# The Role of ADGRA3 in Adipose Thermogenesis: A Potential Therapeutic Target for Obesity

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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.

***Keywords:*** Obesity; Insulin resistance; Thermogenesis; ADGRA3; Hesperetin.

## 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 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 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 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 ⩾4 mice per genotype or treatment were assembled for all in vivo studies. All in vivo studies were repeated 2-3 separated 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) isolation*

SVF from inguinal white adipose tissue (iWAT) 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 adipocytes were removed, 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 pellets were re-suspended in DMEM medium containing 10% fetal bovine serum (FBS).

### *2.3. Cell culture*

3T3-L1 and 293T cell lines were purchased from the Cell Bank of the Chinese Academy of Sciences in Shanghai. Confluent pre-adipocytes (3T3-L1 and SVF) were induced into mature 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 DMEM containing 10% FBS for 2 days, then treated with DMEM containing 5 μg/ml insulin, 1 nM T3, 1 μM rosiglitazone and 10% FBS for 6 days and cultured with DMEM containing 10% FBS for 2 days. The sh*Adgra3*, sh*Gnas* (pLKO.1-U6-sh*Gnas*

plasmid encapsulated in nanomaterials) and shNC were added to mature 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 mature adipocytes or 293T for 48 hours. Hesperetin (10 μM) and PKAi (protein kinase A inhibitor, 20 μM H-89) was added to mature adipocytes for 48 hours.

### *2.4 Construction of plasmid*

The pLV3-CMV-*Adgra3*(mouse)-3×FLAG, 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. The protein transferred to the PVDF membrane was probed with primary antibodies 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.

### *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). Mouse IP1 level was detected using a sensitive ELISA kit (MM-0790M2). Mouse insulin level was detected using a sensitive ELISA kit (MM-0579M1). 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℃. 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. 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 oxygen consumption rate of cells was measured using a BBoxiProbe R01 kit (BB-48211) according to the manufacturers' instructions.

### *2.20. 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/)[22]. All other data associated with this paper can be found in the main text or the Supplementary Materials.

### *2.21. Statistical Analysis*

All data are presented as mean ± *SEM*. 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 and human visceral adipocytes dataset from the human protein atlas database. 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. 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* (Figure 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 (Figure 1F-G and Figure 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 of ADGRA3 in the SVF of WAT and BAT (Figure 1I). Moreover, it was observed that the modulation of the expression levels of *Adgra3* and *Ucp1* exhibited a similar pattern during the differentiation process between WAT and BAT 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 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 (Figure 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 (Figure 2F-G).

Following the overexpression of *Adgra3*, there was an observed up-regulation in the expression of UCP1 in 3T3-L1 mature adipocytes (Figure 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 (Figure 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 (Figure 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 globally knocking down *Adgra3* in mice, there was a significant increase in the weight of sh*Adgra3* mice (mice with global *Adgra3* knockdown) (Figure 3B). Furthermore, the food intake of sh*Adgra3* mice was elevated (Figure S2A). Serum triacylglycerol (TG) levels (Figure S2B), 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 S2C) 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 S2D), eWAT (Figure S2E) and BAT (Figure S2D) but not lead to hepatic steatosis (Figure S2E).

Remarkably, the knockdown of *Adgra3* resulted in a significant reduction in both body temperature (Figure 3D) and BAT temperature (Figure 3E). 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

(Figure 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.

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 (Figure 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). The growth of body weight of *Adgra3* OE mice was alleviated (Figure 4B) during the HFD feeding accompanied with a 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 (Figure 4D and S4B) and BAT temperature (Figure 4E and S4C). Meanwhile, *Adgra3* overexpression decreased the TG level in serum and liver (Figure S5B-C) as well as the area of adipocytes in iWAT and BAT (Figure S4D and S5D).

Moreover, the expression levels of thermogenic and lipolysis-related genes were elevated in iWAT (Figure 4F and S4E) and BAT (Figure 4G and S4F). Western-blot (Figure 4H-I and S4G-H) and immunohistochemical staining of UCP1 (Figure 4J and S4I) 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). These findings indicate that the overexpression of *Adgra3* is capable of inducing the hallmarks of thermogenesis in mice.

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.

### *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 $G_\alpha$ 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 (Figure 5A-B), while no interaction was observed with the other three Gα proteins ($G_i$, $G_q$ and $G_{12}$; Figure 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 (Figure 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 3T3L1 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 (Figure 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 (Figure 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 (Figure 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 (Figure 6G-H).

Moreover, the results showed that the induction effect of hesperetin on UCP1 and p-CREB is attenuated after *Gnas* knocked down (Figure 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 (Figure 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. Hesperetin increased body temperature (Figure 7D) and BAT temperature (Figure 7E) in shNC mice, which were significantly blunted in sh*Adgra3* mice. Meanwhile, hesperetin decreased the area of adipocytes in iWAT (Figure 7F) and BAT (Figure 7G), and the effect disappeared in sh*Adgra3* mice. Moreover, the expression level of UCP1 were elevated in both iWAT (Figure 7H and 7J) and BAT (Figure 7I and 7K) after hesperetin treatment in shNC mice but not 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 7L). The ITT showed that hesperetin alleviated the insulin resistance of HFD mice which showed no significance in sh*Adgra3* mice (Figure 7M). Moreover, the fasting serum insulin level was reduced after hesperetin treatment (Figure 7N) and the HOMA-IR also showed a moderate improvement (Figure 7O), 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.

## 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 [23]. 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 [24, 25]. 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. Our findings suggest that ADGRA3, when overexpressed or stimulated by its known 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 [26]. Previous studies showed that hesperetin improved glycemic control [26, 27] and was involved in adipocyte differentiation

[28], but whether hesperetin induces the biogenesis of beige adipocyte was uncertain. 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 high expression level of ADGRA3 in adipose depots in both mice and humans, additional investigations are warranted to ascertain whether ADGRA3 plays a comparable role in humans. 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.

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**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; 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; GTT: glucose tolerance test; HFD: high-fat diet; 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; NCD: normal-chow diet; 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.

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## Figure and figure legends

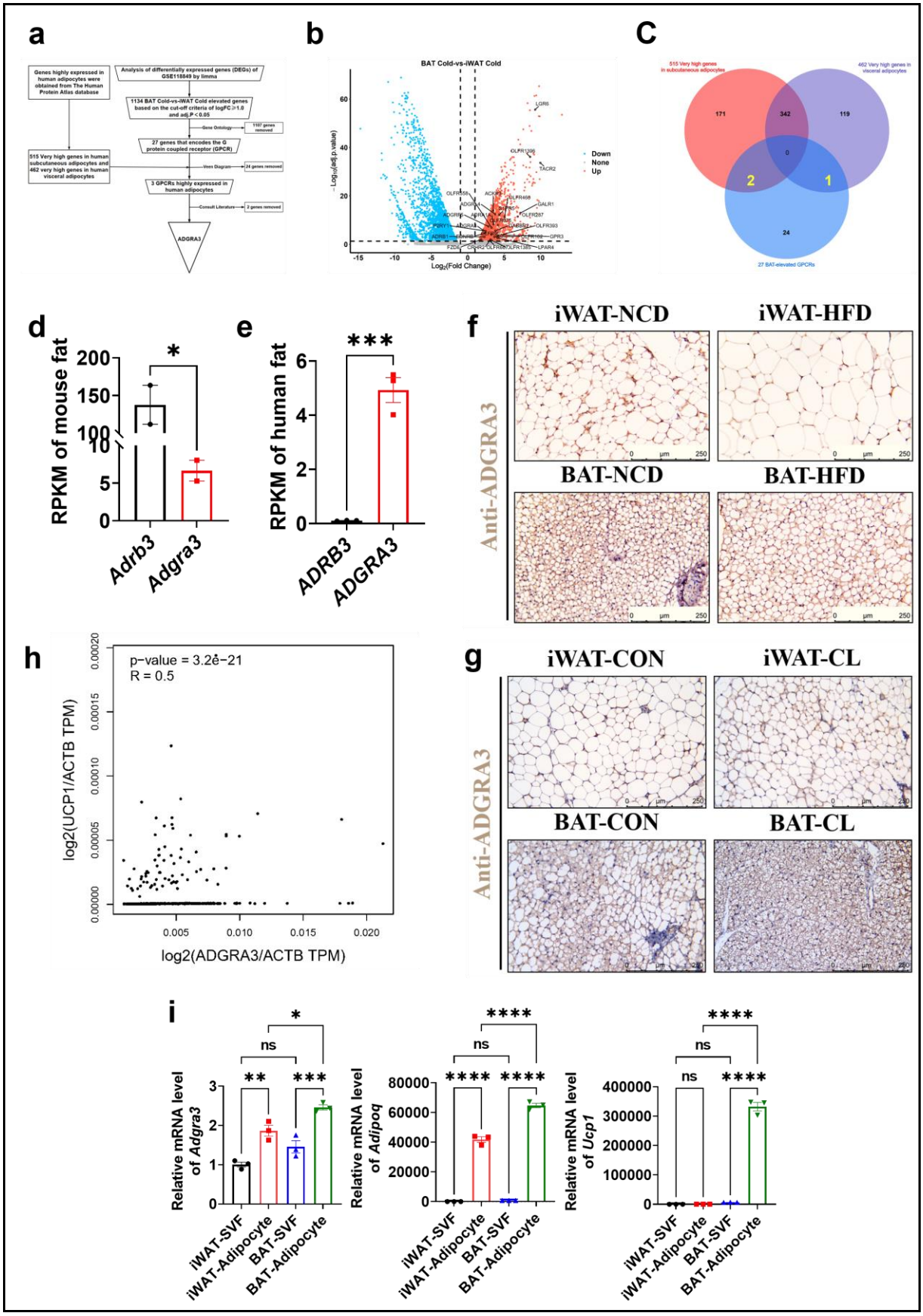
a
Genes highly expressed in human adipocytes were obtained from The Human Protein Atlas database
Analysis of differentially expressed genes (DEGs) of GSE118849 by limma
1134 BAT Cold-vs-iWAT Cold elevated genes based on the cut-off criteria of logFC ≥ 1.0 and adj.P < 0.05
Gene Ontology
1107 genes removed
27 genes that encodes the G protein coupled receptor (GPCR)
515 Very high genes in human subcutaneous adipocytes and 462 very high genes in human visceral adipocytes
Venn Diagram
24 genes removed
3 GPCRs highly expressed in human adipocytes
Consult Literature
2 genes removed
ADGRA3
b
BAT Cold-vs-iWAT Cold
Down
None
Up
Log2(Fold Change)
c
515 Very high genes in subcutaneous adipocytes
462 Very high genes in visceral adipocytes
27 BAT-elevated GPCRs
171
342
119
0
2
1
24
d
RPKM of mouse fat
Adrb3
Adgra3
e
RPKM of human fat
ADRB3
ADGRA3
f
iWAT-NCD
iWAT-HFD
BAT-NCD
BAT-HFD
Anti-ADGRA3
h
p-value = 3.2e−21
R = 0.5
log2(UCP1/ACTB TPM)
log2(ADGRA3/ACTB TPM)
g
iWAT-CON
iWAT-CL
BAT-CON
BAT-CL
Anti-ADGRA3
i
Relative mRNA level of Adgra3
Relative mRNA level of Adipoq
Relative mRNA level of Ucp1
iWAT-SVF
iWAT-Adipocyte
BAT-SVF
BAT-Adipocyte
ns
*
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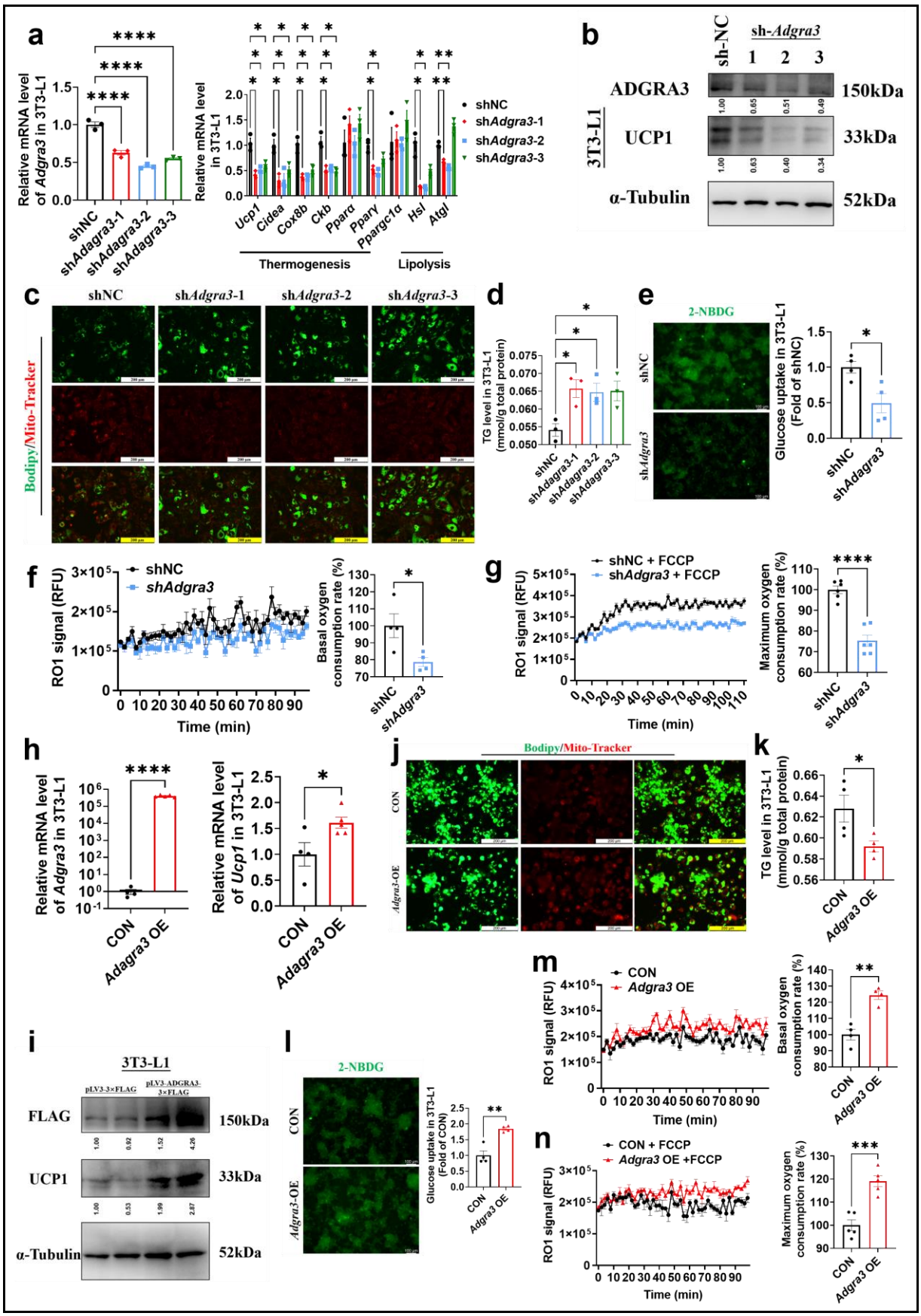
**Figure 1. ADGRA3 is a high-expressed GPCR in human adipocytes and mouse brown fat.** (A-F) ADGRA3 screening as a high-expressed GPCR in human adipocytes and mouse brown fat via comprehensive analysis. Brown adipose tissue and subcutaneous WAT were dissected from mice that were treated in cold (4℃) 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) and human fat (E) from HPA RNA-seq normal tissues (PRJEB4337). (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. (I) qPCR analysis of *Adgra3*, *Adipoq* and *Ucp1* genes in Stromal Vascular Fraction (SVF) and mature adipocyte isolated from iWAT and BAT. 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).


a
Relative mRNA level of Adgra3 in 3T3-L1
shNC
shAdagra3-1
shAdagra3-2
shAdagra3-3
Relative mRNA level in 3T3-L1
Ucp1
Cidea
Cox8b
Ckb
Pparα
Pparγ
Ppargc1α
Hsl
Atgl
Thermogenesis
Lipolysis
shNC
shAdgra3-1
shAdgra3-2
shAdgra3-3
b
sh-NC
sh-Adgra3
1
2
3
ADGRA3
150kDa
3T3-L1
UCP1
33kDa
α-Tubulin
52kDa
c
shNC
shAdgra3-1
shAdgra3-2
shAdgra3-3
Bodipy/Mito-Tracker
d
TG level in 3T3-L1 (mmol/g total protein)
shNC
shAdagra3-1
shAdagra3-2
shAdagra3-3
e
2-NBDG
shNC
shAdgra3
Glucose uptake in 3T3-L1 (Fold of shNC)
f
RO1 signal (RFU)
shNC
shAdgra3
Time (min)
Basal oxygen consumption rate (%)
g
shNC + FCCP
shAdgra3 + FCCP
Maximum oxygen consumption rate (%)
h
Relative mRNA level of Adgra3 in 3T3-L1
CON
Adagra3 OE
Relative mRNA level of Ucp1 in 3T3-L1
j
Bodipy/Mito-Tracker
CON
Adgra3-OE
k
TG level in 3T3-L1 (mmol/g total protein)
CON
Adgra3 OE
i
3T3-L1
pLV3-3×FLAG
pLV3-ADGRA3-3×FLAG
FLAG
150kDa
UCP1
33kDa
α-Tubulin
52kDa
l
2-NBDG
CON
Adgra3-OE
Glucose uptake in 3T3-L1 (Fold of CON)
m
CON
Adgra3 OE
RO1 signal (RFU)
Time (min)
Basal oxygen consumption rate (%)
n
CON + FCCP
Adgra3 OE +FCCP
RO1 signal (RFU)
Time (min)
Maximum oxygen consumption rate (%)

656

657

**Figure 2. *Adgra3* overexpression promotes the biogenesis of beige adipocytes.**

(A, H) qPCR analysis of *Adgra3*, thermogenesis and lipolysis genes in 3T3-L1 mature adipocytes. (B, I) Western blot analysis for level of ADGRA3, UCP1 and ADGRA3-3×FLAG protein in 3T3-L1 mature 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 adipocytes. Scale bars, 200 μm. (D, K) The level of intracellular triglyceride in 3T3-L1 mature adipocytes. (E, I) Glucose uptake assay in 3T3-L1 mature adipocytes and staining intensity analysis diagram (right). (F, M) When 3T3-L1 mature 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. (G, N) When FCCP-treaded 3T3-L1 mature 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. 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).

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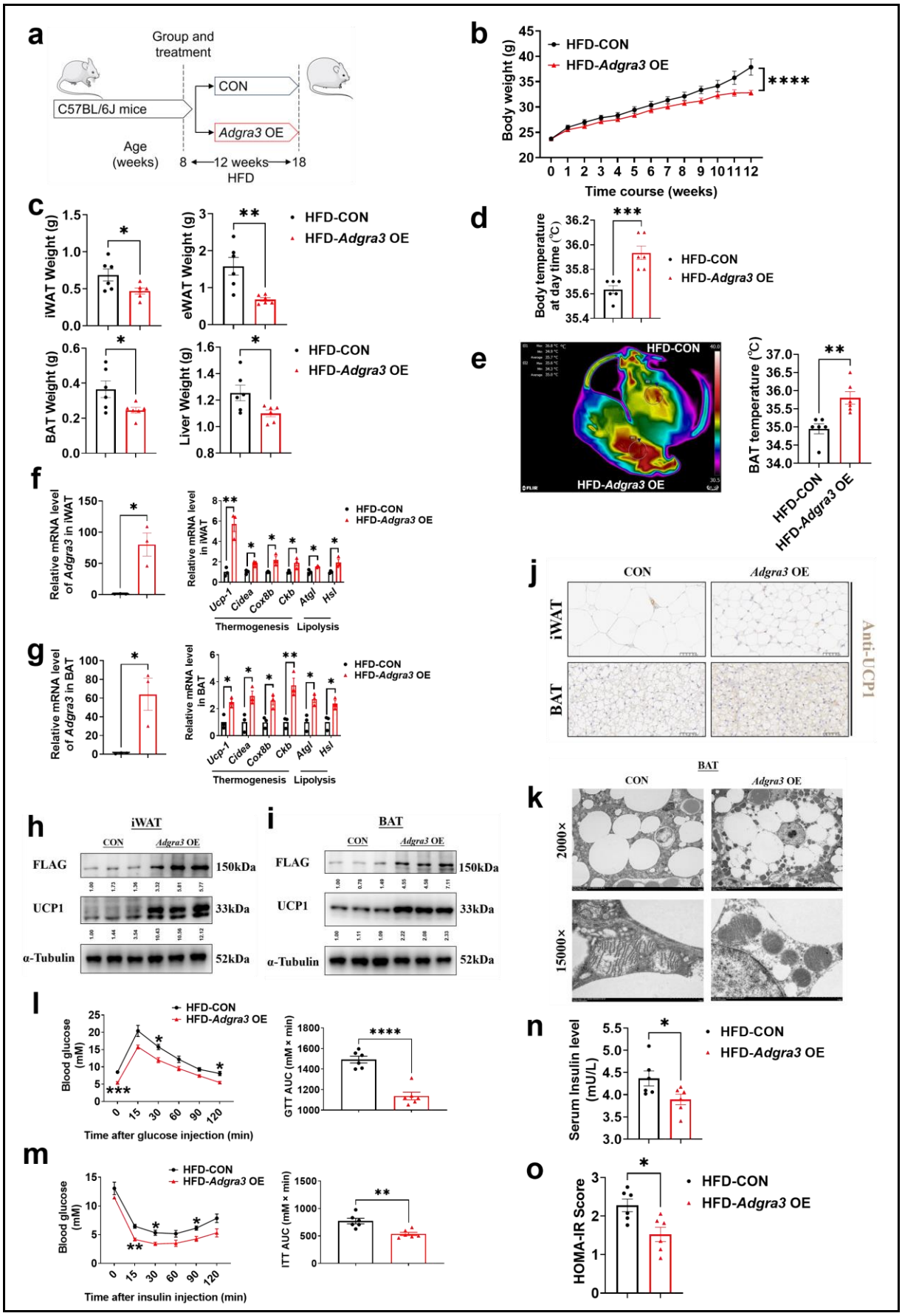
**Figure 3. Knockdown of *Adgra3* suppressed the adipose thermogenic program and impaired metabolic homeostasis in mice.** (A) Experimental schematic. C57BL/6J mice fed with a NCD for eight weeks (n=5 per treatment) were injected with sh*Adgra3* (pLKO.1-U6-sh*Adgra3*-2 plasmid encapsulated in nanomaterials) 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. (E) Thermal image and BAT temperature of mice injected with shNC or sh*Adgra3* for 28 days. (F-G) qPCR analysis of *Adgra3*, genes associated with thermogenesis and lipolysis in iWAT (F) and BAT (G) from different treatment mice. (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. (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-O) The fasting serum insulin (N) and HOMA-IR (O) in mice injected with either shNC or sh*Adgra3* for 28 days. 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-E, H-I and N-O) and two-way ANOVA (B and L-M).


a
Group and treatment
C57BL/6J mice
CON
Adgra3 OE
Age (weeks)
8
12 weeks
18
HFD
b
HFD-CON
HFD-Adgra3 OE
Body weight (g)
Time course (weeks)
****
c
iWAT Weight (g)
eWAT Weight (g)
BAT Weight (g)
Liver Weight (g)
HFD-CON
HFD-Adgra3 OE
d
Body temperature at day time (℃)
HFD-CON
HFD-Adgra3 OE
e
HFD-CON
HFD-Adgra3 OE
BAT temperature (℃)
f
Relative mRNA level of Adgra3 in iWAT
Relative mRNA level in iWAT
Ucp-1
Cidea
Cox8b
Ckb
Atgl
Hsl
Thermogenesis
Lipolysis
g
Relative mRNA level of Adgra3 in BAT
Relative mRNA level in BAT
h
iWAT
CON
Adgra3 OE
FLAG
UCP1
α-Tubulin
150kDa
33kDa
52kDa
i
BAT
j
CON
Adgra3 OE
iWAT
BAT
Anti-UCP1
k
BAT
2000×
15000×
l
Blood glucose (mM)
Time after glucose injection (min)
GTT AUC (mM × min)
m
Time after insulin injection (min)
ITT AUC (mM × min)
n
Serum Insulin level (mU/L)
o
HOMA-IR Score

703

704

**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 (n=6 per treatment) 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. (E) Thermal image and BAT temperature in mice injected with CON or *Adgra3* OE. (F-G) qPCR analysis of *Adgra3*, genes associated with thermogenesis and lipolysis in iWAT (F) and BAT (G) from different treatment mice. (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. (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-O) The fasting serum insulin (N) and HOMA-IR (O) in mice injected with CON or *Adgra3* OE. 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).

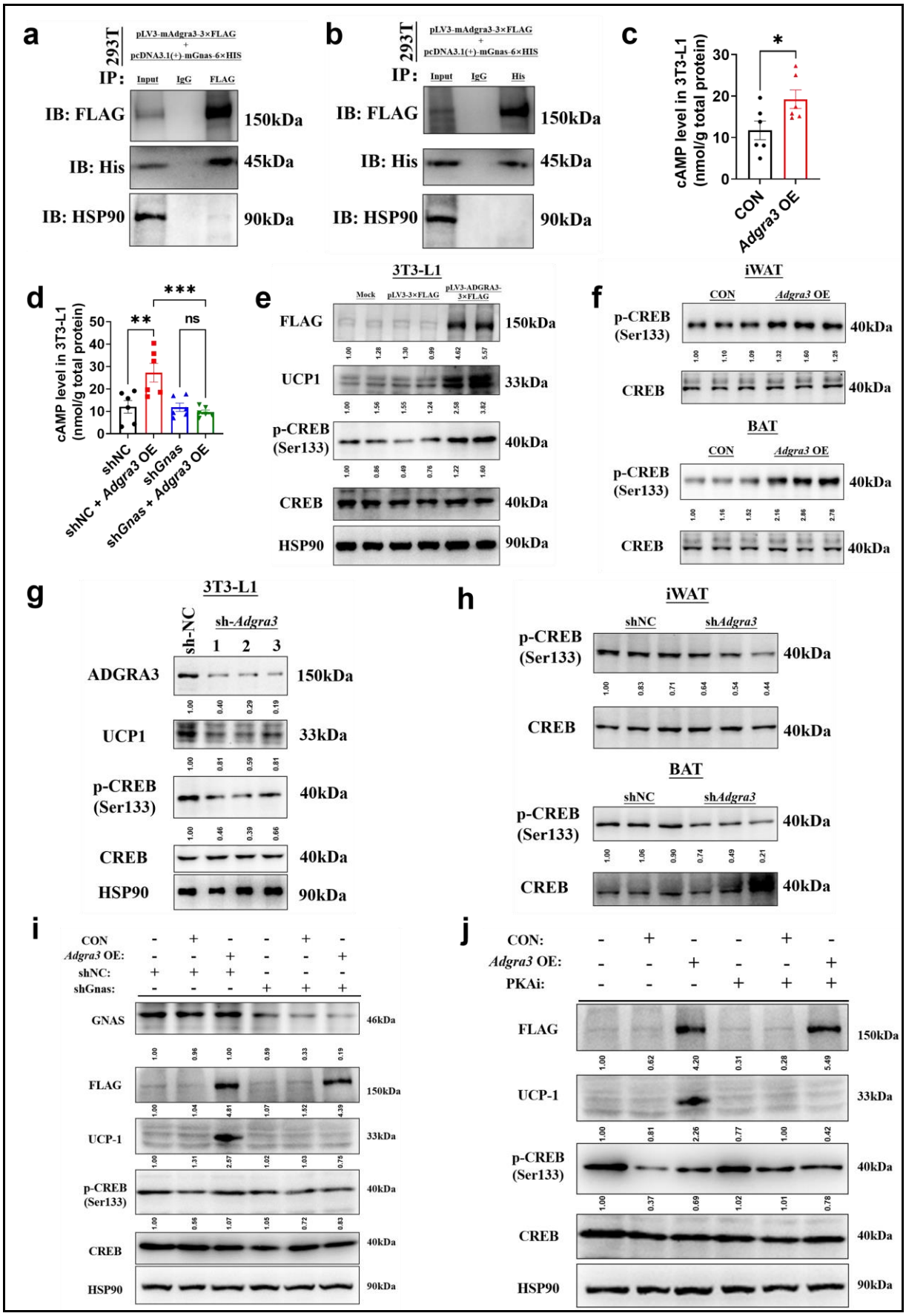
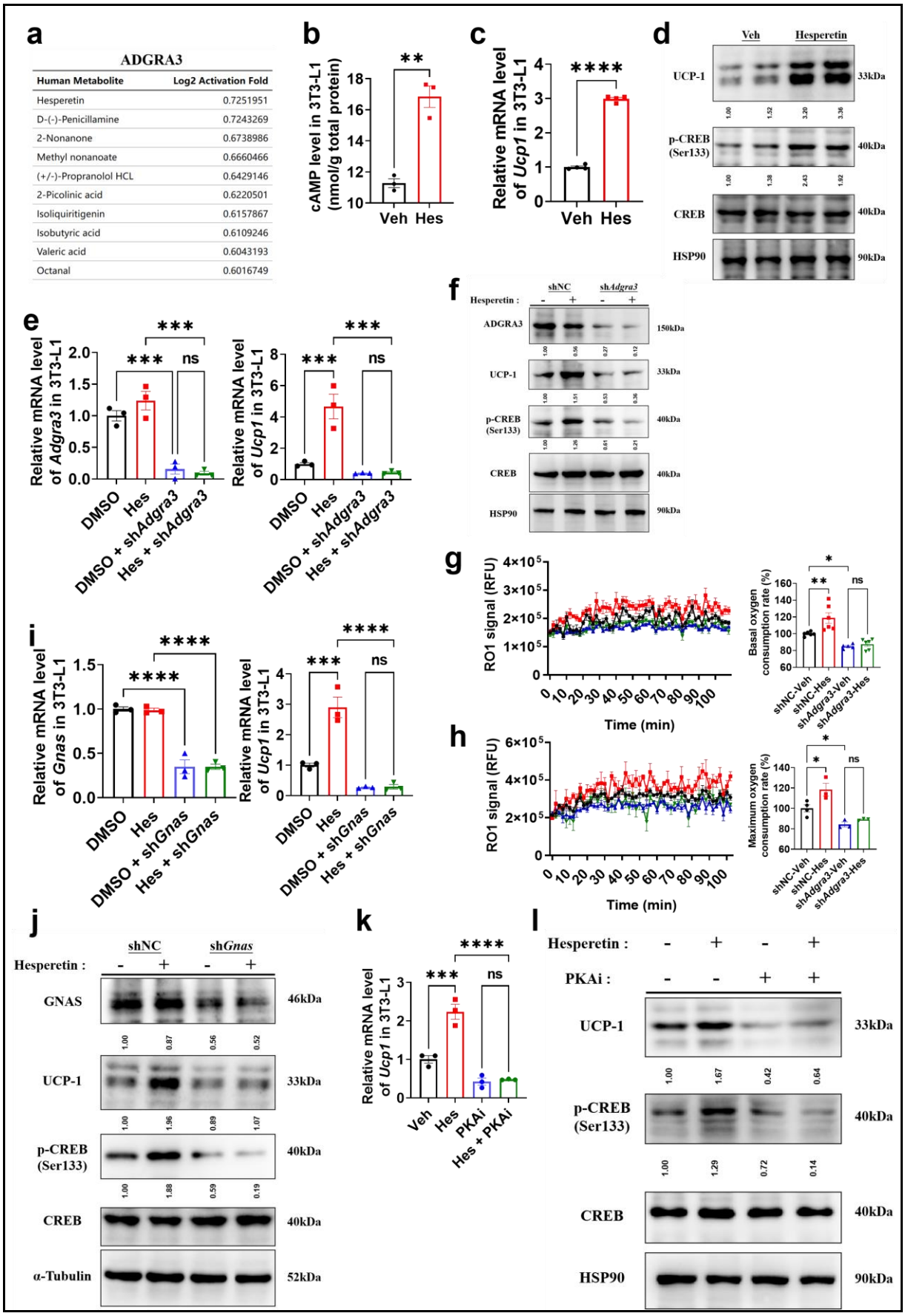

a
293T
pLV3-mAdgra3-3×FLAG
+
pcDNA3.1(+)-mGnas-6×HIS
IP:
Input
IgG
FLAG
IB: FLAG
150kDa
IB: His
45kDa
IB: HSP90
90kDa
b
293T
pLV3-mAdgra3-3×FLAG
+
pcDNA3.1(+)-mGnas-6×HIS
IP:
Input
IgG
His
IB: FLAG
150kDa
IB: His
45kDa
IB: HSP90
90kDa
c
cAMP level in 3T3-L1 (nmol/g total protein)
CON
Adgra3 OE
d
cAMP level in 3T3-L1 (nmol/g total protein)
shNC
shNC + Adgra3 OE
shGnas
shGnas + Adgra3 OE
ns
e
3T3-L1
Mock
pLV3-3×FLAG
pLV3-ADGRA3-3×FLAG
FLAG
150kDa
UCP1
33kDa
p-CREB (Ser133)
40kDa
CREB
40kDa
HSP90
90kDa
f
iWAT
CON
Adgra3 OE
p-CREB (Ser133)
40kDa
CREB
40kDa
BAT
CON
Adgra3 OE
p-CREB (Ser133)
40kDa
CREB
40kDa
g
3T3-L1
sh-NC
sh-Adgra3
1
2
3
ADGRA3
150kDa
UCP1
33kDa
p-CREB (Ser133)
40kDa
CREB
40kDa
HSP90
90kDa
h
iWAT
shNC
shAdgra3
p-CREB (Ser133)
40kDa
CREB
40kDa
BAT
shNC
shAdgra3
p-CREB (Ser133)
40kDa
CREB
40kDa
i
CON
Adgra3 OE:
shNC:
shGnas:
GNAS
46kDa
FLAG
150kDa
UCP-1
33kDa
p-CREB (Ser133)
40kDa
CREB
40kDa
HSP90
90kDa
j
CON:
Adgra3 OE:
PKAi:
FLAG
150kDa
UCP-1
33kDa
p-CREB (Ser133)
40kDa
CREB
40kDa
HSP90
90kDa

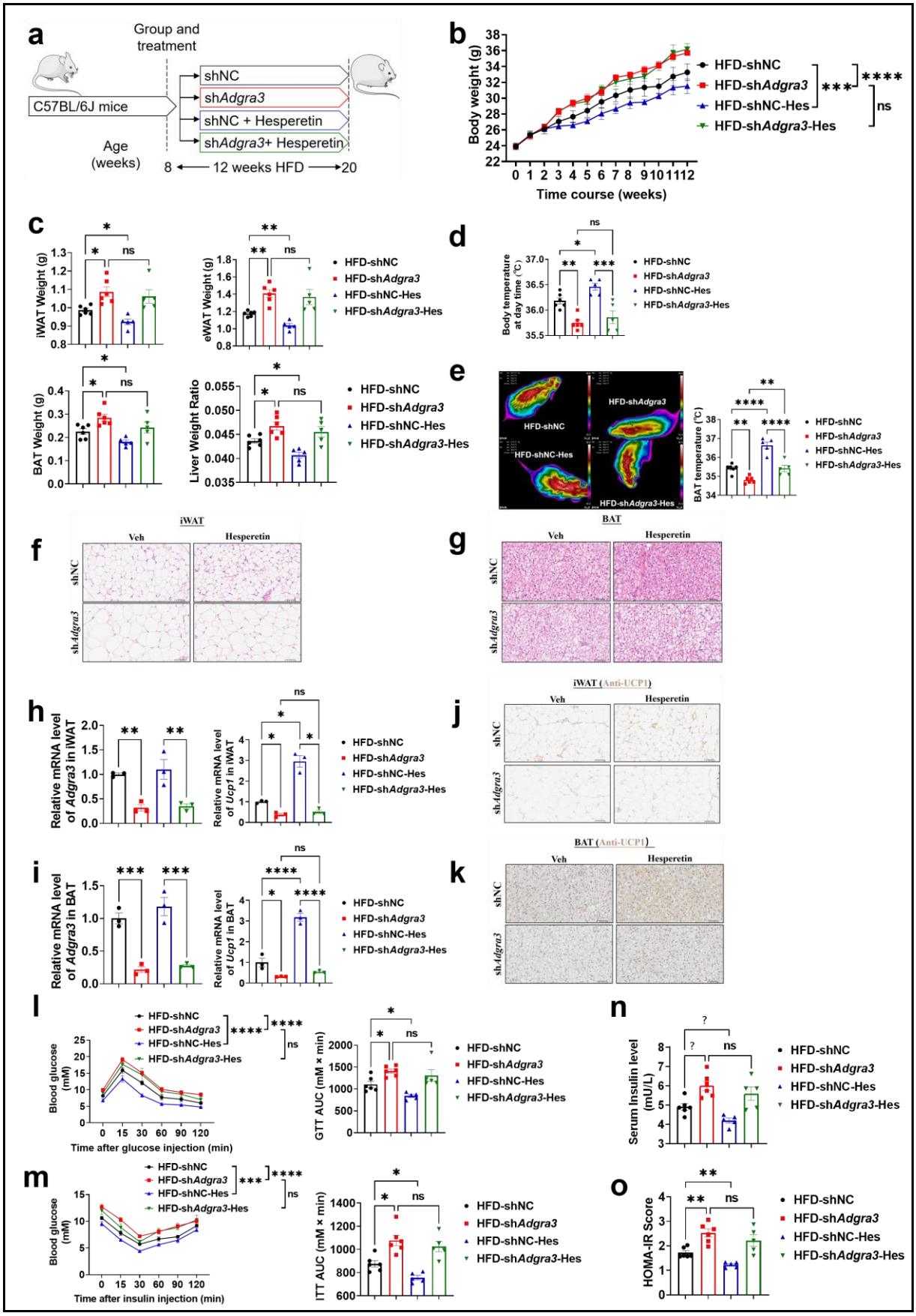
**Figure 5. ADGRA3 promotes the biogenesis of beige adipocytes via the Gs-PKA-CREB axis.** (A-B) Western-blot analysis for level of ADGRA3-3×FLAG, GNAS-6×HIS and HSP90 proteins in 293T transfected with different plasmids. (C-D) The level of cAMP in 3T3-L1. An ELISA kit was used to measure the level of cAMP. (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 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).


a
ADGRA3
Human Metabolite | Log2 Activation Fold
Hesperetin 0.7251951
D-(-)-Penicillamine 0.7243269
2-Nonanone 0.6738986
Methyl nonanoate 0.6660466
(+/-)-Propranolol HCL 0.6429146
2-Picolinic acid 0.6220501
Isoliquiritigenin 0.6157867
Isobutyric acid 0.6109246
Valeric acid 0.6043193
Octanal 0.6016749
b
cAMP level in 3T3-L1 (nmol/g total protein)
Veh Hes
c
Relative mRNA level of Ucp1 in 3T3-L1
Veh Hes
d
Veh Hesperetin
UCP-1 33kDa
p-CREB (Ser133) 40kDa
CREB 40kDa
HSP90 90kDa
e
Relative mRNA level of Adgra3 in 3T3-L1
Relative mRNA level of Ucp1 in 3T3-L1
DMSO Hes DMSO + shAdgra3 Hes + shAdgra3
f
shNC shAdgra3
Hesperetin :
ADGRA3 150kDa
UCP-1 33kDa
p-CREB (Ser133) 40kDa
CREB 40kDa
HSP90 90kDa
g
RO1 signal (RFU)
Time (min)
Basal oxygen consumption rate (%)
shNC-Veh shNC-Hes shAdgra3-Veh shAdgra3-Hes
h
RO1 signal (RFU)
Time (min)
Maximum oxygen consumption rate (%)
shNC-Veh shNC-Hes shAdgra3-Veh shAdgra3-Hes
i
Relative mRNA level of Gnas in 3T3-L1
Relative mRNA level of Ucp1 in 3T3-L1
DMSO Hes DMSO + shGnas Hes + shGnas
j
shNC shGnas
Hesperetin :
GNAS 46kDa
UCP-1 33kDa
p-CREB (Ser133) 40kDa
CREB 40kDa
α-Tubulin 52kDa
k
Relative mRNA level of Ucp1 in 3T3-L1
Veh Hes PKAi Hes + PKAi
l
Hesperetin :
PKAi :
UCP-1 33kDa
p-CREB (Ser133) 40kDa
CREB 40kDa
HSP90 90kDa

741

742

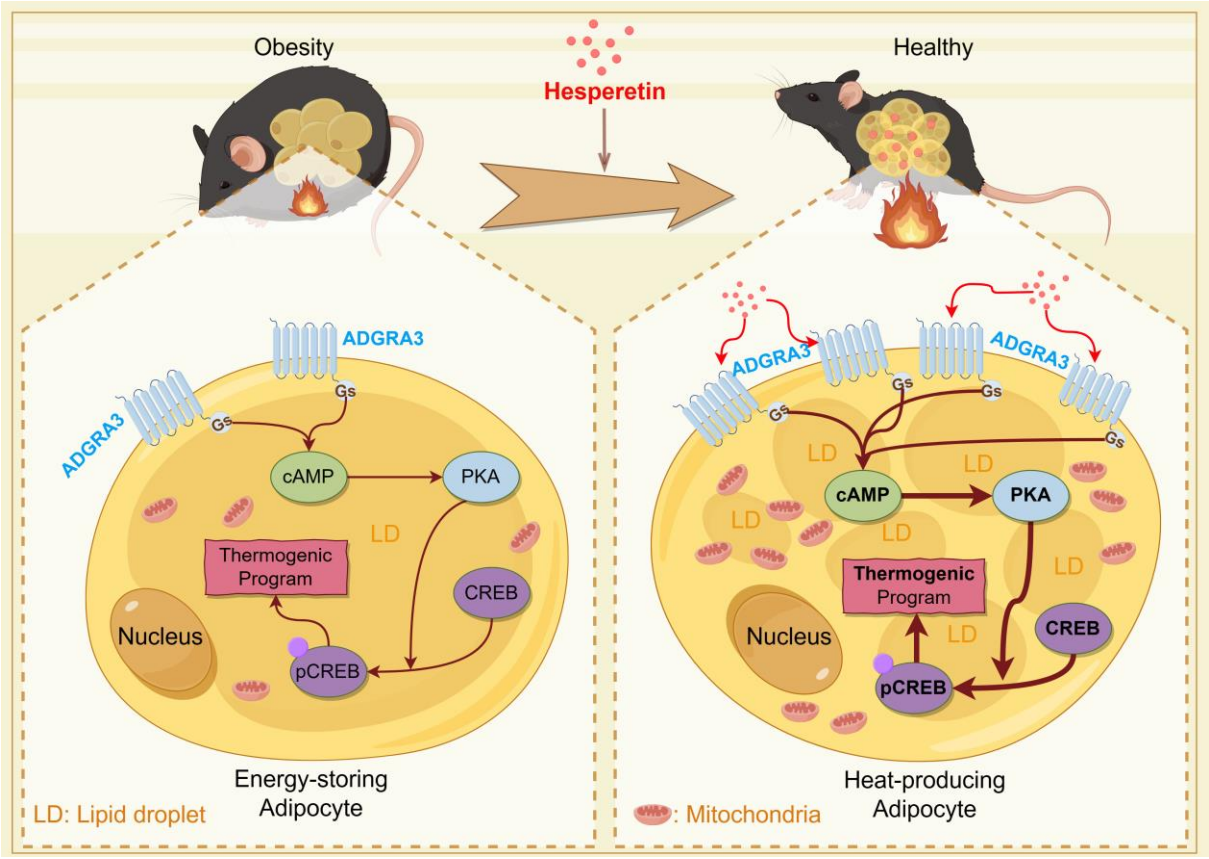
**Figure 6. Hesperetin promotes the biogenesis of beige adipocytes via ADGRA3-$G_s$-PKA-CREB axis.** (A) Table of human metabolites with the ability to activate ADGRA3, from the PRESTO-Salsa database. (B) The level of cAMP in 3T3-L1. ELISA kit was used to measure the level of cAMP. (C, E, I and K) qPCR analysis of *Adgra3*, *Gnas* and *Ucp1* in 3T3-L1 mature adipocytes. (D, F, J and L) Western-blot analysis for level of ADGRA3, GNAS, UCP1, p-CREB and CREB protein in 3T3-L1 mature adipocytes. (G) When 3T3-L1 mature 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. (H) When FCCP-treaded 3T3-L1 mature 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. 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 G-H) and one-way ANOVA (E, I and K).


a
Group and treatment
C57BL/6J mice
shNC
shAdgra3
shNC + Hesperetin
shAdgra3+ Hesperetin
Age (weeks)
8
12 weeks HFD
20
b
Body weight (g)
Time course (weeks)
HFD-shNC
HFD-shAdgra3
HFD-shNC-Hes
HFD-shAdgra3-Hes
c
iWAT Weight (g)
eWAT Weight (g)
BAT Weight (g)
Liver Weight Ratio
d
Body temperature at day time (℃)
e
HFD-shNC
HFD-shAdgra3
HFD-shNC-Hes
HFD-shAdgra3-Hes
BAT temperature (℃)
f
iWAT
Veh
Hesperetin
shNC
shAdgra3
g
BAT
h
Relative mRNA level of Adgra3 in iWAT
Relative mRNA level of Ucp1 in iWAT
j
iWAT (Anti-UCP1)
i
Relative mRNA level of Adgra3 in BAT
Relative mRNA level of Ucp1 in BAT
k
BAT (Anti-UCP1)
l
Blood glucose (mM)
Time after glucose injection (min)
GTT AUC (mM × min)
n
Serum Insulin level (mU/L)
m
Time after insulin injection (min)
ITT AUC (mM × min)
o
HOMA-IR Score

757

758

**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. (E) Thermal image and BAT temperature of different treated mice. (F-G) Representative images of iWAT (F) and BAT (G) stained with HE. Scale bars, 50 μm. (H-I) qPCR analysis of *Adgra3* and *Ucp1* in iWAT (H) and BAT (I) from different treated mice. (J-K) Representative images of iWAT (J; Scale bars, 50 μm.) and BAT (K; Scale bars, 50 μm.) stained with UCP1. (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. (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-O) The fasting serum insulin (N) and HOMA-IR (O) in different treated mice. 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-E, H-I, N and O) and two-way ANOVA (B and L-M).




**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).

**Supplementary materials and methods**

| 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 |
| *β-Actin* | TGTCCACCTTCCAGCAGATGT | AGCTCAGTAACAGTCCGCCTAGA |
| *Hexo* | GCCAGCCTCTCCTGATTTTAGTGT | GGGAACACAAAAGACCTCTTCTGG |
| *mito-16s* | CCGCAAGGGAAAGATGAAAGAC | TCGTTTGGTTTCGGGGTTTC |

**Table S1. Primer sequences used for qPCR.**

| 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 |
| 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. 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.

## Supplementary figure

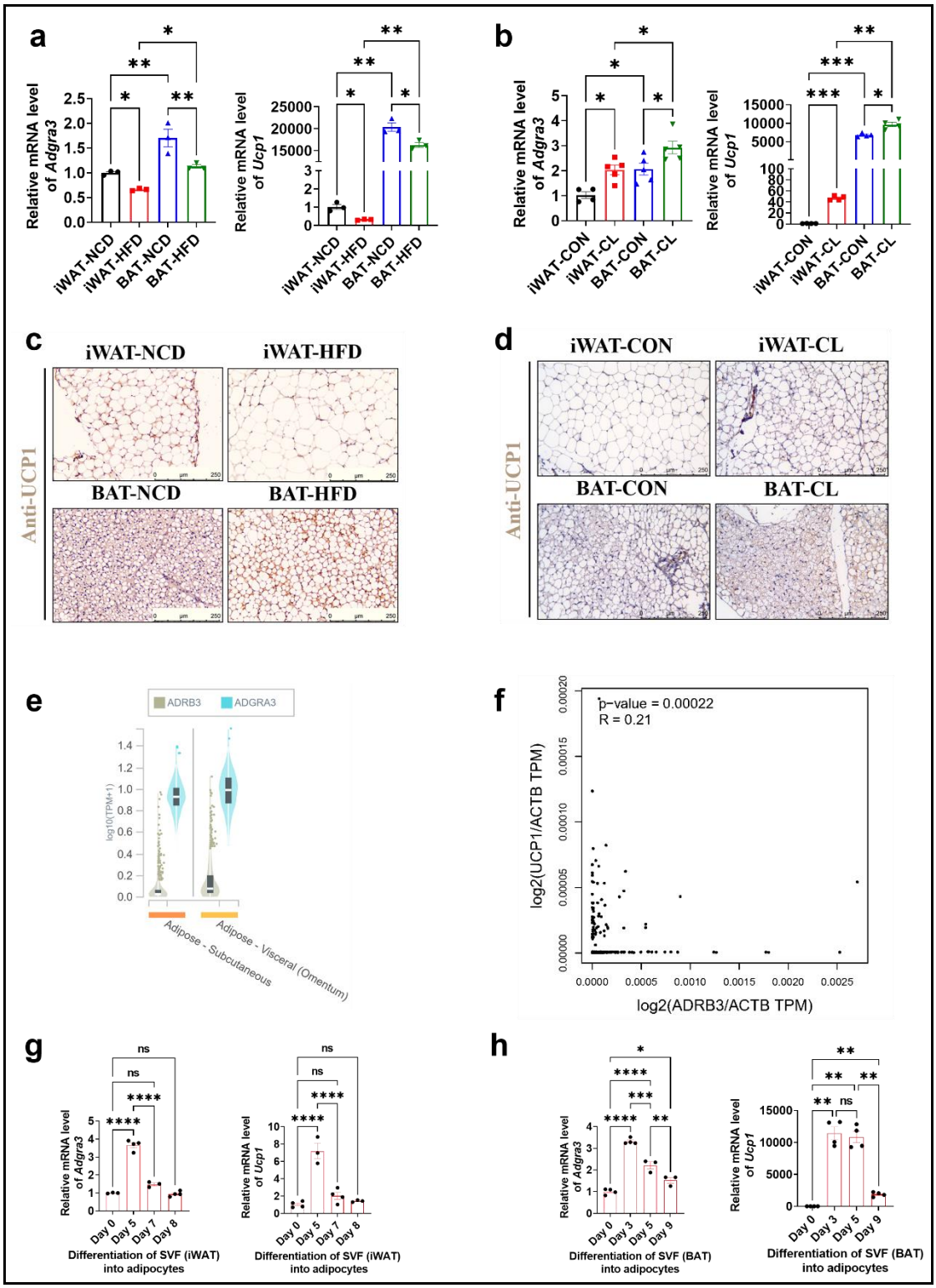



**Figure S1. ADGRA3 positively correlated with beige fat.** (A-B) qPCR analysis of *Adgra3* and *Ucp1* in iWAT and BAT from different treatment mice. (C) C57BL/6J mice fed with a NCD or a HFD for 12 weeks. Representative images of iWAT and BAT stained with UCP1.

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 *ADRB3* and *ADGRA3* genes in human fat 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. (G-H) qPCR analysis of *Adgra3* and *Ucp1* during the differentiation of SVF (iWAT (G) or BAT (H)) into adipocytes. iWAT, inguinal white adipose tissue; BAT, brown adipose tissue; NCD, normal chow diet; HFD, high-fat diet; CL: CL-316,243; qPCR, quantitative real-time PCR. SVF, Stromal Vascular Fraction. All data are presented as mean ± *SEM*. Statistical significance was determined by one-way ANOVA (A-B and G-H) and simple linear regression (F).

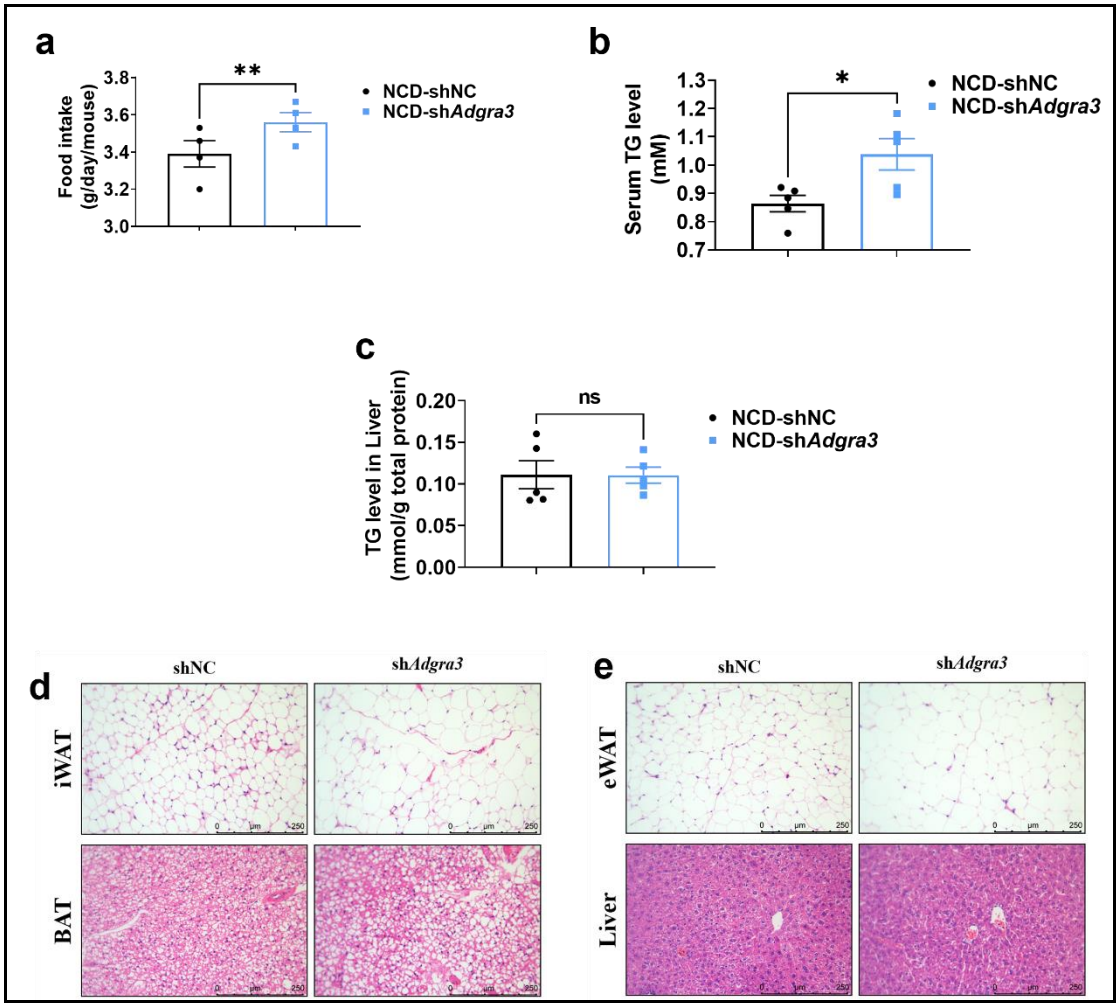



**Figure S2. Characterization of wild-type and *Adgra3*-knockdown mice.** (A-E) C57BL/6J mice fed with a NCD for eight weeks (n=5 per treatment) were injected with sh*Adgra3* (pLKO.1-U6-sh*Adgra3*-2 plasmid encapsulated in nanomaterials) or shNC (pLKO.1-U6-shNC plasmid encapsulated in nanomaterials) twice a week for four weeks. (A) Food intake of different treated mice. (B-C) The TG level of serum(B) and liver (C) from different treated mice. (D) Representative images of iWAT (top) and BAT (bottom) stained with hematoxylin and eosin. Scale bars, 250 μm. (E) Representative images of eWAT (top) and Liver (bottom) stained with hematoxylin and eosin. Scale bars, 250 μm. 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. 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-C).

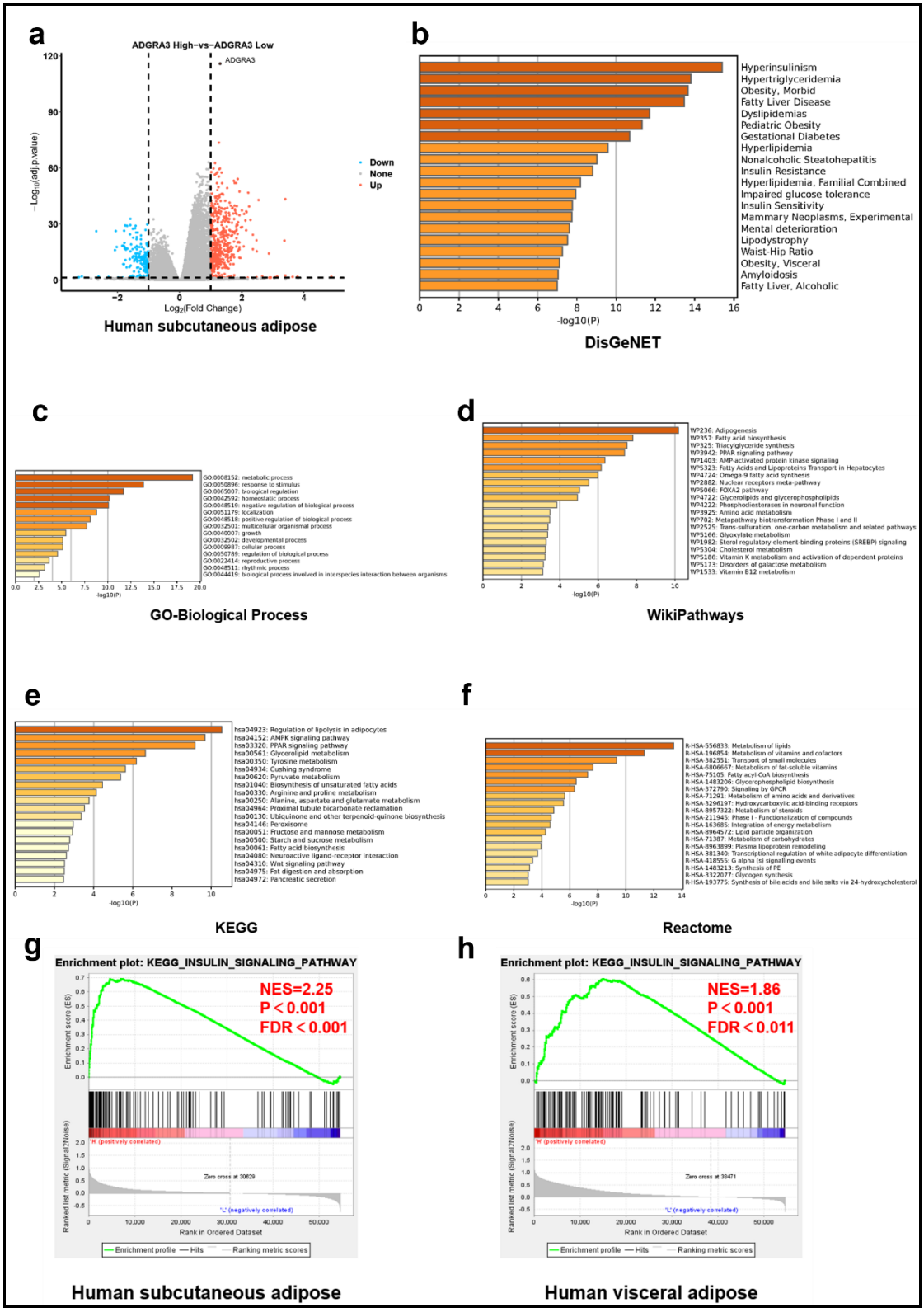



**Figure S3. *ADGRA3* high expressed gene sets in human subcutaneous fat are enriched to lipid metabolism and adipocyte differentiation.** (A) Volcano plot summarizing the

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.

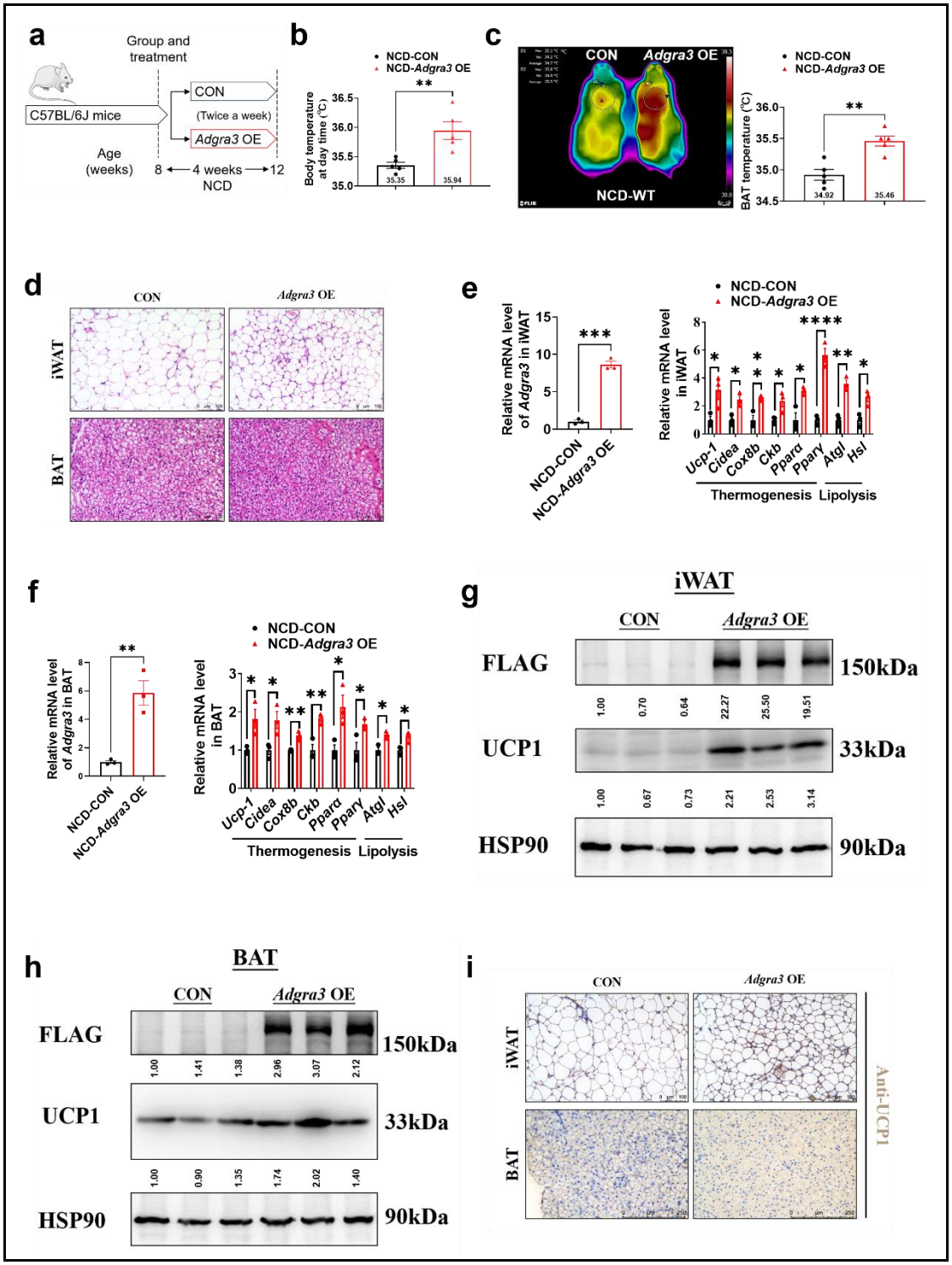



**Figure S4. *Adgra3* overexpression activated the adipose thermogenic program in mice.** (A) Experimental schematic. C57BL/6J mice fed with a NCD for eight weeks (n=5 per treatment) were injected with *Adgra3* OE (pLV3-CMV-*Adgra3*(mouse)-3×FLAG plasmid

encapsulated in nanomaterials) or CON (pLV3-CMV-MCS-3×FLAG plasmid encapsulated in nanomaterials) twice a week for four weeks. (B) Body temperature of mice injected with CON or *Adgra3* OE for 28 days. (C) Thermal image and BAT temperature in mice injected with CON or *Adgra3* OE for 28 days. (D) Representative images of iWAT (top) and BAT (bottom) stained with HE. Scale bars, 100 μm. (E-F) qPCR analysis of *Adgra3*, genes associated with thermogenesis and lipolysis in iWAT (E) and BAT (F) from different treatment mice. (G-H) Western-blot analysis for the level of ADGRA3-3×FLAG and UCP1 protein in iWAT (G) and BAT (H) from differently treated mice. The ImageJ software was used for gray scanning. (I) 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. All data are presented as mean ± *SEM*. Statistical significance was determined by unpaired two-tailed student's t-test (B-C and E-F).

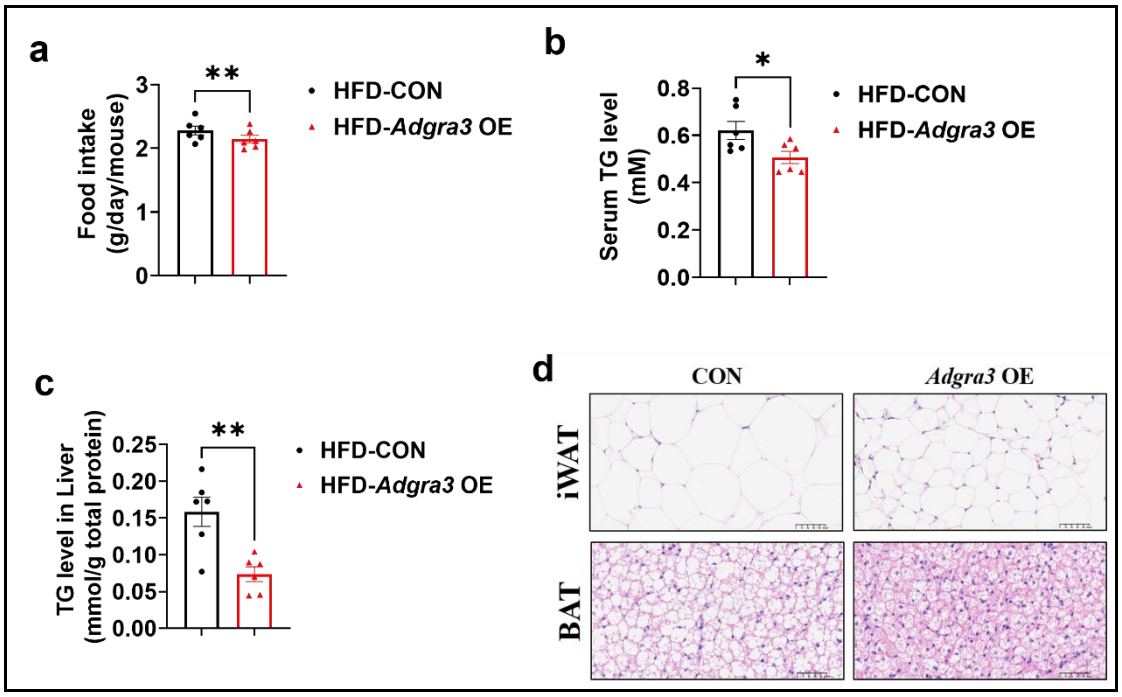



**Figure S5. Characterization of wild-type and Adgra3-overexpressed mice with diet-induced obesity (DIO).** (A-D) C57BL/6J mice (n=6 per treatment) were fed with a HFD and injected with Adgra3 OE (pLV3-CMV-Adgra3 (mouse)-3×FLAG plasmid encapsulated in nanomaterials) or CON (pLV3-CMV-MCS-3×FLAG plasmid encapsulated in nanomaterials) once a week for 12 weeks. (A) Food intake of different treated mice. (B-C) The TG level of serum(B) and liver (C) from different treated mice. (D) Representative images of iWAT (top) and BAT (bottom) stained with hematoxylin and eosin. Scale bars, 50 μm. HFD, high-fat diet; iWAT, inguinal white adipose tissue; BAT, brown adipose tissue. 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-C).

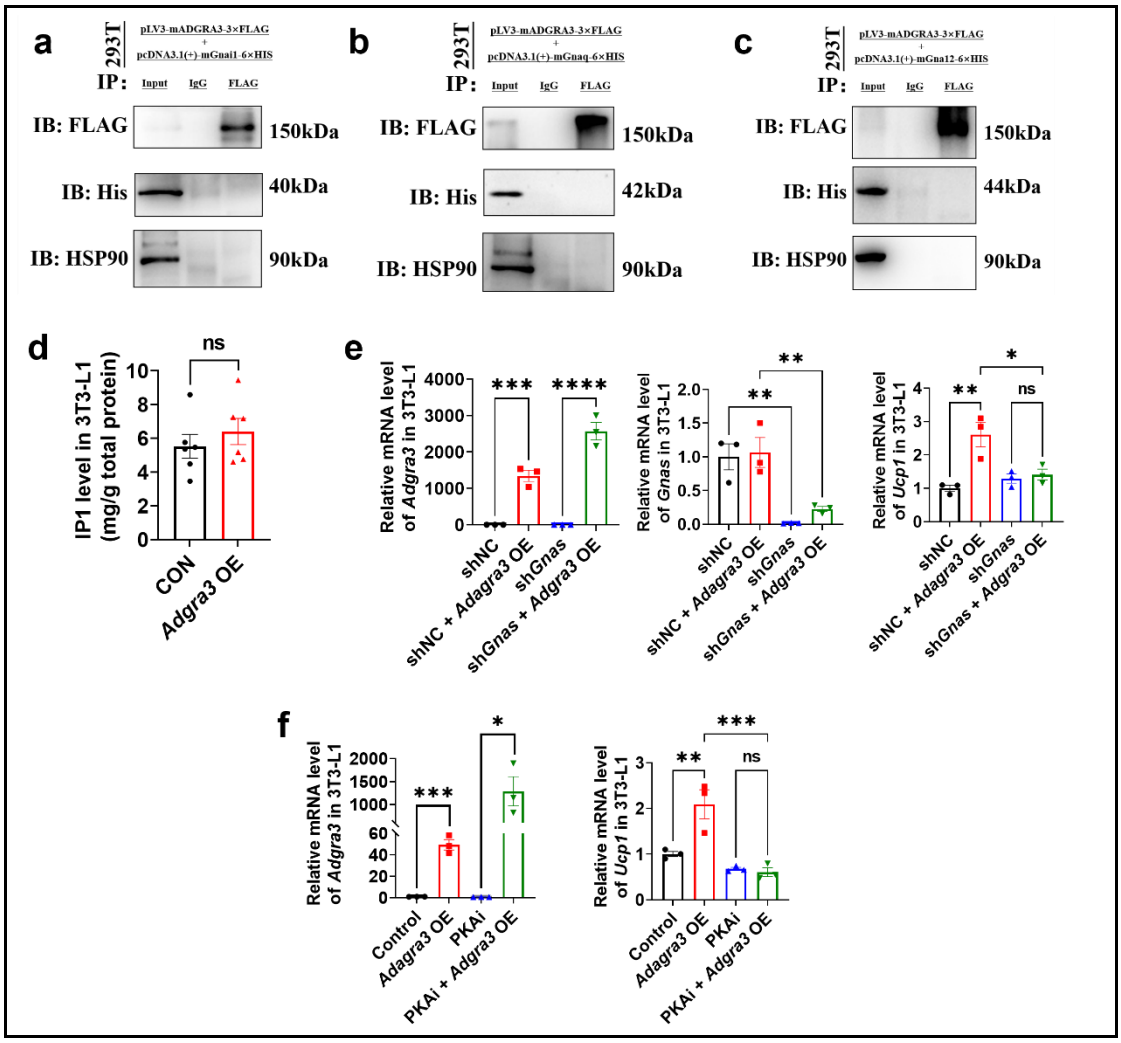



**Figure S6. ADGRA3 was not observed to bind to $G_i$, $G_q$ and $G_{12}$.** (A-C) Western-blot analysis for level of ADGRA3-3×FLAG, GNAI1-6×HIS (A), GNAQ-6×HIS (B), GNA12-6×HIS (C) and HSP90 proteins in 293T transfected with different plasmids. (D) The level of IP1 in 3T3-L1. An ELISA kits was used to measure the level of IP1. (E-F) qPCR analysis of *Gnas*, *Adgra3* and *Ucp1* in 3T3-L1 mature adipocytes. 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).