with a NCD for eight weeks were injected with *Adgra3* OE (pLV3-CMV-*Adgra3*(mouse)-3×FLAG plasmid encapsulated in nanomaterials) or CON (pLV3-CMV-MCS-3×FLAG plasmid encapsulated in nanomaterials) twice a week for four weeks. (B) qPCR analysis of *Adgra3* gene in iWAT, BAT, heart, liver and spleen from different treatment mice (N = 4 for each group). (C) qPCR analysis of *Adgra3* gene in lung, kidney, stomach, skeletal muscle and brain from different treatment mice (N = 4 for each group). (D) Body temperature of mice injected with CON or *Adgra3* OE for 28 days (N = 5 for each group). (E) Thermal image and BAT temperature in mice injected with CON or *Adgra3* OE for 28 days (N = 5 for each group). (F) The fT4 level of serum from different treated mice (N = 5 for each group). (G) Representative images of iWAT (top) and BAT (bottom) stained with HE. Scale bars, 100 μm. (H-I) qPCR analysis of *Adgra3*, genes associated with thermogenesis and lipolysis in iWAT (H) and BAT (I) from different treatment mice (N = 3 for each group). (J-K) Western-blot analysis for the level of ADGRA3-3×FLAG and UCP1 protein in iWAT (J) and BAT (K) from differently treated mice. The ImageJ software was used for gray scanning. (L) Representative images of iWAT (top; Scale bars, 100 μm.) and BAT (bottom; Scale bars, 250 μm.) stained with UCP1. NCD, normal chow diet; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; fT4, free tetraiodothyronine. All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (B-F and H-I).

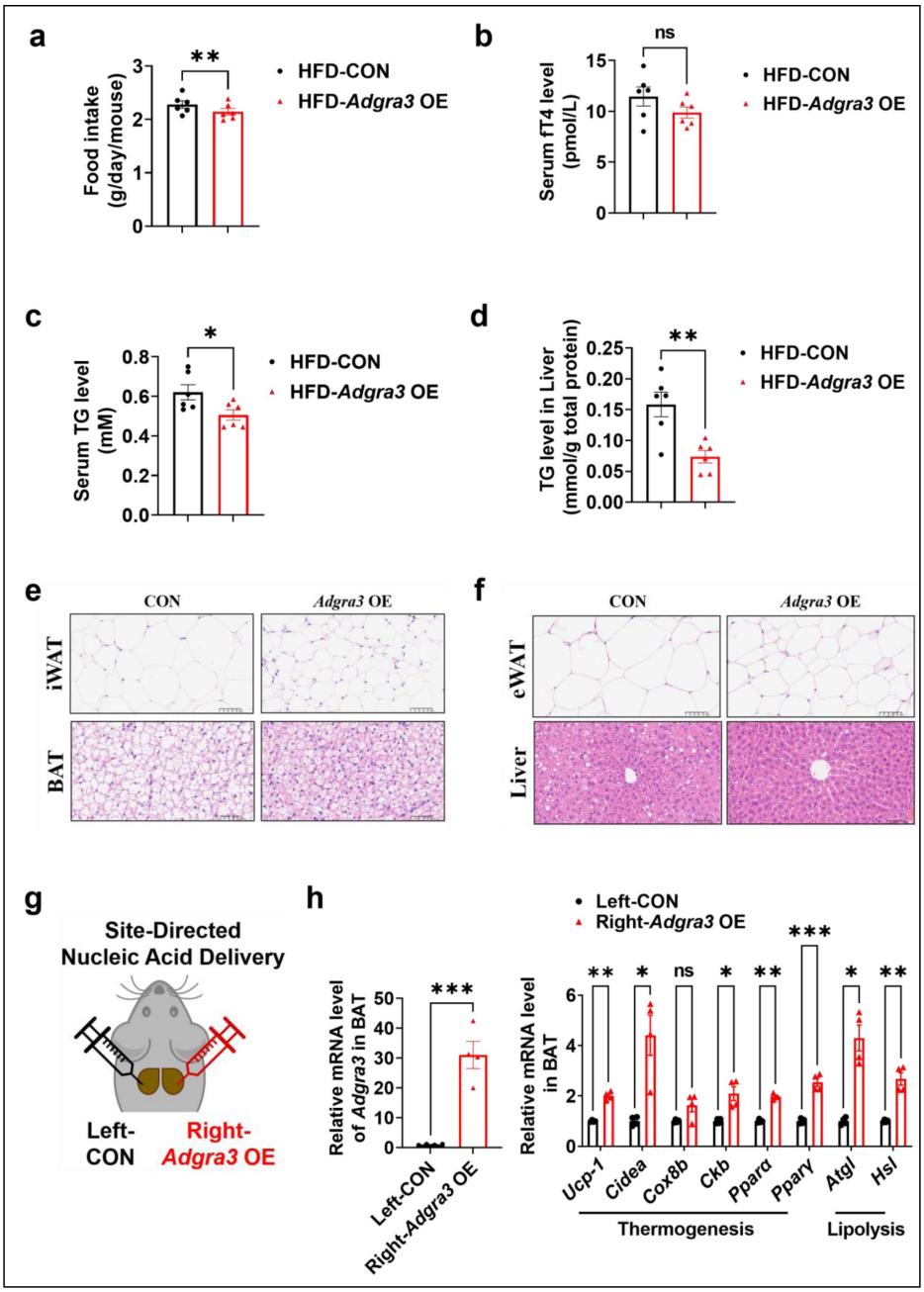

Figure S5.

Characterization of wild-type and *Adgra3*-overexpressed mice.

(A-F) C57BL/6J mice were fed with a HFD and injected with *Adgra3* OE (pLV3-CMV-Adgra3 (mouse)-3×FLAG plasmid encapsulated in nanomaterials) or CON (pLV3-CMV-MCS-3×FLAG plasmid encapsulated in nanomaterials) once a week for 12 weeks. (A) Food intake of different treated mice (N = 6 for each group). (B) The fT4 level of serum from different treated mice (N = 6 for each group). (C-D) The TG level of serum (C) and liver (D) from different treated mice (N = 6 for each group). (E) Representative images of iWAT (top) and BAT (bottom) stained with hematoxylin and eosin. Scale bars, 50 μm. (F) Representative images of eWAT (top) and Liver (bottom) stained with hematoxylin and eosin. Scale bars, 50 μm. (G) Schematic depicting the site-directed nanomaterials-encapsulated nucleic acid injections used to overexpress *Adgra3* in BAT. (H) qPCR analysis of *Adgra3*, genes associated with thermogenesis and lipolysis in different-treated BAT (N = 4 for each group). HFD, high-fat diet; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; fT4, free tetraiodothyronine. All data are presented as mean ± SEM. Statistical significance was determined by paired two-tailed student's t-test (A) and unpaired two-tailed student's t-test (B-D and H).

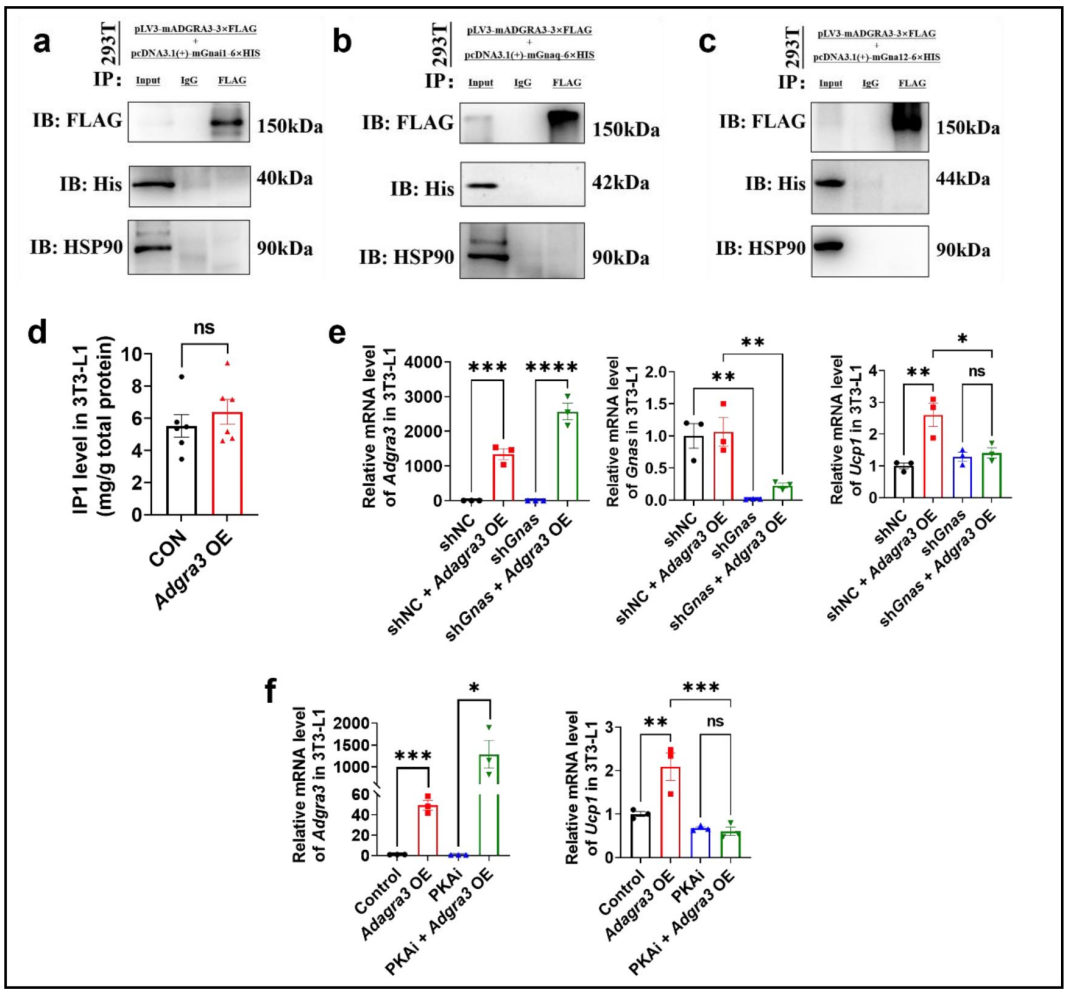

Figure S6.

ADGRA3 was not observed to bind to G_i, G_q and G_{12}.

(A-C) Western-blot analysis for level of ADGRA3-3×FLAG, GNAI1-6×HIS (A), GNAQ-6×HIS (B), GNA12-6×HIS (C) and HSP90 proteins in 293T transfected with different plasmids. (D) The level of IP1 in 3T3-L1 (N = 6 for each group). An ELISA kits was used to measure the level of IP1. (E-F) qPCR analysis of *Gnas*, *Adgra3* and *Ucp1* in 3T3-L1 mature beige-like adipocytes (N = 3 for each group). PKAi, protein kinase A inhibitor, 20 μM H-89. All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (D) and one-way ANOVA (E-F).

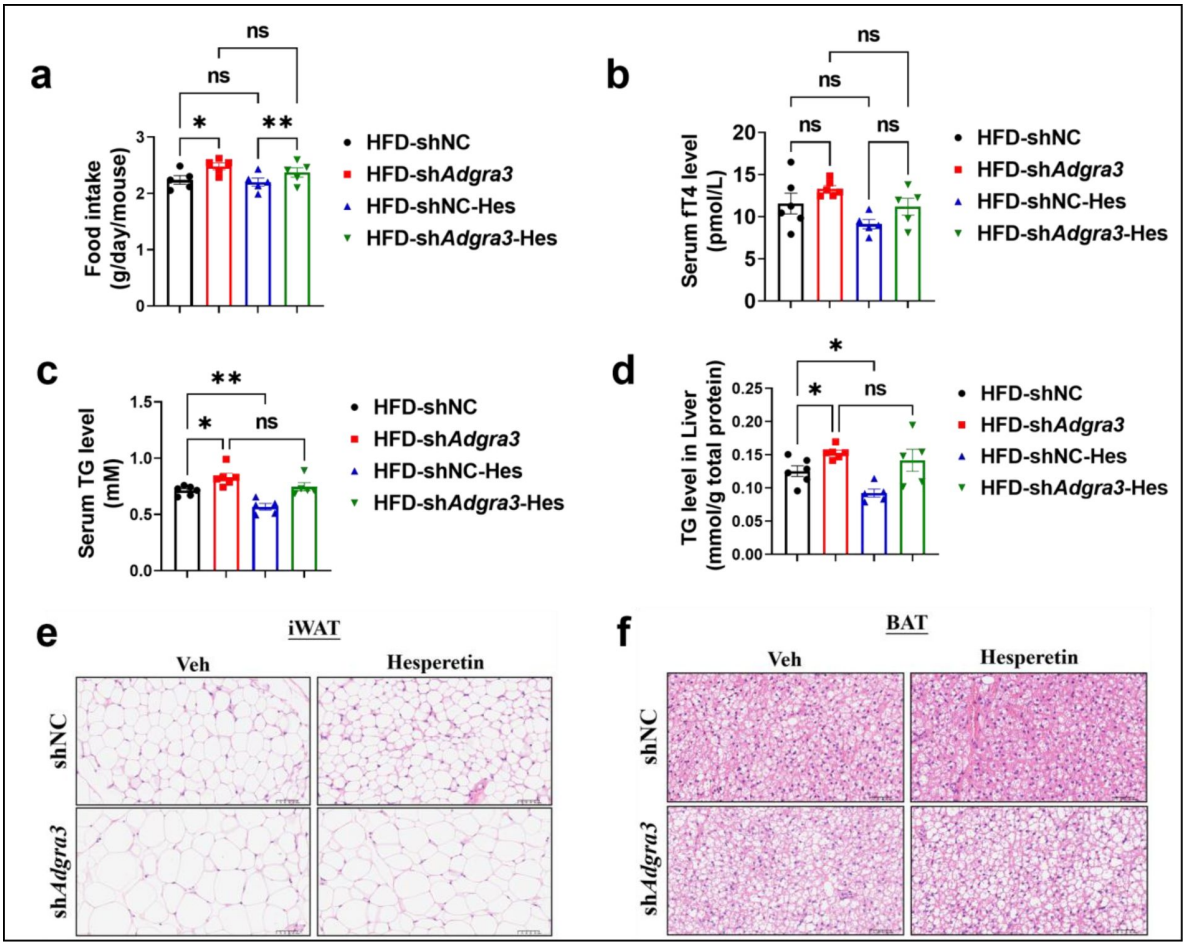

Figure S7.

Characterization of wild-type and *Adgra3*-knockdown mice after hesperetin treatment.

(A) Food intake of different treated mice (N = 5 for each group). (B) The fT4 level of serum from different treated mice (N = 6 for HFD-shNC and HFD-sh*Adgra3*; N = 5 for HFD-shNC-Hes and HFD-sh*Adgra3*-Hes). (C-D) The TG level of serum (C) and liver (D) from different treated mice (N = 6 for HFD-shNC and HFD-sh*Adgra3*; N = 5 for HFD-shNC-Hes and HFD-sh*Adgra3*-Hes). (E-F) Representative images of iWAT (E) and BAT (F) stained with hematoxylin and eosin. Scale bars, 50 μm. HFD, high-fat diet; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; fT4, free tetraiodothyronine, Hes, Hesperetin. All data are presented as mean ± *SEM*. Statistical significance was determined by one-way ANOVA (A-D).

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Author information

Zewei Zhao
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Longyun Hu
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Bigui Song
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Tao Jiang
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Qian Wu
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Jiejing Lin
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Xiaoxiao Li
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Yi Cai
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Jin Li
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Bingxiu Qian
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Siqi Liu
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China

Jilu Lang
Department of Cardiovascular Surgery, Peking University Shenzhen Hospital, Shenzhen, China

For correspondence: langjl1983@126.com

Zhonghan Yang
Shenzhen Key Laboratory of Systems Medicine for inflammatory diseases, School of Medicine, Shenzhen Campus of Sun Yat-Sen University, Sun Yat-Sen University, Shenzhen, China
ORCID iD: 0000-0002-3009-4074

For correspondence: yangzhh@mail.sysu.edu.cn

Editors

Reviewing Editor
Shingo Kajimura
Beth Israel Deaconess Medical Center, Boston, United States of America

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David James
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Reviewer #1 (Public review):

Summary:

This article identifies ADGR3 as a candidate GPCR for mediating beige fat development. The authors use human expression data from Human Protein Atlas and Gtex databases and combine this with experiments performed in mice and a murine cell line. They refer to a GPCR bioactivity screening tool PRESTO-Salsa, with which it was found that Hesperetin activates ADGR3. From their experiments, authors conclude that Hesperetin activates ADGR3, inducing a Gs-PKA-CREB axis resulting in adipose thermogenesis.

Strengths:

The authors analyze human data from public databases and perform functional studies in mouse models. They identify a new GPCR with a role in thermogenic activation of adipocytes.

Considerations:

Selection of ADGRA3 as a candidate GPCR relevant for mediating beiging in humans:

The authors identify GPCRs that are expressed more highly in murine iBAT compared to iWAT in response to cold and assess which of these GPCRs are expressed in human subcutaneous or visceral adipocytes. Although this strategy will identify GPCRs that are expressed at higher levels in brown fat compared to beige and thus possibly more active in thermogenic function, the relevance in choosing GPCRs that also are expressed in unstimulated human white adipocytes should be considered. Thermogenic activity is not normally present in human white adipocytes. It would have strengthened the GPCR selection if the authors instead had assessed the intersection with human brown adipocytes that were activated with norepinephrine.

Strategy to investigate the role of ADGRA3 in WAT beiging:

Having identified ADGRA3 as their candidate receptor, the authors investigated the receptor in mouse models, the murine inguinal adipocyte cell line 3T3 and in human subcutaneous adipose progenitors (HAdsc) differentiated in vitro. Calling the human cells "beige" is a stretch as these cells are derived from a white adipose depot. The authors do observe regulation in UCP1 and abundance of mitochondria following modification of ADGRA3 in the cells. However, in future studies, it should be considered if the receptor rather plays a role in differentiation per se, and perhaps not specifically in thermogenic differentiation/activity.

According to the Human Protein Atlas and Gtex databases, ADGRA3 is not only expressed in adipocytes, but also in other tissues and cell types. The authors address this by measuring the expression in a panel of these tissues, demonstrating a knockdown not only in the adipose tissue, but also in the liver and less pronounced in the muscle (Figure S2). It should thus be emphasized that the decreased TG levels in serum and liver in the mice might in fact depend on Adgra3 overexpression in the liver. Even though this might not have been the purpose of the experiment, it is important to highlight this as it could serve as hypothesis building for future studies of the function of this receptor.

https://doi.org/10.7554/eLife.100205.2.sa3

Reviewer #2 (Public review):

Based on bioinformatics and expression analysis using mouse and human samples, the authors claim that the adhesion G-protein coupled receptor ADGRA3 may be a valuable target for increasing thermogenic activity and metabolic health. Genetic approaches to deplete

ADGRA3 expression in vitro resulted in reduced expression of thermogenic genes including Ucp1, reduced basal respiration and metabolic activity as reflected by reduced glucose uptake and triglyceride accumulation. In line, nanoparticle delivery of shAdgra3 constructs is associated with increased body weight, reduced thermogenic gene expression in white and brown adipose tissue (WAT, BAT), and impaired glucose and insulin tolerance. On the other hand, ADGRA3 overexpression is associated with an improved metabolic profile in vitro and in vivo, which can be explained by increasing the activity of the well-established Gs-PKA-CREB axis. Notably, a computational screen suggested that ADGRA3 is activated by hesperetin. This metabolite is a derivative of the major citrus flavonoid hesperidin and has been described to promote metabolic health. Using appropriate in vitro and in vivo studies, the authors show that hesperitin supplementation is associated with increased thermogenesis, UCP1 levels in WAT and BAT, and improved glucose tolerance, an effect that was attenuated in the absence of ADGRA3 expression.

Comments on revised version:
In my opinion, the critical points I raised were not adequately addressed, neither in the revision nor in the response to the reviewer. Therefore, my initial assessment has not changed, the main claims are only partially supported by the data presented.

https://doi.org/10.7554/eLife.100205.2.sa2

Reviewer #3 (Public review):

Summary:

The manuscript by Zhao et al. explored the function of adhesion G protein-coupled receptor A3 (ADGRA3) in thermogenic fat biology.

Strengths:

Through both in vivo and in vitro studies, the authors found that the gain function of ADGRA3 leads to browning of white fat and ameliorates insulin resistance.

Comments on revised version:

The revised manuscript by Zhao et al. has limited improvement. The authors refused to perform revised experiments using primary cultures even though two reviewers pointed out the same weakness (3T3-L1 adipocytes are unsuitable). Using infrared thermography to measure body temperature is also problematic.

https://doi.org/10.7554/eLife.100205.2.sa1

Author response:

The following is the authors' response to the original reviews.

> ***Public Reviews:***
>
> ***Reviewer #1 (Public Review):***
>
> *Summary:*
>
> *This article identifies ADGR3 as a candidate GPCR for mediating beige fat development. The authors use human expression data from the Human protein atlas and Gtex databases and combine this with experiments performed in mice and a murine cell line. They refer to a GPCR bioactivity screening tool PRESTO-Salsa, with which it was found*

> *that Hesperetin activates ADGR3. From their experiments, authors conclude that Hesperetin activates ADGR3, inducing a Gs-PKA-CREB axis resulting in adipose thermogenesis.*
>
> *Strengths:*
>
> *The authors analyze human data from public databases and perform functional studies in mouse models. They identify a new GPCR with a role in the thermogenic activation of adipocytes.*
>
> *Weaknesses:*
>
> *(1) Selection of ADGRA3 as a candidate GPCR relevant for mediating beiging in humans:*
>
> *The authors identify genes upregulated in iBAT compared to iWAT in response to cold, and among these differentially expressed genes, they identify highly expressed GPCRs in human white adipocytes (visceral or subcutaneous). Finally, among these genes, they select a GPCR not previously studied in the literature.*
>
> *If the authors are interested in beiging, why do they not focus on genes upregulated in iWAT (the depot where beiging is described to occur in mice), comparing thermoneutral to cold-induced genes? I would expect that genes induced in iWAT in response to cold would be extremely relevant targets for beiging. With their strategy, the authors exclude receptors that are induced in the tissue where beiging is actually described to occur.*
>
> *Furthermore, the authors are comparing genes upregulated in cold in BAT (but not WAT) to highly expressed genes in human white adipocytes during thermoneutrality. Overall, the authors fail to discuss the logic behind their strategy and the obvious limitations of it.*

Thanks for your valuable advice. In this study, we focus on genes that exhibited higher expression in BAT compared to iWAT under cold stimulation conditions, as these genes might play a role in adipose thermogenesis. Regarding the genes you mentioned that iWAT upregulates following cold stimulation, we did identify other intriguing targets in these genes in another ongoing study, albeit not encompassed within the scope of this study. Moreover, instead of making a comparison, we intersected 27 GPCR coding genes that were highly expressed in BAT compared to iWAT with genes that were highly expressed in human adipocytes (Figure 1C).

With your suggestions, we realized that the description of the screening strategy in the manuscript was not clear enough, so we made the following supplement:

"…dataset obtained from the Gene Expression Omnibus (GEO) database. Additionally, we utilized the human subcutaneous adipocytes dataset (Figure 1C, red) and human visceral adipocytes dataset (Figure 1C, purple) from the human protein atlas database to obtain genes that are highly expressed in human white adipocytes. The GSE118849 dataset comprises samples of brown adipose tissue (BAT) and inguinal white adipose tissue (iWAT) obtained from mice subjected to a 72-hour cold exposure at a temperature of 4℃.

A total of 1134 differentially expressed genes (DEGs) that exhibited up-regulation in BAT compared to iWAT under cold stimulation were identified in the analysis, which might play a role in adipose thermogenesis. These DEGs were further screened to identify highly…"

> *(2) Relevance of ADGRA3 and comparison to established literature:*

There has been a lot of literature and discussion about which receptor should be targeted in humans to recruit thermogenic fat. The current article unfortunately does not discuss this literature nor explain how it relates to their findings. For example, O'Mara et al (PMID:

31961826) demonstrated that chronic stimulation with the B3 adrenergic agonist, Mirabegron, resulted in the recruitment of thermogenic fat and improvement in insulin sensitivity and cholesterol. Later, Blondin et al (PMID: 32755608), highlighted the B2 adrenergic receptor as the main activation path of thermogenic fat in humans. There is also a recent report on an agonist activating B2 and B3 simultaneously (PMID: 38796310). Thus, to bring the literature forward, it would be beneficial if the current manuscript compared their identified activation path with the activation of these already established receptors and discussed their findings in relation to previous studies.

Thanks to your suggestion. We have included a supplementary discussion on the relevant human adipose thermogenic receptors in the discussion section, as presented below:

"The induction of beige fat has been investigated as a potentially effective therapeutic approach in combating obesity [23]. A clinical trial revealed that treatment with the chronic β3-AR agonist mirabegron leads to an increase in human brown fat, HDL cholesterol, and insulin sensitivity [24]. Subsequently, Blondin et al discovered that oral administration of mirabegron only elicits an increase in BAT thermogenesis when administered at the maximal allowable dose, indicating that human brown adipocyte thermogenesis is primarily driven by β2-adrenoceptor (β2-AR) stimulation [11]. Consistent with this finding, we found much higher levels of ADRB2 expression in human white adipose tissue than ADRB3 (Figure S1E). Furthermore, a recent study has demonstrated that simultaneous activation of β2-AR and β3-AR enhances whole-body metabolism through beneficial effects on skeletal muscle and BAT [25]."

> *In Figures 1d and e, the authors show the expression of ADGRA3 in comparison to the expression of ADRB3. In human brown adipocytes, ADRB2 has been shown to be the main receptor through which adrenergic activation occurs (PMID: 32755608), thus authors should show the relative expression of this gene as well.*

We wholeheartedly endorse the proposal to augment the *ADRB2* expression data in Figures 1D and E. However, it is regrettable to note that the pertinent databases (PRJNA66167 and PRJEB4337) are deficient in *ADRB2* expression information. Fortunately, the GTEx database houses the *ADRB2* expression data. Consequently, we have integrated these crucial data into Figure S1E.

> *(3) Strategy to investigate the role of ADGRA3 in WAT beiging:*

Having identified ADGRA3 as their candidate receptor, the authors proceed with investigations of this receptor in mouse models and the murine inguinal adipocyte cell line 3T3.

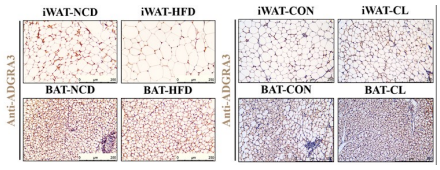
> *First of all, in Figure 1D, the authors show a substantially lower expression of ADGRA3 compared to ADRB3. It could thus be argued that a mouse would not be the best model system for studying this receptor. It would be interesting to see data from experiments in human adipocytes.*

Thanks for your helpful advice. We induced human adipose-derived mesenchymal stem cells (hADSCs) into adipocytes to evaluate the effect of ADGRA3 on human adipocytes (Figure 8).

> *Moreover, if the authors are interested in inducing beiging, why do they show expression in iBAT and not iWAT?*

Maybe the description of this article wasn't clear enough, but we did show the expression and effects of ADGRA3 in iWAT and BAT (Author response image 1, Figure 3F-J and Figure 4F-J).

Author response image 1.



> *The authors perform in vivo experiments using intraperitoneal injections of shRNA or overexpression CMV-driven vectors and report effects on body temperature and glucose metabolism. It is here important to note that ADGRA3 is not uniquely expressed in adipocytes. A major advantage of databases like the Human Protein Atlas and Gtex, is that they give an overview of the gene expression across tissues and cell types. When looking up ADGRA3 in these databases, it is expressed in subcutaneous and visceral adipocytes. However, other cell types and tissues demonstrate an even higher expression. In the Human protein atlas, the enhanced cell types are astrocytes and hepatocytes. In the Gtex database tissues with the highest expression are Brain, Liver, and Thyroid.*
>
> *With this information in mind, IP injections for modification of ADGRA3 receptor expression could be expected to affect any of these tissues and cells.*
>
> *The manuscript report changes body temperature. However, temperature is regulated by the brain and also affected by thyroid activity. Did the authors measure the levels of circulating thyroid hormones? Gene expression changes in the brain? The authors report that Adgra3 overexpression decreased the TG level in serum and liver. The liver could be the primary targeted organ here, and the adipose effects might be secondary. The data would be easier to interpret if authors reported the effects on the liver, thyroid, and brain, and the gene expression across tissues should be discussed in the article.*

Thank you for your valuable advice. We supplemented the results of the effect of local BAT injection of *Adgra3* OE on thermogenic genes (Figures S5G-H), the levels of circulating thyroid hormones (Figures S2H, S4F and S5B) and the effects of *Adgra3* overexpression/knockdown on *Adgra3* expression levels (Figures S2A-B and S4B-C) in multiple tissues as well as discussed in the article, as follows:

“Given the consideration that the non-targeted nanoparticle approach utilized in this study for modulating *Adgra3* expression levels in vivo alter *Adgra3* expression in tissues beyond adipose tissue (Figures S2A-B and S4B-C), notably the liver and skeletal muscle, the construction of *Adgra3* adipose tissue-specific knockout/overexpression mouse models is imperative for a more nuanced understanding of the precise mechanisms underlying the influence of on adipose thermogenesis. We will employ more sophisticated models in subsequent studies to further elucidate the effects of ADGRA3 on adipose thermogenesis and metabolic homeostasis. Nevertheless, our findings underlie a potential therapeutic feature of…”

> *Finally, the identification of Hesperetin using the PRESTO-Salsa tool, and how specific the effect of Hesperetin is on ADGRA3, is currently unclear. This should be better discussed, and authors should consider measuring the established effects of Hesperetin in their model systems, including apoptosis.*

Thanks for your suggestion. We have further discussed the relevant content and added it in the discussion section as follows:

"Previously, the influence of hesperetin on ADGRA3 has remained unreported. In this study, we screened hesperetin as a potential agonist for ADGRA3 by using the PRESTO-Salsa tool as well as discovered that hesperetin has an agonist effect on ADGRA3 through a series of experiments. This study focuses on the regulatory effect of hesperetin on adipose thermogenesis and explores whether this effect is dependent upon ADGRA3. As such, we refrained from conducting further investigations into other potential effects of hesperidin, including its potential role in antioxidant and in apoptosis."

> ***Reviewer #2 (Public Review):***
>
> *Based on bioinformatics and expression analysis using mouse and human samples, the authors claim that the adhesion G-protein coupled receptor ADGRA3 may be a valuable target for increasing thermogenic activity and metabolic health. Genetic approaches to deplete ADGRA3 expression in vitro resulted in reduced expression of thermogenic genes including Ucp1, reduced basal respiration, and metabolic activity as reflected by reduced glucose uptake and triglyceride accumulation. In line, nanoparticle delivery of shAdgra3 constructs is associated with increased body weight, reduced thermogenic gene expression in white and brown adipose tissue (WAT, BAT), and impaired glucose and insulin tolerance. On the other hand, ADGRA3 overexpression is associated with an improved metabolic profile in vitro and in vivo, which can be explained by increasing the activity of the well-established Gs-PKA-CREB axis. Notably, a computational screen suggested that ADGRA3 is activated by hesperetin. This metabolite is a derivative of the major citrus flavonoid hesperidin and has been described to promote metabolic health. Using appropriate in vitro and in vivo studies, the authors show that hesperetin supplementation is associated with increased thermogenesis, UCP1 levels in WAT and BAT, and improved glucose tolerance, an effect that was attenuated in the absence of ADGRA3 expression.*
>
> *Overall, the data suggest that ADGRA3 is a constitutively active Gs-coupled receptor that improves metabolism by activating adaptive thermogenesis in WAT and BAT. The conclusions of the paper are partly supported by the data, but some experimental approaches need further clarification.*
>
> *(1) The in vivo approaches to modulate Adgra3 expression in mice are carried out using non-targeted nanoparticle-based approaches. The authors do not provide details of the composition of the nanomaterials, but it is highly likely that other metabolically active organs such as the liver are targeted. This is critical because Adgre3 is expressed in many organs, including the liver, adrenal glands, and gastrointestinal system. Therefore, many of the observed metabolic effects could be indirect, for example by modulating bile acids or corticosterone levels. Consistent with this, after digestion in the gastrointestinal tract, hesperetin is rapidly metabolized in intestinal and liver cells. Thus, hesperetin levels in the systemic circulation are likely to be insufficient to activate Adgra3 in thermogenic adipocytes/precursors. Overall, the authors need to repeat the key metabolic experiments in adipose-specific Adgra3 knockout/overexpression models to validate the reliability of the in vivo results. In addition, to validate the relevance of hesperetin supplementation for adaptive thermogenesis in BAT and WAT vivo, the levels of hesperetin present in the systemic circulation should be quantified.*

Thank you for your valuable advice. Unfortunately, we could not perform quantitative determination of hesperetin concentration in the systemic circulation because we had used the serum of hesperetin-treated mice for the quantitative determination of serum insulin, fT4 and TG. According to your other suggestions, we supplemented the results of the effect of local BAT injection of *Adgra3* OE on thermogenic genes (Figures S5G-H), the levels of circulating thyroid hormones (Figures S2H, S4F and S5B) and the effects of *Adgra3*

overexpression/knockdown on *Adgra3* expression levels (Figures S2A-B and S4B-C) in multiple tissues as well as discussed in the article, as follows:

"Given the consideration that the non-targeted nanoparticle approach utilized in this study for modulating *Adgra3* expression levels in vivo alter *Adgra3* expression in tissues beyond adipose tissue (Figures S2A-B and S4B-C), notably the liver and skeletal muscle, the construction of *Adgra3* adipose tissue-specific knockout/overexpression mouse models is imperative for a more nuanced understanding of the precise mechanisms underlying the influence of on adipose thermogenesis. We will employ more sophisticated models in subsequent studies to further elucidate the effects of ADGRA3 on adipose thermogenesis and metabolic homeostasis. Nevertheless, our findings underlie a potential therapeutic feature of…"

> *(2) Standard measurements for energy balance are not presented. Quantitative data on energy expenditure, e.g. by indirect calorimetry, and food intake are missing and need to be included to validate the authors' claims.*

We are in full agreement with your proposal. Regrettably, owing to the constraints of experimental facilities, we are presently unable to access quantitative data pertaining to the energy expenditure of animals. However, we believe that the present results can also partially support the idea that ADGRA3 promotes energy metabolism and the results of the effect of ADGRA3 on food intake were shown in Figure S2C and Figure S5A respectively.

> *(3) The thermographic images used to determine the BAT temperature are not very convincing. The distance and angle between the thermal camera and the BAT have a significant effect on the determination of the temperature, which is not taken into account, at least in the images presented.*

Thank you very much for pointing out the lack of our method description. According to the methods of literatures (Xia, Bo et al. PLoS biology. 2020. doi:10.1371/journal.pbio.3000688) and (Warner, Amy et al. PNAS. 2013. doi:10.1073/pnas.1310300110), the same batch of representative infrared images of mice were all captured using a thermal imaging camera (FLIR ONE PRO), measured at the same distance perpendicular to the plane on which the mice were located. We have supplemented this description in the Materials and Methods section, as shown below:

"2.20. Infrared Thermography.

BAT temperature was measured at room temperature by infrared thermography according to previous publications [22, 23]. The same batch of representative infrared images of mice were all captured using a thermal imaging camera (FLIR ONE PRO), measured at the same distance perpendicular to the plane on which the mice were located. To quantify interscapular region temperature, the average surface temperature from a region of the interscapular BAT was taken with FLIR Tools software."

> *(4) The 3T3-L1 cell line is not an adequate cell culture model to study thermogenic adipocyte differentiation. To validate their results, the key experiments showing that ADGRA3 expression modulates thermogenic marker expression in a hesperetin-dependent manner need to be performed in a reliable model, e.g. primary murine adipocytes.*

Induction of 3T3L1 cell line into white adipocytes is indeed not suitable for studying thermogenic adipocyte differentiation. However, with reference to previous studies (Wei, Gang et al. Cell metabolism. 2021. doi: 10.1016/j.cmet.2021.08.012) and (Bae IS, Kim SH. Int J Mol Sci. 2019. doi: 10.3390/ijms20246128), 3T3-L1 cell line was used to differentiate into beige-

like adipocytes in this study, and many studies believe that this method is suitable for studying the thermogenic effect of adipocytes in vitro. Meanwhile, we provided a more detailed description of the induction of beige-like adipocytes by 3T3-L1 in the Materials and Methods section and induced human adipose-derived stem cells (hADSC) into adipocytes to evaluate the effect of ADGRA3 on human adipocytes (Figure 8).

"...supplemented with 10% FBS. Confluent 3T3-L1 pre-adipocytes were induced into mature beige-like adipocytes with 0.5 mM isobutyl methylxanthine (IBMX), 1 μM dexamethasone, 5 μg/ml insulin, 1 nM 3, 3', 5-Triiodo-L-thyronine (T3), 125 μM indomethacin and 1 μM rosiglitazone in high-glucose DMEM containing 10% FBS for 2 days, then treated with high-glucose DMEM containing 5 μg/ml insulin, 1 nM T3, 1 μM rosiglitazone and 10% FBS for 6 days and cultured with high-glucose DMEM containing 10% FBS for 2 days. hADSCs were seeded on plates coated with 0.1% gelatin and culture and grown to confluence in human mesenchymal stem cells (hMSCs) specialized culture medium (ZQ-1320). Confluent hADSCs were induced into mature human adipocytes with adipogenic induction medium (PCM-I-004) according to the manufacturer's instructions."

> *(5) The experimental setup only allows the measurement of basal cellular respiration. More advanced approaches are needed to define the contribution of ADGRA3 versus classical adrenergic receptors to UCP1-dependent thermogenesis.*

Thanks for your suggestion. The maximum oxygen consumption rate of the cells was also measured (Figures 2G and 2N) by adding FCCP, an uncoupler of oxidative phosphorylation (OXPHOS) in mitochondria.

> ***Reviewer #3 (Public Review):***
>
> *Summary:*
>
> *The manuscript by Zhao et al. explored the function of adhesion G protein-coupled receptor A3 (ADGRA3) in thermogenic fat biology.*
>
> *Strengths:*
>
> *Through both in vivo and in vitro studies, the authors found that the gain function of ADGRA3 leads to browning of white fat and ameliorates insulin resistance.*
>
> *Weaknesses:*
>
> *There are several lines of weak methodologies such as using 3T3-L1 adipocytes and intraperitoneal(i.p.) injection of virus. Moreover, as the authors stated that ADGRA3 is constitutively active, how could the authors then identify a chemical ligand?*
>
> *(1) Primary cultured cells should be used to perform gain and loss function analysis of ADGRA3, instead of using 3T3-L1. It is impossible to detect Ucp1 expression in 3T3-L1 cells.*

Induction of 3T3L1 cell line into white adipocytes is indeed difficult for detecting UCP1 expression. However, with reference to previous studies (Wei, Gang et al. Cell metabolism. 2021. doi:10.1016/j.cmet.2021.08.012) and (Bae IS, Kim SH. Int J Mol Sci. 2019. doi:10.3390/ijms20246128), 3T3-L1 cell line was used to differentiate into beige-like adipocytes in this study, and many studies believe that this method is suitable for studying the thermogenic effect of adipocytes in vitro. Meanwhile, we provided a more detailed description of the induction of beige-like adipocytes by 3T3-L1 in the Materials and Methods section and induced human adipose-derived stem cells (hADSC) into adipocytes to evaluate the effect of ADGRA3 on human adipocytes (Figure 8).

"…supplemented with 10% FBS. Confluent 3T3-L1 pre-adipocytes were induced into mature beige-like adipocytes with 0.5 mM isobutyl methylxanthine (IBMX), 1 μM dexamethasone, 5 μg/ml insulin, 1 nM 3, 3', 5-Triiodo-L-thyronine (T3), 125 μM indomethacin and 1 μM rosiglitazone in high-glucose DMEM containing 10% FBS for 2 days, then treated with high-glucose DMEM containing 5 μg/ml insulin, 1 nM T3, 1 μM rosiglitazone and 10% FBS for 6 days and cultured with high-glucose DMEM containing 10% FBS for 2 days. hADSCs were seeded on plates coated with 0.1% gelatin and culture and grown to confluence in human mesenchymal stem cells (hMSCs) specialized culture medium (ZQ-1320). Confluent hADSCs were induced into mature human adipocytes with adipogenic induction medium (PCM-I-004) according to the manufacturer's instructions."

> *(2) For virus treatment, the authors should consider performing local tissue injection, rather than IP injection. If it is IP injection, have the authors checked other tissues to validate whether the phenotype is fat-specific?*

Thank you for your valuable advice. We supplemented the results of the effect of local BAT injection of *Adgra3* OE on thermogenic genes (Figures S5G-H) and the effects of *Adgra3* overexpression/knockdown on *Adgra3* expression levels (Figures S2A-B and S4B-C) in other tissues.

> *(3) The authors should clarify how constitutively active GPCR needs further ligands.*

Thank you for your suggestion. In fact, we only identified hesperetin as a potential agonist of ADGRA3 rather than a ligand. The results also indicate that overexpression of ADGRA3 without additional hesperetin is sufficient to activate downstream PKA signaling pathways through constitutive activity (Figure 5). Recently, Chen et al identified oleic ethanolamine (OEA) as a potential endogenous agonist of GPR3, which is also a constitutively active GPCR. Overall, the high constitutive activity of constitutively active GPCRs arises from the combined effects of stimulation by endogenous agonists and their basal coupling with Gs.

As for why we screened and identified potential agonists of ADGRA3, we hope to find more convenient pathways for its clinical application than gene overexpression, as described in the article:

"Considering the difficulty of overexpressing ADGRA3 in clinical application, hesperetin was screened as a potential agonist of ADGRA3 by PRESTO-Salsa database (Figure 6A). The…"

> ***Recommendations for the authors:***
>
> ***Reviewer #1 (Recommendations For The Authors):***
>
> *Minor comments*
>
> *The title appears to be overstated as no clinical trials were performed and experiments were not even performed in human brown adipocytes.*

Thank you for your critical suggestion, therefore we have added the experimental results of human adipocytes (Figure 8) and revised the title to "Constitutively active receptor ADGRA3 signaling induces adipose thermogenesis".

> *Please specify n-number and what are replicates or independent experiments. Please also state if any outliers were excluded and why.*

Thanks for your valuable suggestion. We have added a description of the n-number in the Figure legends section, number of independent experiments and exclusion criteria for

outliers in the Materials and Methods section, as follows:

"…of tissue samples. Cohorts of ≥4 mice per genotype or treatment were assembled for all in vivo studies. All in vivo studies were repeated 2-3 independent times. All procedures related to…"

"…μM H-89) was added to 3T3-L1 mature beige-like adipocytes for 48 hours. All in vitro studies were repeated 2-3 independent times."

"All data are presented as mean ± *SEM*. In this study, outliers that met the three-sigma rule were excluded from analysis, with the exception of those presented in Figure S1E. Given the possibility that the outliers in Figure S1E represent extreme expressions of the inherent variability within the population sample, we have chosen to retain these specific outliers for further analysis. Student's t-test was used to compare two groups. One-way analysis of…"

> *Authors use Infrared Thermography to measure body temperature. Depending on the distance between the mouse and the camera, the mouse needs to be at the same spot.*

Thank you very much for pointing out the lack of our method description. According to the methods of literatures (Xia, Bo et al. PLoS biology. 2020. doi:10.1371/journal.pbio.3000688) and (Warner, Amy et al. PNAS. 2013. doi:10.1073/pnas.1310300110), the same batch of representative infrared images of mice were all captured using a thermal imaging camera (FLIR ONE PRO), measured at the same distance perpendicular to the plane on which the mice were located. We have supplemented this description in the Materials and Methods section, as shown below:

"2.20. Infrared Thermography.

BAT temperature was measured at room temperature by infrared thermography according to previous publications [22, 23]. The same batch of representative infrared images of mice were all captured using a thermal imaging camera (FLIR ONE PRO), measured at the same distance perpendicular to the plane on which the mice were located. To quantify interscapular region temperature, the average surface temperature from a region of the interscapular BAT was taken with FLIR Tools software."

> *Please discuss the limitations of the experiments and discuss the relevant literature.*

Thanks for your recommendations. We discussed the limitations of the experiments and the relevant literature in the discussion section, as follows:

"The induction of beige fat has been investigated as a potentially effective therapeutic approach in combating obesity [23]. A clinical trial revealed that treatment with the chronic β3-AR agonist mirabegron leads to an increase in human brown fat, HDL cholesterol, and insulin sensitivity [24]. Subsequently, Blondin et al discovered that oral administration of mirabegron only elicits an increase in BAT thermogenesis when administered at the maximal allowable dose, indicating that human brown adipocyte thermogenesis is primarily driven by β2-adrenoceptor (β2-AR) stimulation [11]. Consistent with this finding, we found much higher levels of ADRB2 expression in human white adipose tissue than ADRB3 (Figure S1E). Furthermore, a recent study has demonstrated that simultaneous activation of β2-AR and β3-AR enhances whole-body metabolism through beneficial effects on skeletal muscle and BAT [25]."

"Given the consideration that the non-targeted nanoparticle approach utilized in this study for modulating *Adgra3* expression levels in vivo alter *Adgra3* expression in tissues beyond adipose tissue (Figures S2A-B and S4B-C), notably the liver and skeletal muscle, the construction of *Adgra3* adipose tissue-specific knockout/overexpression mouse models is

imperative for a more nuanced understanding of the precise mechanisms underlying the influence of on adipose thermogenesis. We will employ more sophisticated models in subsequent studies to further elucidate the effects of ADGRA3 on adipose thermogenesis and metabolic homeostasis. Nevertheless, our findings underlie a potential therapeutic feature of…”

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