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CORRESPONDENCE

In Situ Saturating Mutagenesis Screening Identifies a Functional Genomic Locus that Regulates *Ucp1* Expression

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Abstract

A better understanding of the molecular mechanisms that control the UCP1 expression in brown and beige adipocytes is essential for us to modulate adipose cell fate and promote thermogenesis, which may provide a therapeutic view for the treatment of obesity and obesity-related diseases. To systematically identify *cis*-element(s) that transcriptionally regulates *Ucp1,* we here took advantage of the high-throughput CRIPSR-Cas9 screening system, and performed an *in situ* saturating mutagenesis screen, by using a customized sgRNA library targeting the ~ 20 kb genomic region near *Ucp1*. Through the screening, we have identified several genomic loci that may contain key regulatory element for *Ucp1* expression in cultured brown and white adipocytes *in vitro*, and in inguinal white adipose tissue *in vivo*. Our study highlights a broadly useful approach for studying *cis*-regulatory elements in a high-throughput manner.

Keywords *In situ* saturating mutagenesis screen · Brown and beige adipocytes · UCP1 · sgRNA-9768

Uncoupling protein 1 (UCP1) is a mitochondrial protein, which allows electrons to be released and uncouples oxidative respiration from ATP synthesis, resulting in heat generation (Harms and Seale 2013). UCP1 expression is largely controlled at the transcriptional level, presenting a molecular hallmark of thermogenic adipocytes (Ricquier 2011). Better dissection of the regulatory elements for UCP1 transcription will certainly help to understand the regulation of thermogenesis in adipocytes, and develop treatments to combat obesity. Although the available information on the regulation of UCP1 transcription is rather extensive (Montanari et al. 2017; Bonet and Oliver 1831; Collins et al. 2010; Inagaki et al. 2016), to our knowledge, a systematic genetic screen for cis-element(s) that transcriptionally regulates UCP1 expression has not been performed. Here, we took advantage of the CRISPR technology, in combination with our previously established *Ucp1-GFP* reporter system (Qiu et al. 2018), and performed an *in situ* saturating mutagenesis screening, with the aim to identify potential DNA regulatory elements for UCP1 regulation.

Recent advances in genome engineering technologies allow *in situ* saturating mutagenesis studies for potential regulatory elements of a specific genomic region in a high-throughput manner (Canver et al. 2015). To elucidate potential DNA regulatory element(s) involved in *Ucp1* transcription, we customized an sgRNA library spreading the ~ 20 kb genomic region, covering the 5′ region of the *Ucp1* gene as well as all the introns (Fig. 1a). The ~ 20 kb genomic region was intensive for epigenetic signals of enhancers (H3K4me and H3K27ac) or promoters (H3K4me3), according to the ChIP-seq results carried out in brown adipose tissues (ENCODE data) (Fig. 1a). We then performed screenings with this customized sgRNA library using our established screening platform, which was generated with the immortalized *Ucp1-GFP* primary mouse brown preadipocytes, with GFP signal reflecting the endogenous UCP1 protein level (Qiu et al. 2018; Li et al. 2018) (Fig. 1b). *Ucp1-GFP* brown preadipocytes ($UCP1_{pre}$) were transduced with the lentiCRISPR library and differentiated following a standard protocol. Mature brown adipocytes ($UCP1_{all}$) were then sorted to obtain high GFP + (top 5%) ($UCP1_{pos}$) and GFP- (bottom 5%) ($UCP1_{neg}$) populations. Two other cell groups, preadipocytes $UCP1_{pre}$ and presorted $UCP1_{all}$ cells, were

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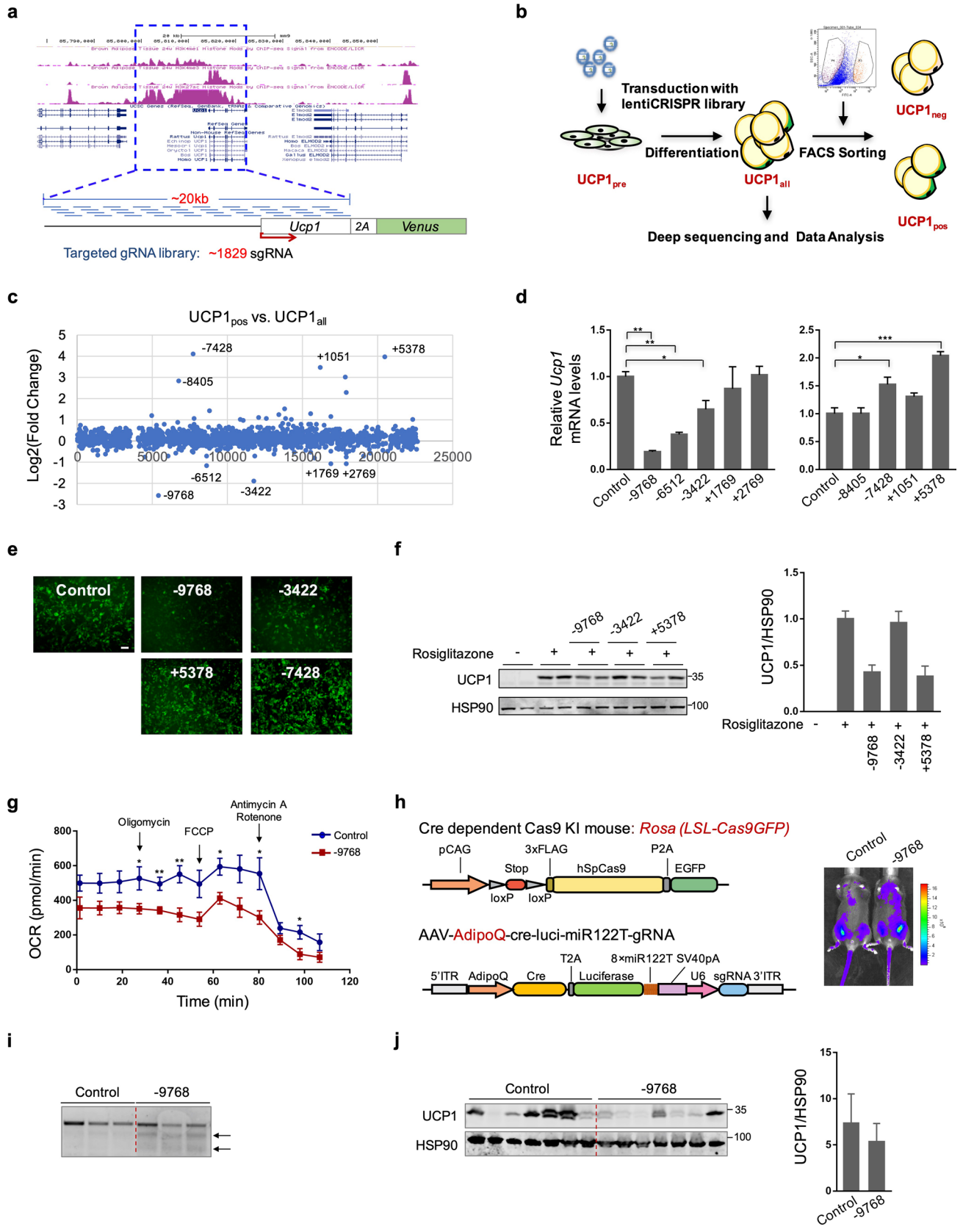
a
~20kb
Ucp1
2A
Venus
Targeted gRNA library: ~1829 sgRNA
b
Transduction with lentiCRISPR library
Differentiation
FACS Sorting
UCP1pre
UCP1all
UCP1neg
UCP1pos
Deep sequencing and Data Analysis
c
UCP1pos vs. UCP1all
Log2(Fold Change)
-7428
-8405
+1051
+5378
-6512
-9768
-3422
+1769 +2769
d
Relative Ucp1 mRNA levels
Control
-9768
-6512
-3422
+1769
+2769
-8405
-7428
+1051
+5378
e
Control
-9768
-3422
+5378
-7428
f
Rosiglitazone
UCP1
HSP90
-9768
-3422
+5378
UCP1/HSP90
g
Oligomycin
FCCP
Antimycin A
Rotenone
Control
-9768
OCR (pmol/min)
Time (min)
h
Cre dependent Cas9 KI mouse: Rosa (LSL-Cas9GFP)
pCAG
3xFLAG
Stop
hSpCas9
P2A
EGFP
loxP
loxP
AAV-AdipoQ-cre-luci-miR122T-gRNA
5'ITR
AdipoQ
Cre
T2A
Luciferase
8×miR122T
SV40pA
U6
sgRNA
3'ITR
Control
-9768
i
Control
-9768
j
Control
-9768
UCP1
HSP90
UCP1/HSP90

◂**Fig. 1** *In situ* saturating mutagenesis screening identifies a functional genomic locus that regulates *Ucp1* expression. **a** Schematic view of *Ucp1* sgRNA library design for *in situ* cis-element screen with the *Ucp1-GFP* reporter cell line. **b** Workflow of the screening strategy. **c** Scatterplot displaying enriched or depleted sgRNAs in $UCP1_{pos}$ group as compared to $UCP1_{all}$ group. TSS, transcriptional start site. **d** *Ucp1* mRNA expression analyses in brown adipocytes treated with individual sgRNAs as indicated. $n=4$. **e** Representative images of GFP intensity in brown adipocytes treated with individual sgRNAs. Scale bar = 50 μm. **f** UCP1 protein expression analyses in white adipocytes under basal level or upon rosiglitazone stimulation, treated with or without indicated sgRNAs. **g** Oxygen consumption analysis in brown adipocytes under basal level treated with sgRNA -9768 or control vectors. $n=5$. **h** Schematic illustration of the CRISPR-Cas9 KI mice and AAV constructs used in this study (left). *In vivo* luminescence analysis after 2 weeks' injection of AAV vectors (right). **i** Genome editing activity in inguinal white adipose tissues from mice treated with sgRNA −9768 or control vectors. **j** UCP1 protein expression analyses in inguinal white adipose tissues from control or sgRNA −9768-treated mice after exposure to cold (4 °C) for 2 days. $n=7$

also collected. Each group of cells was subjected to genomic DNA extraction and deep sequencing of integrated sgRNAs. Each sgRNA is labeled as a relative targeting locus, with the transcriptional start site (TSS) of *Ucp1* designated as 0. Comparisons were primarily carried out between $UCP1_{pos}$ and $UCP1_{all}$ groups to mainly avoid disturbances from cells that may have low UCP1 expression due to unsuccessful differentiation. Three independent screenings were performed, and fold changes of sgRNA counts in $UCP1_{pos}$ vs. $UCP1_{all}$ were averaged between three results. Using a cut-off of $\log_2$(Fold Change [$UCP1_{pos}$ /$UCP1_{all}$]) ≥ 1 or ≤ - −1, we have identified in total 13 sgRNAs that have significant enrichment in $UCP1_{pos}$, as well as 5 sgRNAs that have significant depletion in $UCP1_{pos.}$ (Fig. 1c and Table S1). We then validated four of the top-enriched and five of top-depleted sgRNAs individually in brown adipocytes. Consistent with the screening results, cells treated by the sgRNAs −9768, −6512, and −3422 showed significantly downregulated *Ucp1* expression, suggesting existing enhancers in these targeted regions, whereas cells treated by sgRNAs -7428 and + 5378 loci resulted in significantly upregulated *Ucp1* expression, which indicated the possible repressors in the targeted regions (Fig. 1d and e). No significant difference in *Ucp1* expression was observed with sgRNA + 1769, + 2769, −8405, or + 1051, a reflection of non-specific noise in the screening (Fig. 1d). Further tests using the established primary mouse *Ucp1-GFP* white adipocytes showed that sgRNA −9768 and + 5378 treatment led to obvious downregulation of UCP1 expression in the white adipocytes under rosiglitazone stimulation, whereas no significant differences with sgRNA −3422 and slightly increased UCP1 expression caused by sgRNA −6512 and −7428 treatment (Fig. 1f and Fig. S1). Consistently, the oxygen consumption rate (OCR) in sgRNA −9768-treated cells was significantly lower than that in control cells (Fig. 1g).

We then went on to further validate the effect of sgRNA −9768 *in vivo*. We delivered sgRNA -9768 to the adipose tissue of adult CRISPR-Cas9 knockin mice (Platt et al. 2014) via *in situ* injection of adeno-associated virus (AAV) expressing cre-recombinase and sgRNA (Fig. 1h). To specifically target adipose tissue, cre-recombinase was expressed under an adipocyte-specific adipoQ promoter, and eight copies of miR122 targeting sequences were added at the 3′ to limit hepatocyte expression. A firefly luciferase expression cassette was also included in the vector to assist *in vivo* evaluation of delivery efficiency (Fig. 1h). Three weeks after virus injection, mice were exposed to cold (4 °C) for 2 days to stimulate UCP1 expression in white adipose tissues. Consistent with the results in *in vitro* cultured cells, we observed clear genome-editing efficiency (Fig. 1i), and a significant downregulation of UCP1 expression in white adipose depots from mice treated with sgRNA −9768 (Fig. 1j). We also examined possible physiological change after treatment with sgRNA −9768. Results showed that sgRNA −9768 treatment did not affect mice body weight or iWAT tissue mass (Fig. S2A and S2B), however, caused decreased UCP1 expression, less multilocular adipocytes, larger adipocyte size, and a decreased trend of the ratio of mitDNA to nuDNA, as well. No significant change in the mRNA levels of other thermogenic genes or lipolysis-related genes was observed, indicating a specific regulation to *Ucp1* expression (Fig. S2C–F). Altogether, these results suggest that the −9768 locus carries functional regulatory elements that are necessary for UCP1 expression in adipocytes.

In this study, we performed an unbiased screening with customized sgRNA library covering potential regulatory regions for UCP1 transcriptional activity, and identified several genomic loci especially the −9768 locus, that may contain key regulatory elements for *Ucp1* expression. However, the exact regulatory sequences and potential transcription factor(s) or epigenetic regulator(s) that bind to these sequences remain to be defined. Our results also showed some inconsistencies in primary mouse brown and white adipocytes. For example, sgRNA −6512 and + 5378 displayed different functional effects in brown and white adipocytes, suggesting different regulatory mechanisms governing *Ucp1* expression in brown and white adipocytes, which warrant further investigation. The most direct physiological change after treatment with sgRNA −9768 should be the change of body temperature, especially the local temperature of white adipose tissue targeted with sgRNA −9768, which can be measured by infrared camera. sgRNA targeting known regulatory regions for *Ucp1* transcription were included in the designed sgRNA library; however, these sgRNAs were not screened out in this study. This is possibly due to low targeting efficiencies of these sgRNAs, background disturbances

from library handling, adipocyte differentiation, etc. Indeed, there exist some limitations in our screening system: (1) as mature adipocytes cannot proliferate, the selection step with the fluorescence activating cell sorting (FACS) is less able to enrich for the desired sgRNAs, compared with screenings in which enhancement of proliferation or acquisition of drug resistance is the desired outcome. The inability for further enrichment in our screening is causing high background disturbances; (2) the lengthy and multi-step adipocyte differentiation procedures are also bringing about a significant amount of variations from differentiation efficiency, which itself leads to variations in expression levels of UCP1, also another big source of background disturbance. Further optimization of the screening strategy may help to reduce false-positive or false-negative results. Nonetheless, by combining a reporter cell line and a CRISPR-mediated genetic screening, our study highlights a useful approach to identify possible regulatory elements for UCP1 transcriptional activity.

Materials and Methods

Animals

All mice were housed under standard conditions at Shanghai Institutes for Biological Sciences (SIBS), China. The animals presented a healthy status and male mice were employed for all experiments.

The colony of Cre-dependent Cas9 knockin mouse (Jackson Laboratory) was maintained by crossing with wild-type C57BL/6 J mice (Shanghai Laboratory Animal Co. Ltd, China). For adipocyte-specific genomic deletions, 7~8 weeks' old heterozygous Cas9 knockin animals ($Rosa^{Cas9\pm}$) were recorded for body weight. Animals were then randomly divided into groups, and administrated via *in situ* injection of adeno-associated virus 8 (AAV-8) expressing cre-recombinase and sgRNA targeting specific genomic loci. A firefly luciferase expression cassette was included in the AAV vector to assist *in vivo* evaluation of delivery efficiency. AAV vectors with cre-recombinase and luciferase cassettes and no sgRNA were used as control viruses.

For *in situ* AAV virus injection, viruses were dissolved in phosphate-buffered saline (PBS) and mice were injected bilaterally with a dose of $1\sim2\times10^{12}$ vg into the inguinal fat pads. After virus injection, animals were fed with normal chow diet (NCD) (Shanghai Laboratory Animal Co. Ltd, P1103F). *In vivo* bioluminescence imaging of AAV vectors was performed in mice administrated with AAV vectors 2 weeks later. And 3 weeks after virus injection, mice were exposed to cold (4 °C) for 2 days and dissected for further analysis.

Cell Culture and Differentiation

HEK 293 T and NIH 3T3 cell lines (Cell Bank, Type Culture Collection Committee, Chinese Academy of Sciences, Shanghai) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Gibco, 8,117,254) containing 10% fetal bovine serum (FBS, Gibco, 16,000,044) and 1% penicillin/streptomycin in a humidified incubator with 5% CO_2 at 37 °C. The *Ucp1-GFP* brown and white adipocytes were used as previously described (Qiu et al. 2018, S1). Briefly, the *Ucp1-GFP* brown and white adipocytes were isolated and immortalized from the stromal vascular fraction from interscapular brown fat pads or inguinal white fat pads of postnatal day 2 *Ucp1-GFP* male mice (Qiu et al. 2018). *Ucp1-GFP* brown and white preadipocytes were cultured at 37 °C in 5% CO_2 in primary culture medium (high-glucose Dulbecco's modified Eagle's medium (DMEM) with 20% fetal bovine serum (FBS) and 1% penicillin/streptomycin). The differentiation of brown and white preadipocytes were following a standard protocol.

Design and Synthesis of Mouse Lentiviral sgRNA Library

We generated a customized sgRNA library targeting the ~ 20 kb genomic region, including the 5′ region of mouse *Ucp1* gene as well as all the introns. Every 20 bp sequence upstream of an NGG PAM sequence on the plus or minus strand was identified as the sequence of a sgRNA. With the removal of sgRNAs with other targets in the genome that match exactly or differ by only 1 base, this customized library is comprised of 1,829 different guides, and they synthesized in CustomeArray (WA, USA).

To produce lentivirus sgRNA library, HEK293T cells in each 15 cm dish were transfected with 14.7 μg pMDLG, 7.9 μg pVSVG, 5.7 μg pREV, and 22.5 μg CRISPR plasmids using polyethylenimine (Polysciences, 23,966–1). Medium was changed 4~6 h after transfection. Lentiviral supernatants were collected at 48 and 72 h post-transfection and centrifuged at 20,000 rpm at 4 °C for 2 h. Viral pellets were then re-suspended in DMEM at 4 °C overnight and titer was calculated using a PCR-based titration kit (Applied Biological Materials Inc, LV900).

In Situ Saturating Mutagenesis Screen for UCP1 Regulators

The *Ucp1-GFP* brown preadipocytes ($UCP1_{pre}$) were infected by the lentiviral library at MOI of 2 ~ 3 to achieve an infection efficiency of around ~ 30%. Cells were then treated with 1.5 μg/ml puromycin (Beyotime, ST551) for 2 days to remove non-infected cells. Cells were recovered for one more day after puromycin treatment, and followed

by a standard differentiation protocol to obtain mature brown adipocytes ($UCP1_{all}$). Cells were harvested at day 8 at maturation stage and high GFP + cells (top 5%) or GFP- cells (bottom 5%) were collected by FACS sorting (FACSAria II; BD Biosciences). Control cells, including $UCP1_{all}$ cells (collected before FACS sorting) and infected $UCP1_{pre}$ cells (collected after puromycin treatment), were obtained at indicated time points.

Genomic DNA of cells from different groups was extracted and the sgRNAs were amplified by PCR method using KOD DNA polymerase (TOYOBO, KOD-401). Briefly, in total, 2 μg (200 ng per PCR reaction; 10 separate reactions for each sample) of genomic DNA from each group were used as DNA template; the PCR program was 94 °C 5 min, 98 °C 20 s, 58 °C 30 s, 68 °C 12 s, 32 cycles. Products (158 bp) were gel-purified and quantified. In total, 1.5 μg PCR products from each group were pooled together and sent for deep sequencing (Illumina HiSeq4000 system) by using the pair-ended 150 bp sequencing protocol. PCR primers used for amplification were: 5′-TGAAAGTATTTC GATTTCTTGGCTT-3′, 5′-CGGTGCCACTTTTTCAAG TT-3′. An 8 bp barcode for multiplexing of different biological samples was added at 5′ of each primer.

For data analysis, the sequencing reads of sgRNAs from different samples were first identified by barcode using cutadapt (v1.9) with default parameters. Build-index function of Bowtie [S2] was applied on the sgRNA sequences of sgRNA library to generate Burrows–Wheeler index. The sgRNA sequences were then retrieved and counted by aligning processed reads of each sample to the sgRNA library using Bowtie. Maximum two mismatches were allowed and only the reads with unique alignment were reported. Total reads were normalized to library sequencing depth. sgRNA enrichment score was determined by calculating (1) the ratio of normalized reads in the $UCP1_{pos}$ group as compared to $UCP1_{all}$ group; and (2) $\log_2$ transformation. Three independent screenings were performed and the average of three sgRNA enrichment scores were used for identification of interested sgRNAs.

Validation of Individual sgRNAs in Brown and White Adipocytes

The 20 bp sequence of sgRNA targeting specific genomic loci was inserted to lentiCRISPR V2 plasmid and used for lentivirus packaging. The target sequences used are listed in Supplemental Table 1. Lentiviruses carrying CRISPR-sgRNA or empty lentiCRISPR V2 vector as control viruses were packaged. The *Ucp1-GFP* brown preadipocytes were then infected and selected with 1.5 μg/ml puromycin (Beyotime, ST551) for 2 days to remove non-infected cells. Cells were then recovered for one more day, and subjected to adipocyte differentiation. Brown adipocytes were collected at day 8, and white adipocytes were collected at day 10 for later mRNA or protein expression analyses.

CRISPR Construct and AAV Packaging

The pAAV-GFP plasmid (Cell Biolabs. Inc) was modified, with the original CMV promoter replaced by the adipoQ promoter for adipocyte-specific expression between NotI and EcoRI restriction enzyme sites, the original GFP cassette replaced with the Cre-T2A-Luciferase-sgRNA cassette between EcoRI and XbaI restriction enzyme sites for expression of Cre-recombinase, luciferase, and sgRNA. And an 8 × miR122 targeting sequence at the 3′ region was added after luciferase cassette to limit the AAV expression in the liver tissue. The sgRNA scaffold was inherited from the lentiCRISPR V2 plasmid (Addgene, #52961), and pieces containing Cre-recombinase, T2A-luciferase, 8 × miR122 targeting sequence, and sgRNA were ligated via PCR. The resultant construct was named AAV-AdipoQ-Cre-Luci-8 × miR122T-gRNA construct, which was further used to insert sgRNA sequences targeting specific genomic loci or directly as control vector.

For adipocyte tissue-specific genomic disruption with sgRNA -9768, mouse *Ucp1* sgRNA −9768 (5′-GAATGA AAAAAAAAGGTGAC + *AGG*-3′) was inserted into the sgRNA scaffold in AAV-AdipoQ-Cre-Luci-8 × miR122T-gRNA construct. And construct was verified by sequencing before using. Genome-editing efficiency by CRISPR targeting in adipose tissue was examined with genomic DNA by T7EI analysis (NEB, E3321). Primers used in T7EI analysis were as follows: 5′-AAAAGAGTCCATGGCCCTGA-3′ and 5′-GATACACAACACAGGCCCAG-3’.

Adeno-associated viruses were generated using packaging plasmids AAV-helper and AAV-8 (Cell Biolabs. Inc) together with AAV-AdipoQ-Cre-Luci-8 × miR122T-gRNA constructs. Viruses were administrated via *in situ* injection at a dose of $1 \sim 2 \times 10^{12}$ vg per mouse for adipose tissue-specific CRISPR genomic loci mutation.

RNA Isolation and Quantitative RT-PCR

Total RNA was isolated from brown adipocytes, white adipocytes, or subcutaneous adipose tissue using Trizol reagent (ThermoFisher, 15,596,018) according to the manufacturer’s instructions. Reverse transcription of isolated RNA was performed using the reverse transcription kit (Takara, RR047A). Quantitative real-time PCR was carried out on the 7900 System (ABI) using SYBR Green supermix (ABI, 4,472,908). Primers used in this study are listed in Table S2.

Western Analysis

Protein from cells or tissues was extracted by the RIPA buffer (Millipore, 20188) and subjected to regular western procedure. The primary antibodies used in the experiments were antibodies to UCP1 (Abcam, ab10983) and HSP90 (Cell Signaling Technology, 4874S).

Cold Challenge Experiment

Heterozygous Cas9 knockin male mice treated with AAV vectors were individually caged with food and water at a cold room (4 °C) for 2 days.

Measurement of Mitochondria Number

Genomic DNA from inguinal white adipose tissues of mice treated with AAV vectors was extracted using the TIANamp Genomic DNA kit (TIANGEN, DP304-03). The presence of amplifiable mitDNA and nuDNA in the extract was assayed through real-time PCR.

Seahorse Assay

The oxygen consumption rate (OCR) was measured using an XF24 Extracellular Flux Analyzer (Seahorse Bioscience). The *Ucp1-GFP* brown preadipocytes treated with lentiviruses were plated in the XF24 V28 cell culture microplate (Seahorse Bioscience) and subjected to adipocyte differentiation for 8 days. Cells were treated with 2 μM Oligomycin, 1.5 μM FCCP, and 1 μM Rotenone/Antimycin A from the Agilent Seahorse XF Cell Mito Stress Test Kit (Agilent Technologies, 103,015–100) during fixed time intervals.

Histology

Mouse tissues were fixed and embedded in paraffin (for HE staining) or frozen (for UCP1 staining). Sections were stained with hematoxylin and eosin or UCP1 antibody (1:100, Abcam, ab10983) according to standard protocols (Wuhan Servicebio Technology).

Statistics

The unpaired, two-tailed Student's *t* test was used for experiments with two groups' comparison. All data are represented as means with SEM. $*P<0.05$, $**P<0.01$, $***P<0.001$.

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Author Contributions YQ and QD designed experiment. YQ performed all experiments. XL performed data analysis for CRIPSR screening. YS and SL assisted with the screening. YW and CT modified the pAAV-GFP plasmid. YQ and QD analyzed the data, prepared figures, and wrote the manuscript. QD supervised the project.

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Availability of Data and Materials All unique materials generated from this study are available from the corresponding author, Qiurong Ding (qrding@sibs.ac.cn).

Compliance with Ethical Standards

Conflict of Interest All authors declare no conflicts of interest.

Ethics Approval All mice were housed under standard conditions at the Shanghai Institutes for Biological Sciences (SIBS), China. All animal procedures were performed according to guidelines, and were approved by the Institutional Animal Care and Use Committee of the Shanghai Institutes for Biological Sciences.

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