Supplementary Information

Identification of a distal enhancer of *Ucp1* essential for thermogenesis and mitochondrial function in brown fat

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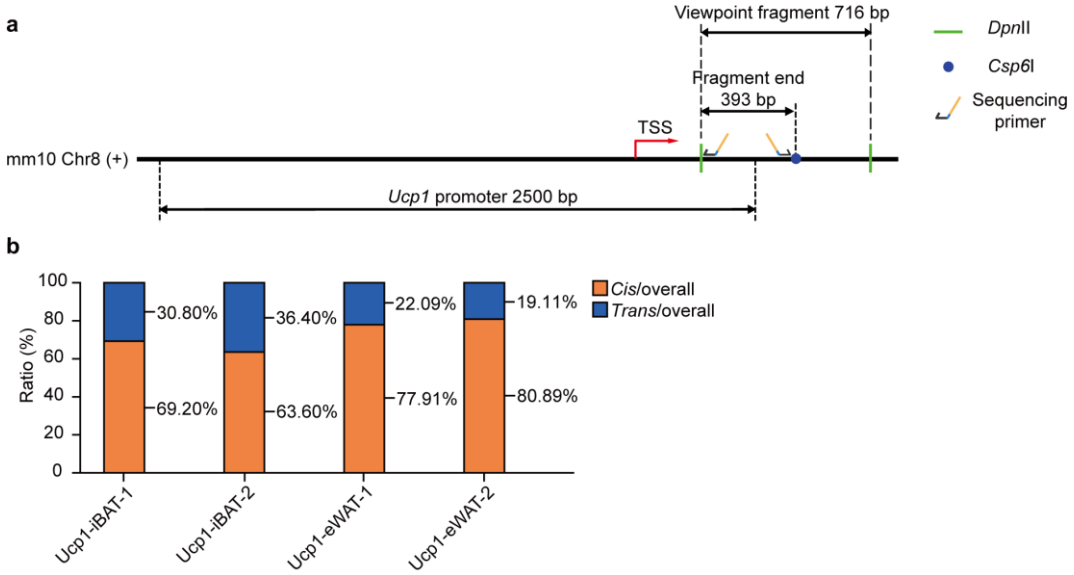
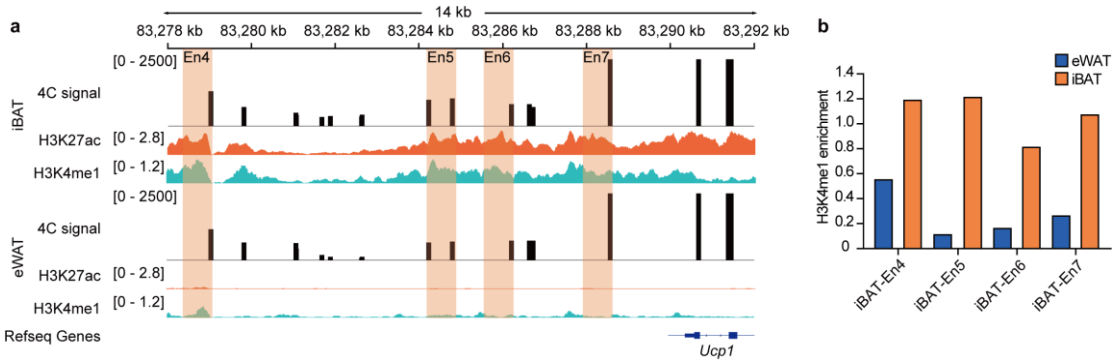
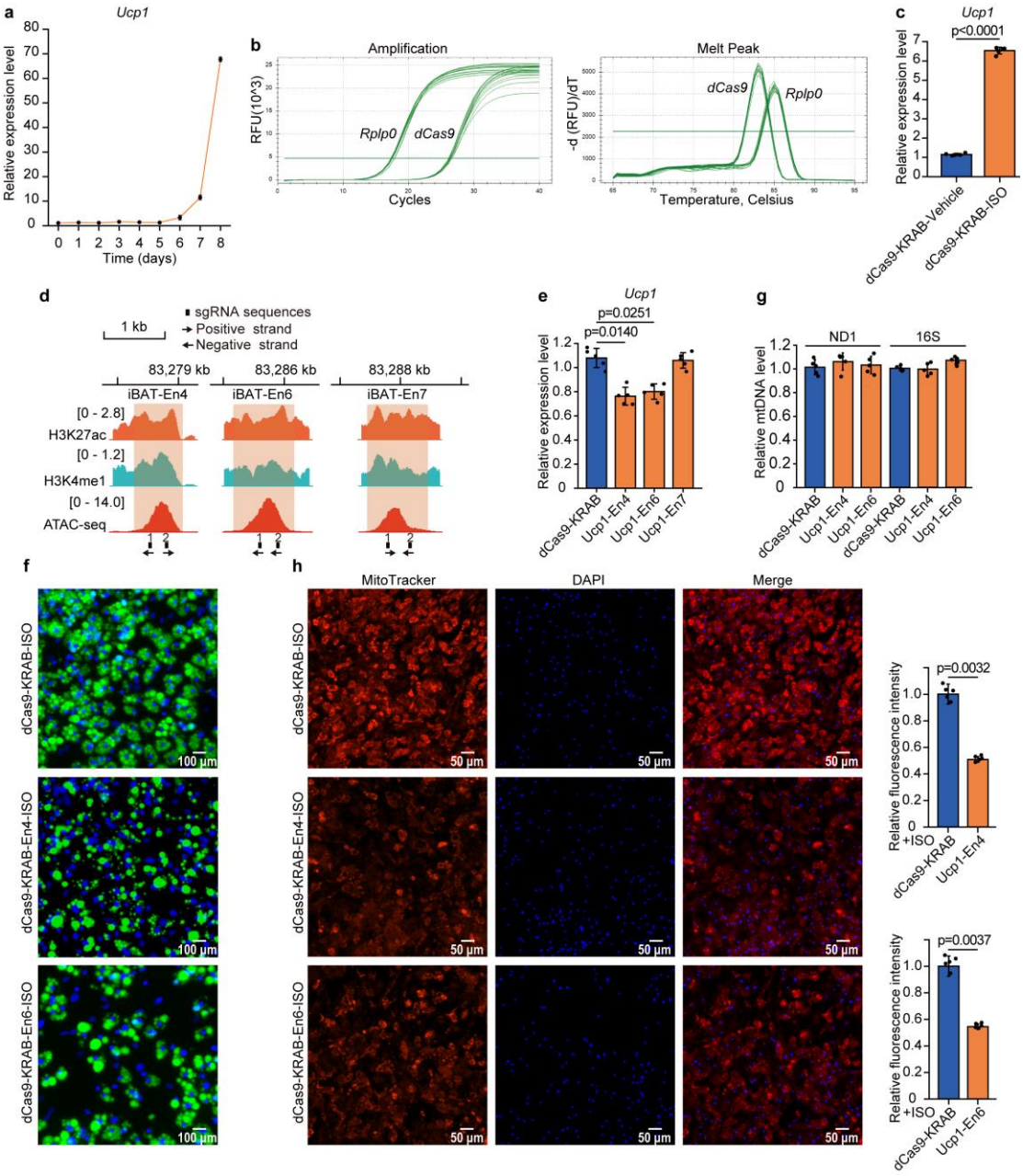
Supplementary Figures



Supplementary Fig. 1 Construction and quality control of the 4C-seq library. a Viewpoint selection and primer design at *Ucp1* promoter region between 2000 bp upstream to 500 bp downstream of the transcription start site for the 4C-seq experiment. **b** Bar plots showing the percentage of mapped reads in *cis*-chromosome and *trans*-chromosome for each 4C dataset.

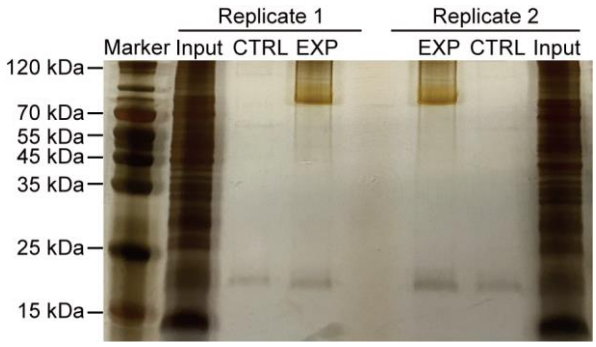


Supplementary Fig. 2 Chromatin interactions and histone modifications of the active enhancer regions. a Chromatin interactions (4C-seq) and histone modification (H3K27ac and H3K4me1) of active enhancer regions in iBAT and eWAT. The orange rectangles represent active enhancer regions of iBAT. **b** Signal values for H3K4me1 histone enrichment at active enhancer regions in iBAT and eWAT were obtained from bigwig files on IGV.



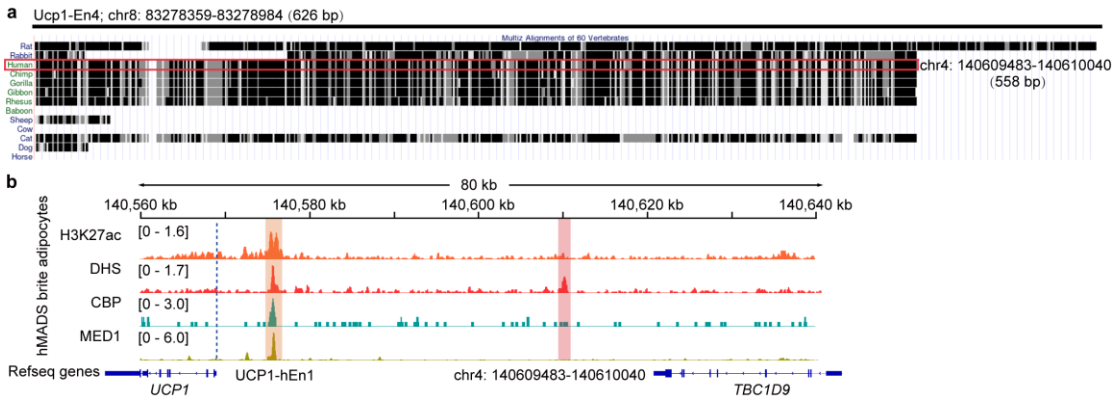
Supplementary Fig. 3 Ucp1-En4 and Ucp1-En6 regulate *Ucp1* expression, and affect mitochondrial mass under cold stimulation. a qRT-PCR analysis of *Ucp1* mRNA expression in brown adipocytes during differentiation. **b** Amplification curve of dCas9 and *Rplp0* (left) and melting curve of dCas9 and *Rplp0* (right) in dCas9-KRAB brown adipocytes on day 8 of differentiation. **c** qRT-PCR analysis of *Ucp1* mRNA expression in dCas9-KRAB brown adipocytes on day 8 of differentiation (n = 5). **d** The dCas9-KRAB system repression the chromatin of Ucp1-En4, Ucp1-En6, and Ucp1-En7 regions. ChIP-seq of active enhancer-associated histone modification (H3K27ac, H3K4me1) and ATAC-seq profiles show the chromatin status of enhancer regions.

Enhancer regions are shown as orange rectangles. The single guide RNA (sgRNA) target locations are indicated in black; the arrow direction indicates the targeting of the forward or reverse DNA strand. **e** qRT-PCR analysis of *Ucp1* mRNA expression in brown adipocytes on day 8 of differentiation (n = 5). **f** BODIPY (green) staining of lipid droplets in brown adipocytes on day 8 of differentiation. The nuclei were stained with DAPI (blue). Scale bar = 100 μm. **g** Mitochondrial DNA (mtDNA) content in brown adipocytes on day 8 of differentiation (n = 5). **h** Mito Tracker (red) staining of mitochondria in brown adipocytes on day 8 of differentiation. The nuclei were stained with DAPI (blue). Relative fluorescence intensity was quantified in five randomly chosen non-overlapping fields. Scale bar = 50 μm. On day 8 of differentiation, brown adipocytes were treated with 10 μM ISO or vehicle for 4 h before harvest for further experiments. Data are presented as mean ± SEM. *P* values were calculated by two-tailed unpaired Student's *t*-tests.

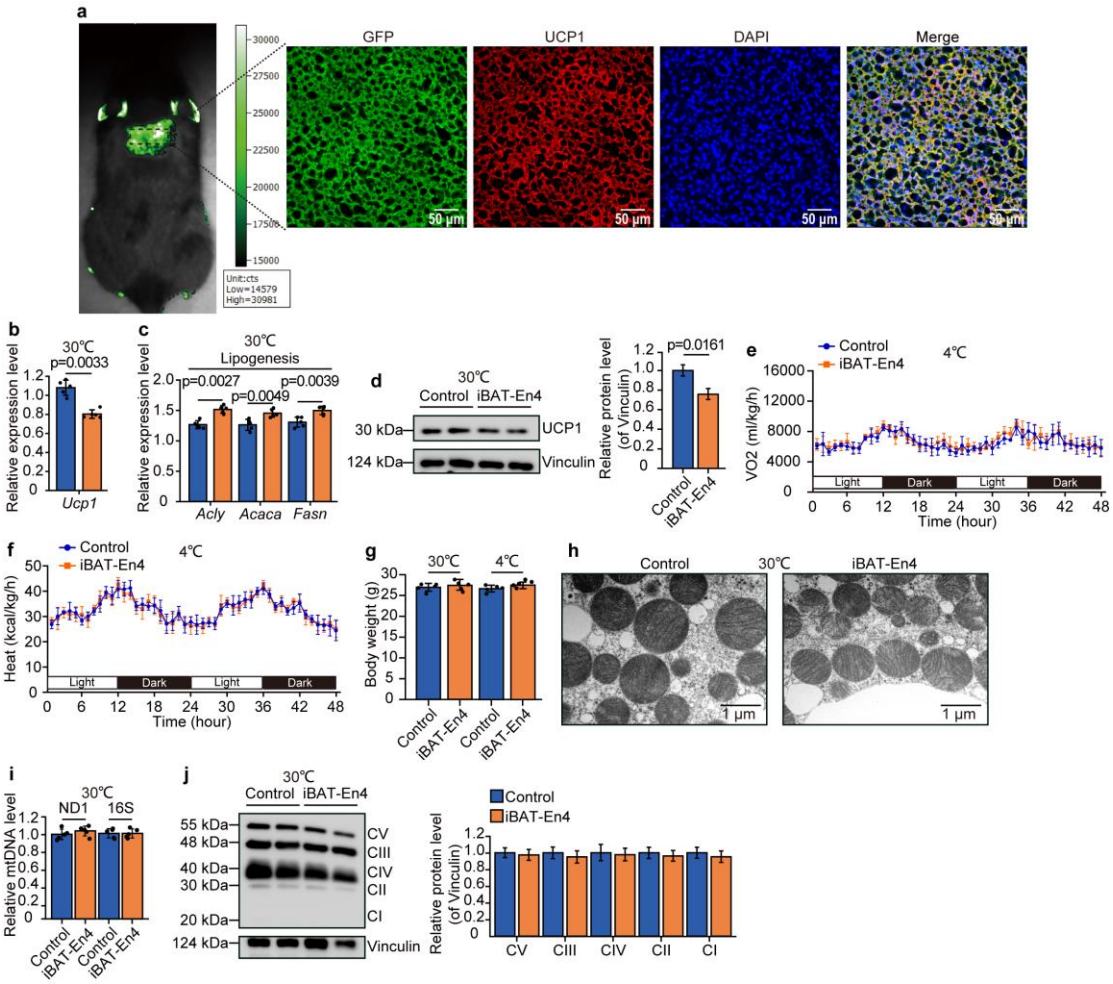


Supplementary Fig. 4 Identification of nucleoproteins binding to Ucp1-En4 region.

Silver staining of the nucleoproteins identified in DNA pull-down assay of Ucp1-En4. CTRL: control group. EXP: Ucp1-En4 probe group.

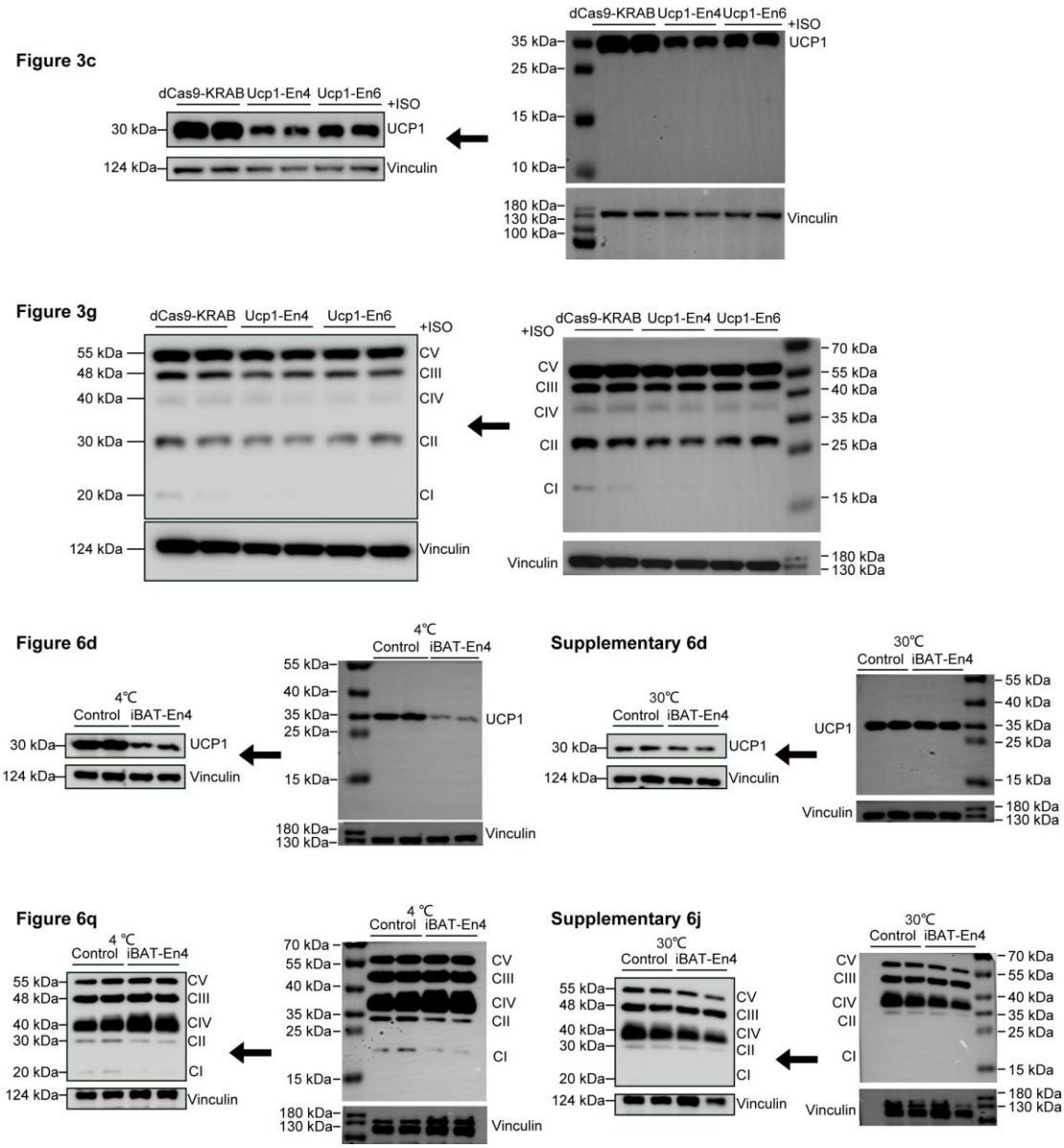


Supplementary Fig. 5 Conservation analysis of Ucp1-En4 enhancer sequence. a Sequence conservation analysis of Ucp1-En4 in selected species using the UCSC genome browser. The solid line represents the mouse Ucp1-En4 enhancer sequence. **b** ChIP-seq (including H3K27ac, CBP, and MED1) and DHS-seq profiles defined putative active enhancers of UCP1 in beige adipocytes differentiated from human multipotent adipose-derived stem (hMADS) cells. Putative active enhancer is represented by an orange rectangle. The conserved sequence with mouse Ucp1-En4 enhancer sequence is represented by a red rectangle.



Supplementary Fig. 6 Repression of Ucp1-En4 did not significantly affect mitochondrial function in iBAT under thermoneutrality conditions. a Representative images of iBAT in mice two weeks after injection of dCas9-KRAB-GFP lentivirus (left). GFP expression (no antibody staining) and immunofluorescence staining of UCP1 (red) and nuclei (blue) are shown (right). Scale bar = 50 μm. **b, c** qRT-PCR analysis of *Ucp1* and lipogenesis-related genes (*Acly*, *Acaca*, and *Fasn*) mRNA expression in iBAT from control and iBAT-En4 mice at 30°C (n = 5). **d** Western blot analysis of UCP1 in iBAT from control and iBAT-En4 mice at 30°C (n = 4). Gray values of protein bands were quantified using Image J software, with Vinculin as a normalizer. **e, f** Oxygen consumption (VO2) and heat production of control and iBAT-En4 mice in metabolic cages at 4°C (n = 5). **g** Body weight of control and iBAT-En4 mice at 30°C or 4°C (n = 5). **h** Transmission electron microscopy images of control and iBAT-En4 mice at 30°C. Scale bar = 1 μm. **i** Assessment of mitochondrial DNA content by qPCR for iBAT from control and iBAT-En4 mice at 30°C (n = 5). **j** Western blot analysis of

mitochondrial OXPHOS complex subunits in iBAT from control and iBAT-En4 mice at 30℃ (n = 4). Gray values of protein bands were quantified using Image J software, with Vinculin as a normalizer. Data are presented as mean ± SEM. *P* values were calculated by two-tailed unpaired Student's *t*-tests.



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