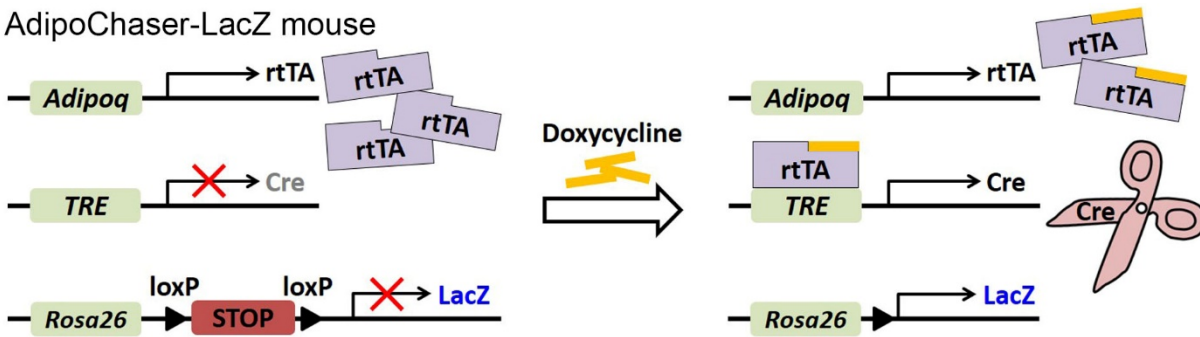


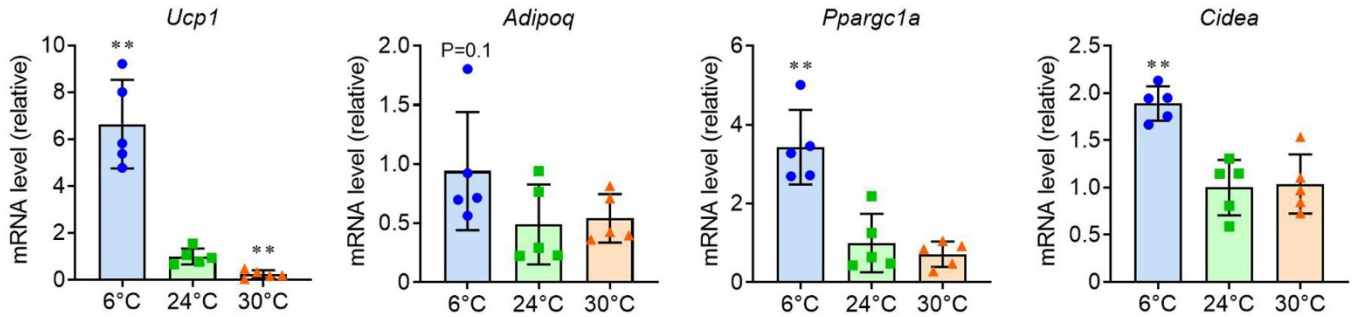
Supplemental Figures

Supplemental Figure 1

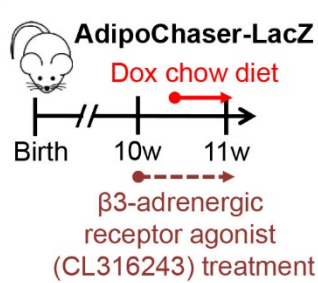
**A** AdipoChaser-LacZ mouse



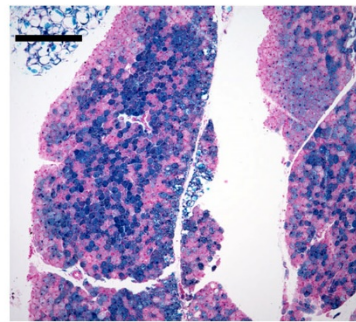
**B**



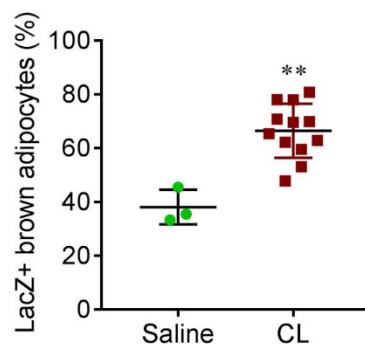
**C**



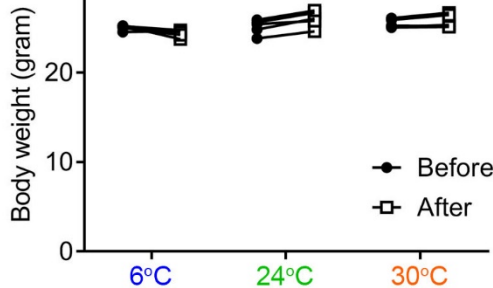
**D** CL treatment



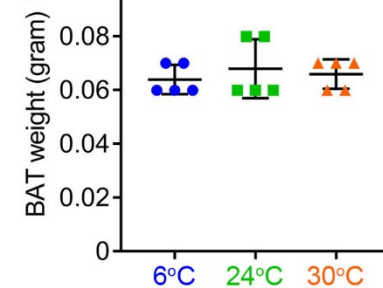
**E**



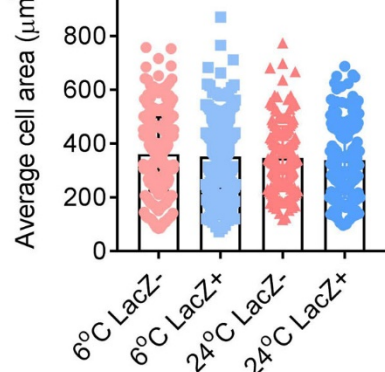
**F**



**G**



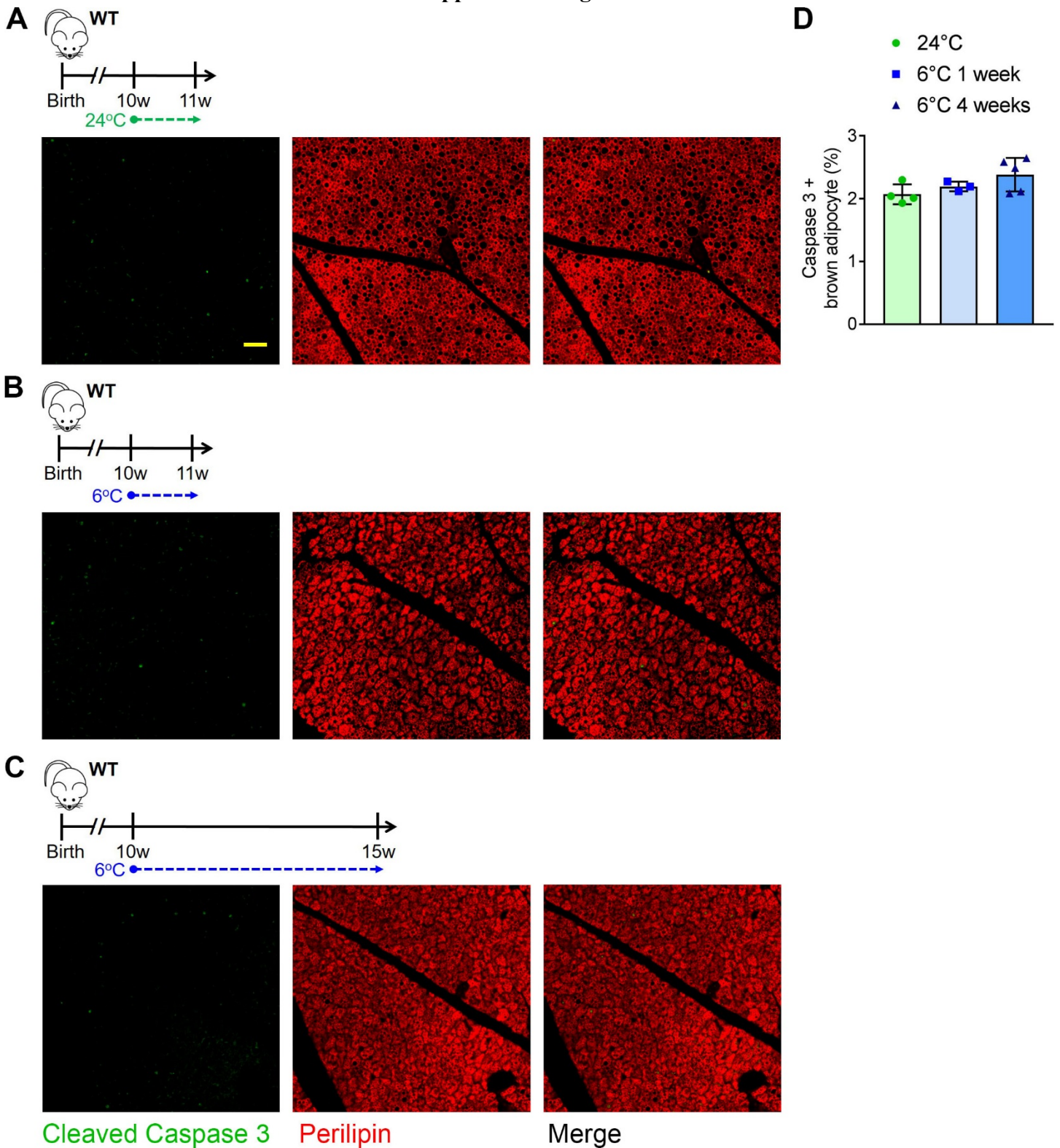
**H**



**Supplemental Figure 1. AdipoChaser mouse models used for cold and thermoneutral exposures.** (A) AdipoChaser-LacZ mouse model, the dox-based, tet-responsive, Cre-loxP labeling system. AdipoChaser-LacZ mice are derived from interbreeding three transgenic strains: 1) mice expressing the “tet-on” transcription factor *rtTA* under the control of the adiponectin gene promoter (*Adn-rtTA*); 2) mice expressing tet-responsive CRE (*TRE-Cre*) that is activated by *rtTA* in the presence of dox; and 3) reporter mice expressing a *LacZ* reporter gene (encodes  $\beta$ -galactosidase) from the *Rosa26* locus in a Cre-dependent manner (*Rosa26-loxP-STOP-loxP-LacZ*).

AdipoChaser-LacZ mouse expresses rtTA in adiponectin expressing cells (e.g. all the white adipocytes), but does not express LacZ in any cell type while maintained on food not containing dox. When dox is included in the food, cells that express rtTA will have the *TRE* promoter activated so that Cre expression is induced. The Cre protein will specifically cut out the floxed transcriptional stop cassette and then turn on LacZ expression. Even after the withdrawal of dox from the food, these LacZ positive cells will permanently express LacZ. **(B)** 10-week-old Wild-type (WT) male mice were housed at 24°C, 6°C, or 30°C for 1 week. qPCR analysis shows the mRNA expression levels of *Ucp1*, *Adipoq*, *Ppargc1a*, as well as *Cidea* in BAT of WT mice. n = 5 male mice. Data represent mean ± s.d. of biologically independent samples. \*\*P < 0.01. Statistical significance was assessed using one-way ANOVA followed by Tukey's multiple comparisons test. **(C)** AdipoChaser-LacZ male mice were kept on normal chow until 10 weeks of age. Mice were then treated with β3-adrenergic receptor agonist for 7 days, during the last 4 days, mice were switched to dox-containing chow diet. **(D)** Representative X-gal staining of BAT from AdipoChaser-LacZ mice treated with β3-adrenergic receptor (CL-316243) agonist. Image is representative of two independent experiments. **(E)** Quantification of the percentage of LacZ-positive brown adipocytes in the total brown adipocytes. n = 3 mice (saline); 12 mice (CL). Data represent mean ± s.d. of biologically independent samples, \*\*P < 0.01. Statistical significance was assessed using a Mann Whitney test. **(F)** Body weight of 10-week-old AdipoChaser-LacZ male mice before and after 7 days of temperature switch. n = 5 mice/group. **(G)** BAT weight of AdipoChaser-LacZ male mice after 7 days of temperature switch. n = 5 mice/group. These images are representative of three independent experiments. **(H)** The average cell size of LacZ negative and positive brown adipocytes in AdipoChaser-LacZ male mice after 7 days of temperature switch. n = 333 cells (6°C LacZ-); 324 cells (6°C LacZ+); 200 cells (24°C LacZ-); 161 cells (24°C LacZ+).

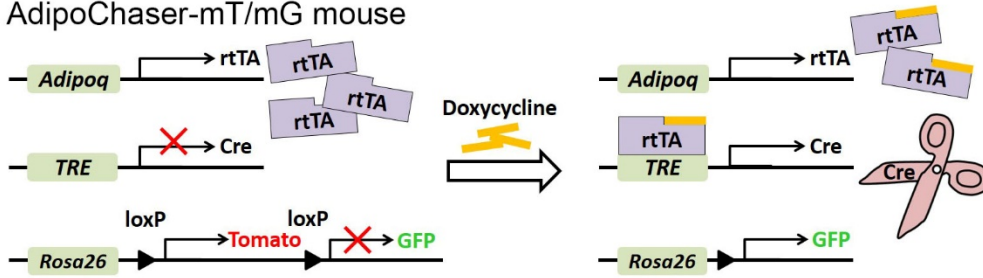
## Supplemental Figure 2



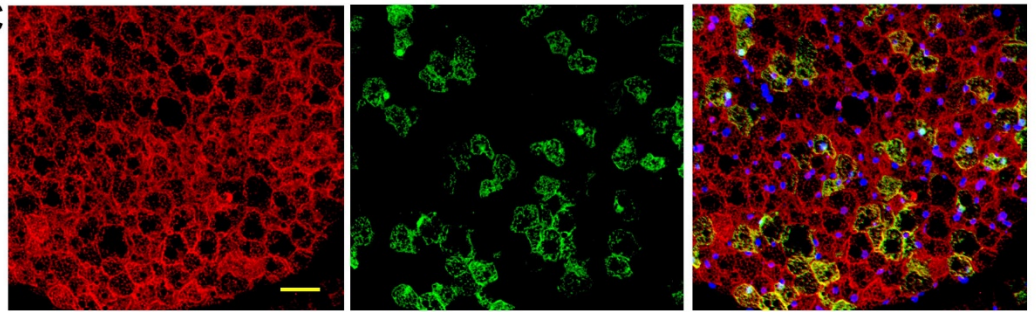
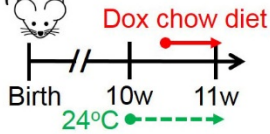
**Supplemental Figure 2. Brown adipocytes do not undergo apoptosis even after prolonged cold exposure.** (A–C) Cleaved (activated) Caspase 3 (green) and Perilipin (red) immunofluorescence staining in BAT of WT mice kept at 24°C or expose to 6°C for indicated time. Scale bar: 50  $\mu$ m. (D) Quantification of the percentage of cleaved Caspase 3+ brown adipocytes in the total brown adipocytes. n = 4 mice (24°C); 3 mice (6°C 1 week); 5 mice (6°C 4 weeks). All images are representative of three independent experiments. Data represent mean  $\pm$  s.d. of biologically independent samples.

### Supplemental Figure 3

#### A AdipoChaser-mT/mG mouse



#### B AdipoChaser-mT/mG

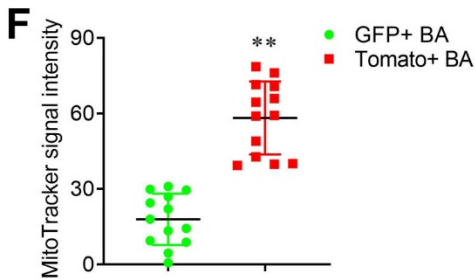
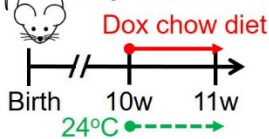


Perilipin

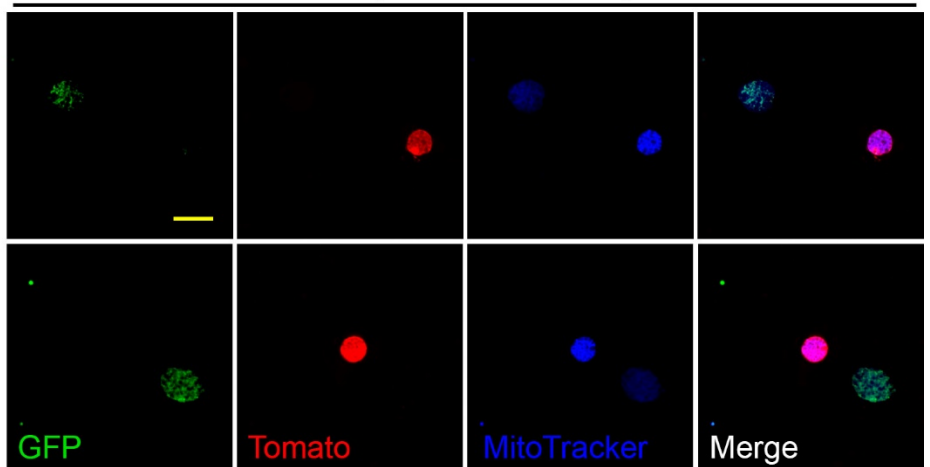
GFP

Merge (DAPI)

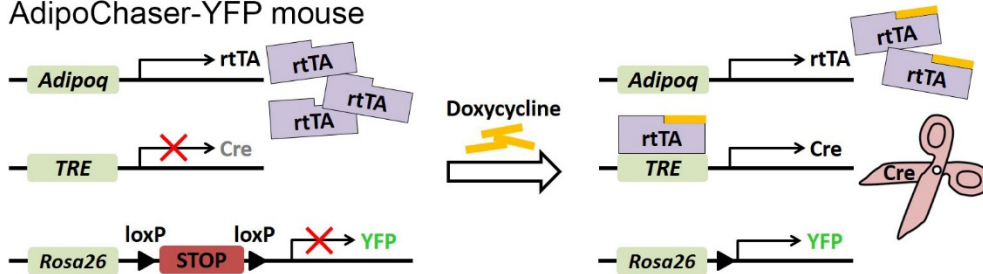
#### D AdipoChaser-mT/mG



#### E Primary brown adipocytes



#### G AdipoChaser-YFP mouse



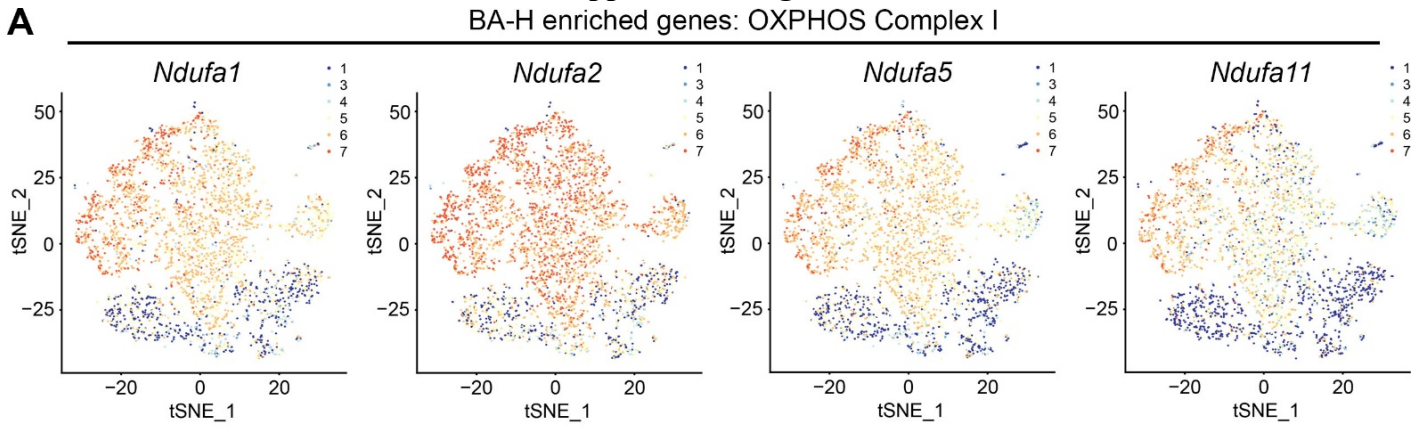
**Supplemental Figure 3. Adiponectin low expressing brown adipocytes have higher mitochondrial membrane potential** (A) AdipoChaser-mT/mG mouse model, similar to the AdipoChaser-LacZ mouse, is produced by crossing *Adn-rtTA* transgenic mice with *TRE-Cre* and *Rosa26-loxP-STOP-loxP-mT/mG* transgenic mice. All cells in AdipoChaser-mT/mG mouse express tdTomato. When dox is included in the food, adiponectin expressing cells will have the *TRE* promoter activated so that Cre expression is induced. The Cre protein will specifically cut out the floxed tdTomato gene and thus turn on eGFP expression. (B) AdipoChaser-

mT/mG male mice were kept on normal chow until 10 weeks of age. Mice were then fed with dox-containing chow for 4 days. (C) Representative immunofluorescence staining for Perilipin (red), GFP (green) and DAPI (blue) of BAT from AdipoChaser-mT/mG mice. Scale bar: 50  $\mu$ m. These images are representative of three independent experiments. (D) AdipoChaser-mT/mG male mice were kept on normal chow until 10 weeks of age. Mice were then treated with dox-containing chow diet for one week. (E) Representative immunofluorescence signal from GFP+ (green) or Tomato+ (red) primary brown adipocytes from AdipoChaser-mT/mG mice kept at 24°C, stained with MitoTraker Deep Red (labels mitochondria and reflects mitochondrial membrane potential) (blue). Scale bar: 50  $\mu$ m. (F) Quantification of MitoTraker Deep Red fluorescent signal in GFP+ or Tomato+ cells. n = 13 cells for each group. Data represent mean  $\pm$  s.d. of biologically independent samples, \*\*P < 0.01. Statistical significance was assessed using a two-tailed Student's t-test (F). All images are representative of three independent experiments. (G) AdipoChaser-YFP mouse model, similar to the AdipoChaser-LacZ and AdipoChaser-mT/mG mice, this mouse model is produced by crossing *Adn-rtTA* transgenic mice with *TRE-Cre* and *Rosa26-loxP-STOP-loxP-YFP* transgenic mice. When dox is included in the food, adiponectin expressing cells will have the *TRE* promoter activated so that Cre expression is induced. The Cre protein will specifically cut out the floxed transcriptional stop cassette and then turn on YFP expression.

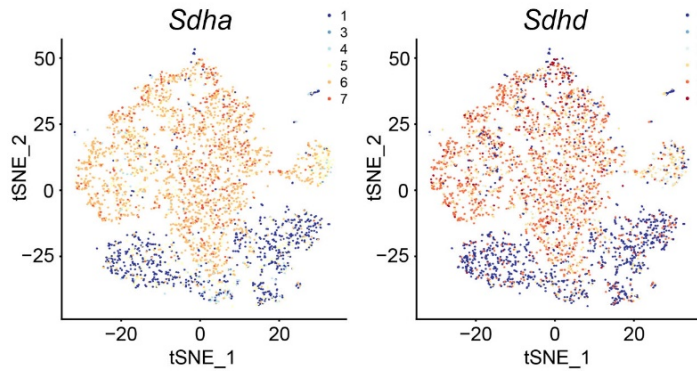


### Supplemental Figure 5

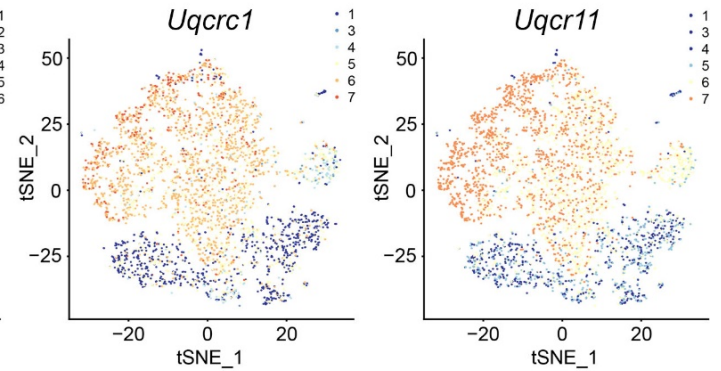
BA-H enriched genes: OXPPOS Complex I



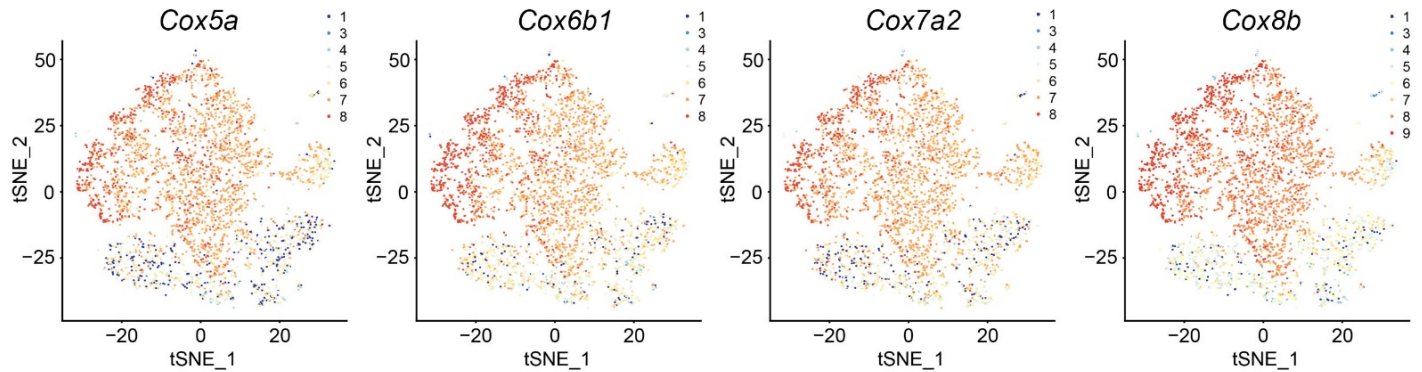
**B** BA-H enriched genes: OXPPOS Complex II



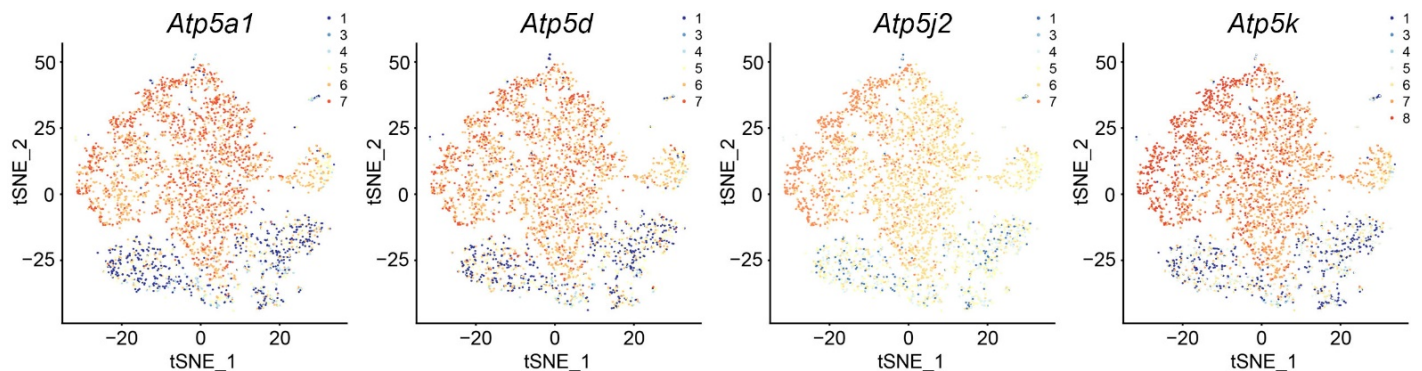
**C** BA-H enriched genes: OXPPOS Complex III



**D** BA-H enriched genes: OXPPOS Complex IV



**E** BA-H enriched genes: OXPPOS Complex V

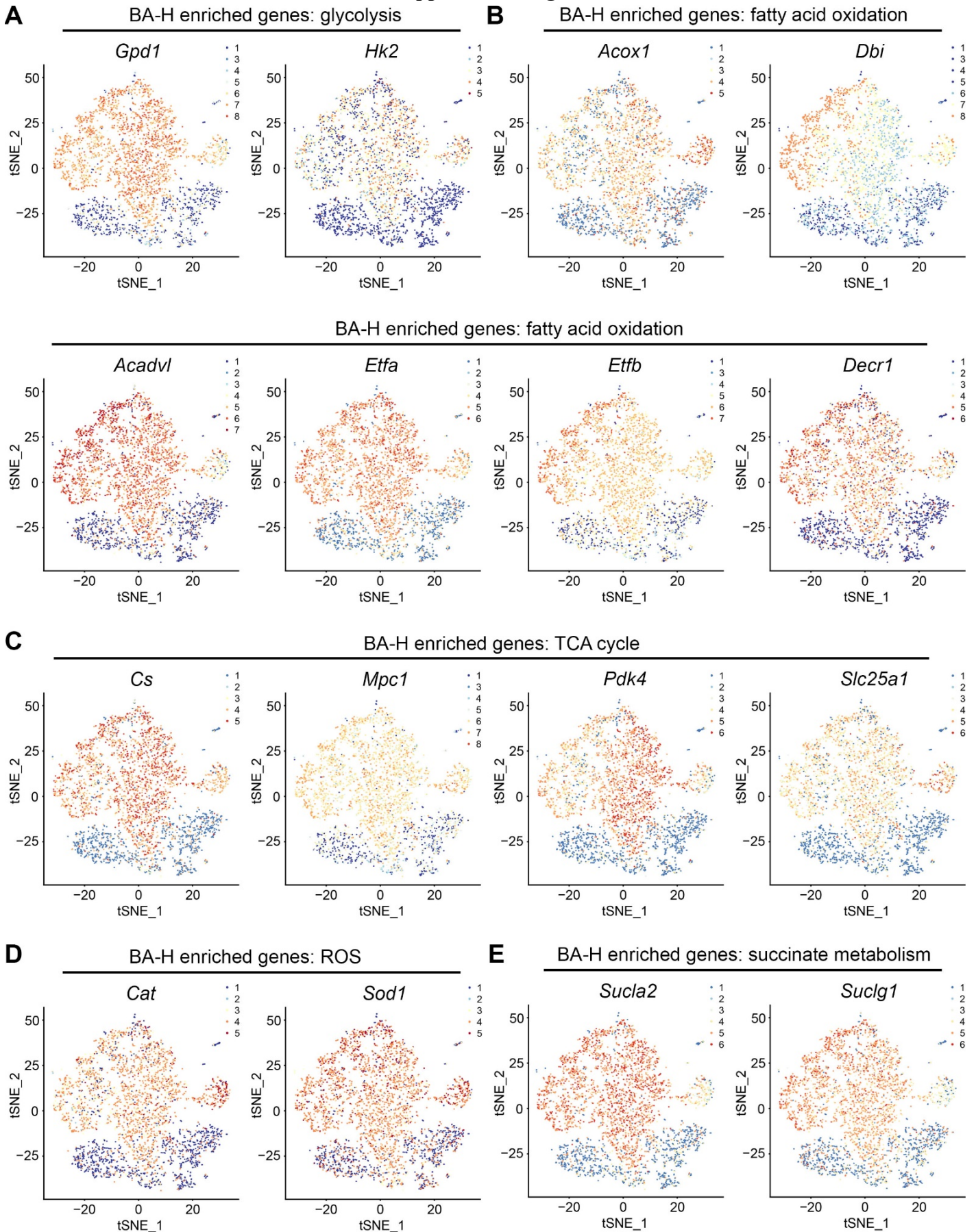


Supplemental Figure 5. Single-cell RNA sequencing reveals genes enriched in BA-H cluster: OXPPOS.

(A) Distribution of *Ndufa1*, *Ndufa2*, *Ndufa5*, and *Ndufa11* expression within tSNE plot, showing genes related to OXPHOS complex I are enriched within the BA-H cluster. (B) Distribution of *Sdha* and *Sdhb* expression within tSNE plot, showing genes related to OXPHOS complex II are enriched within the BA-H cluster. (C) Distribution of *Uqcrc1* and *Uqcr11* expression within tSNE plot, showing genes related to OXPHOS complex III are enriched within the BA-H cluster. (D) Distribution of *Cox5a*, *Cox6b1*, *Cox7a2*, and *Cox8b* expression within tSNE plot, showing genes related to OXPHOS complex IV are enriched within the BA-H cluster. (E) Distribution of *Atp5a1*, *Atp5d*, *Atp5j2*, and *Atp5k* expression within tSNE plot, showing genes related to OXPHOS complex V are enriched within the BA-H cluster.



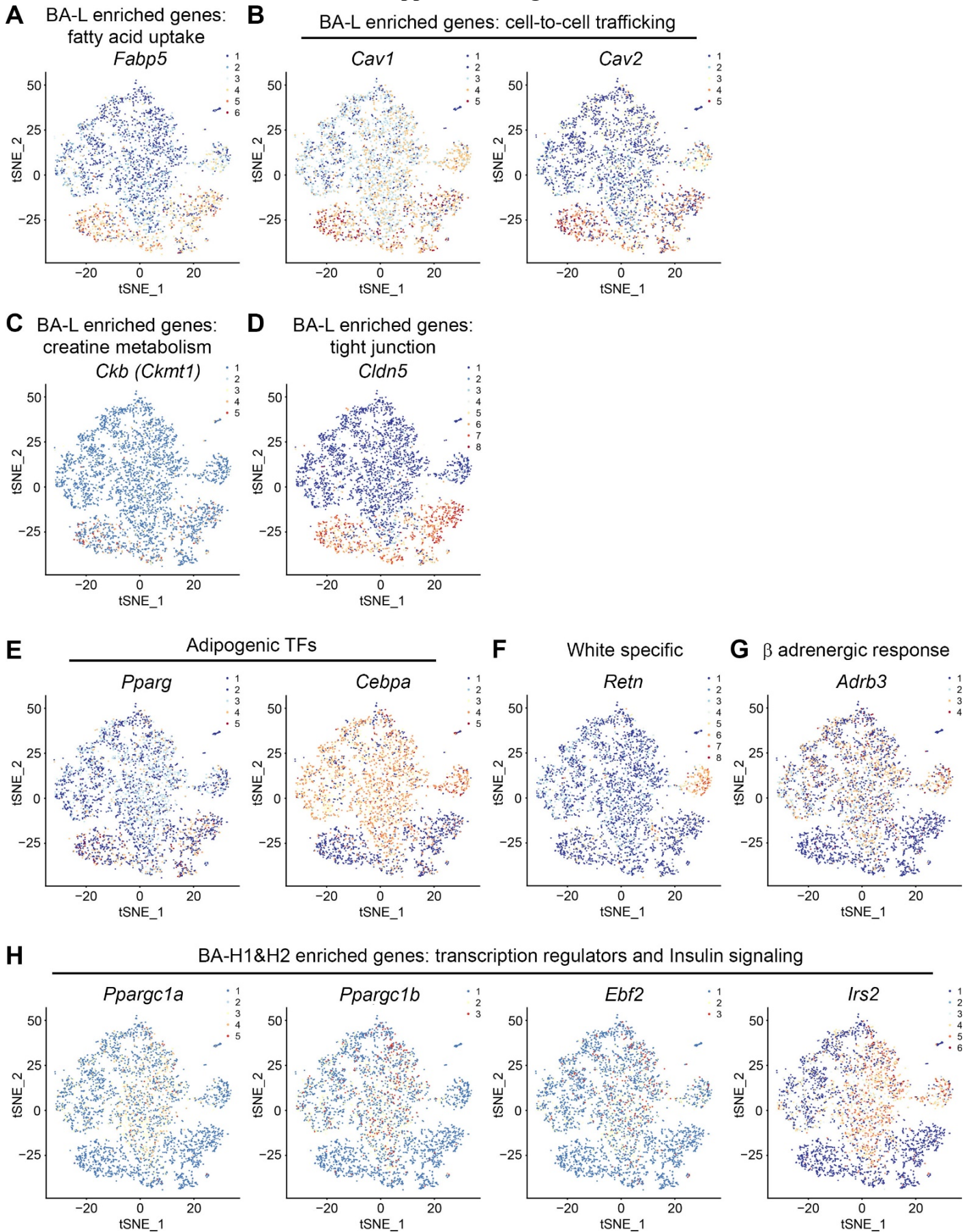
### Supplemental Figure 6



Supplemental Figure 6. Single-cell RNA sequencing reveals gene enriched in BA-H cluster: glycolysis,

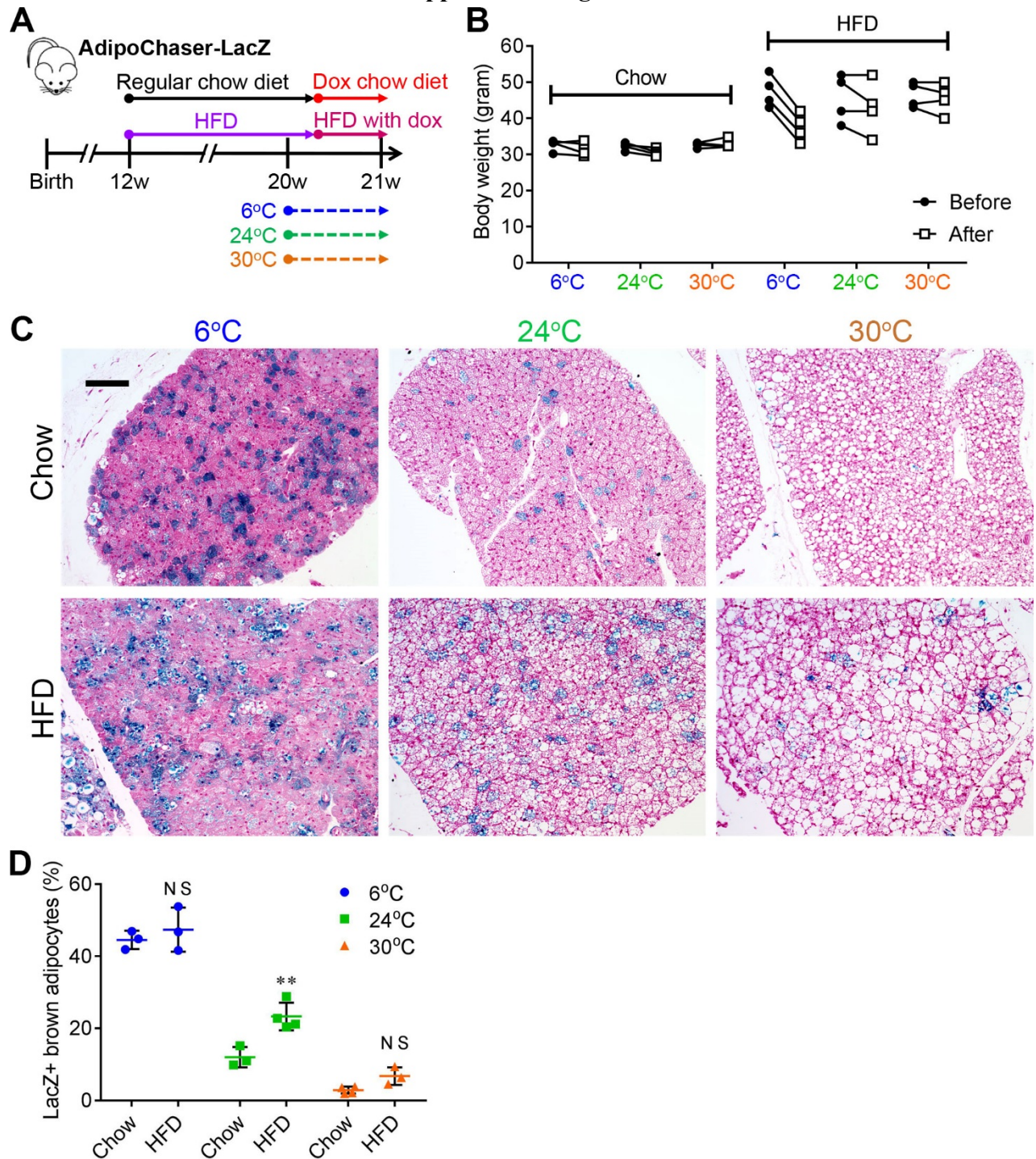
**fatty acid oxidation, TCA cycle, ROS, and succinate metabolism.** (A) Distribution of *Gpd1* and *Hk2* expression within tSNE plot, showing genes related to glycolysis are enriched within the BA-H cluster. (B) Distribution of *Acox1*, *Dbi*, *Acadvl*, *Etfb*, *Etfb*, and *Decr1* expression within tSNE plot, showing genes related to fatty acid oxidation are enriched within the BA-H cluster. (C) Distribution of *Cs*, *Mpc1*, *Pdk4*, and *Slc25a1* expression within tSNE plot, showing genes related to TCA cycle are enriched within the BA-H cluster. (D) Distribution of *Cat* and *Sod1* expression within tSNE plot, showing genes related to ROS are enriched within the BA-H cluster. (E) Distribution of *Sucla2* and *Suclg1* expression within tSNE plot, showing genes related to succinate metabolism are enriched within the BA-H cluster.

### Supplemental Figure 7



**Supplemental Figure 7. Single-cell RNA sequencing reveals gene enriched in BA-L cluster and BA-H sub-clusters.** (A) Distribution of *Fabp5* expression within tSNE plot. (B) Distribution of *Cav1* and *Cav2* expression within tSNE plot, these membrane-bound structural and signaling proteins are essential for cell-to-cell trafficking. *Cav1* and *Cav2* expression are enriched within the BA-L cluster. (C) Distribution of *Ckb* expression within tSNE plot, a key enzyme in creatine metabolism is enriched within the BA-L cluster. (D) Distribution of *Cldn5* expression within tSNE plot, a key regulator of tight junction is enriched within the BA-L cluster. (E) Distribution of *Pparg* and *Cebpa*, the master regulators of adipogenesis and adipocyte function expression within tSNE plot. (F) Distribution of *Retn* expression within tSNE plot, showing the white adipocyte-specific gene is enriched within the white adipocyte cluster. (G) Distribution of *Adrb3* expression within tSNE plot. (H) Distribution of *Ppargc1a*, *Ppargc1b*, *Ebf2*, and *Irs2*, which are genes enriched in the BA-H1, BA-H2 sub-clusters.

### Supplemental Figure 8



**Supplemental Figure 8. Recruiting of adiponectin high-expressing brown adipocytes during cold exposure is not affected by high fat diet feeding.** (A) 12-week-old AdipoChaser-LacZ male mice were kept on chow or HFD for 8 weeks. Mice were then exposed to 6°C, 30°C, or kept at 24°C for 7 days, during the last 4 days, mice were switched to dox-containing chow diet or HFD. (B) Body weight of these mice before and after 7 days of temperature switch. n = 4 mice (chow and HFD, all temperatures). (C) Representative X-gal staining of BAT from chow diet or HFD fed mice kept at 6°C, 24°C, or 30°C. Scale bar: 100  $\mu$ m. (D) Quantification of the percentage of LacZ-positive brown adipocytes in the total brown adipocytes. n = 3 mice (6°C, chow and HFD); 3 mice (24°C, chow); 4 mice (24°C, HFD); 4 mice (30°C, chow); 3 mice (30°C, HFD). In D, data represent mean  $\pm$  s.d. of biologically independent samples, \*\*P < 0.01 between the two diets of mice exposed to the same

temperature. Statistical significance was assessed using a two-way ANOVA followed by Tukey's multiple comparisons test. NS, not significant. All images are representative of three independent experiments.

## Supplemental Video

**Supplemental Video 1. Mouse brown adipose tissue UCP1 and sympathetic neuron 3D imaging.** 10 weeks old UCP1-GFP mice were treated with dox-containing diet for 4 days before tissue harvest. PACT-cleared BAT from UCP1-GFP mice and immunolabeled with GFP (green) and tyrosine hydroxylase (TH) (purple) antibody.

## Supplemental Method

### Mice

Mice were maintained in a 12-h dark/light cycle and housed in groups of three to five with unlimited access to water and food (chow diet, number 5058, lab diet; High fat diet, number D12492, Research Diets; doxycycline chow diet [600 mg/kg], S4107, Bio-Serv; or doxycycline high-fat diet [600 mg/kg], S5867, Bio-Serv, as described for individual experiments). All mice were on a pure C57BL/6J background. *Adn-rtTA* and *Ucp1-rtTA* mouse lines were generated as previously described (48, 49). *TRE-Cre* (Jax stock no. 006234), *TRE-GFP* (Jax stock no. 018913), *Rosa26-loxP-STOP-loxP-LacZ* (Jax stock no. 003309), and *Rosa26-loxP-STOP-loxP-mT/mG* (Jax stock no. 007676) mouse lines were obtained from the Jackson Laboratories. *Rosa26-loxP-STOP-loxP-YFP* (Jax stock no. 006148) mouse line was obtained from Dr. Sangeeta Dhawan. The Institutional Animal Care and Use Committees of City of Hope, Duarte, have approved all animal experiments.

### Thermoneutral or cold exposure and $\beta$ 3-adrenoceptor agonist treatment

Mice were placed in individual cages in the temperature and light controlled rodent incubator (Powers Scientific) at thermoneutrality (30 °C) or cold (6 °C), with free access to food and water. For the  $\beta$ 3-adrenoceptor agonist treatment experiment, mice were treated with CL-316243 (C5976, Sigma) in PBS at 1 mg/kg body weight daily through intraperitoneal injection for 7 days.

### X-gal staining of BAT

Mice were anesthetized and transcardially perfused with 0.2% glutaraldehyde in PBS. Inter-scapular BAT was carefully dissected, immersed in a 6-cell culture dish containing 0.2% glutaraldehyde and then chopped into small slices. The sliced BAT was washed with rinse buffer (100 mM sodium phosphate, 2 mM MgCl<sub>2</sub>, 0.01% sodium deoxycholate and 0.02% NP-40) three times and then soaked in  $\beta$ -gal staining buffer (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide and 1 mg/ml  $\beta$ -gal substrate in rinse buffer) for 48 h at room temperature with mild shaking. After staining, the tissue sliced tissue was moved into 10% formalin overnight for post-fixation. The post-fixed tissue was processed with a standard paraffin tissue-embedding protocol to produce



sections. After paraffin embedding and sectioning, tissue sections were counterstained with nuclear fast red, the staining was imaged using an inverted fluorescence phase contrast microscope (BZ-X710, Keyence).

### **Transmission electron microscopy**

X-gal stained BAT tissue slices were washed in H<sub>2</sub>O three times for 5 min and then fixed in 2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M Cacodylate buffer (Na(CH<sub>3</sub>)<sub>2</sub>AsO<sub>2</sub> · 3H<sub>2</sub>O), pH7.4, overnight at 4 °C. The tissue was washed three times with 0.1 M cacodylate buffer, pH7.4 and post-fixed with 1% OsO<sub>4</sub> in 0.1 M Cacodylate buffer for 30 min and washed three times with 0.1 M Cacodylate buffer. The tissue was then dehydrated through ethanol series (30%~100%), embedded with Eponate and polymerized at ~64 °C for 48 hours. Ultra-thin sections (~70 nm thick) were cut and stained with 2% uranyl acetate for 10 minutes followed Reynold's lead citrate staining for 1 minute. Electron microscopy images were taken on an FEI Tecnai 12 transmission electron microscope equipped with a Gatan Ultrascan 2K CCD camera.

### **H&E and immunofluorescence staining**

For H&E staining, BATs were excised and fixed in 10% phosphate-buffered saline (PBS)-buffered formalin. Following paraffin embedding and sectioning, tissue sections were stained with hematoxylin and eosin stain (H&E). For immunofluorescence staining, formalin-fixed, paraffin-embedded sections from the BAT of AdipoChaser-mT/mG and AdipoChaser-YFP mice were deparaffinized in xylene and rehydrated in a graded series of ethanol to ddH<sub>2</sub>O. Slides were placed in 10 mM sodium citrate buffer (C9999, Sigma) and boiled for 30 min, then blocked in PBST with 5% BSA. Primary antibody used was perilipin 1:500 (NB100-60554, Novus), Cleaved-Caspase3 1:200 (5A1E, #9664, Cell Signaling Technology), COX IV 1:200 (3E11, #4850, Cell Signaling Technology), SDHA 1:200 (D6J9M, #11998, Cell Signaling Technology), PPAR $\gamma$  1:200 (X26H12, #2435, Cell Signaling Technology), FABP4 1:200 (D25B3, #3544, Cell Signaling Technology), ATGL 1:200 (30A4, #2439, Cell Signaling Technology), ELOVL6 1:200 (ab69857, Abcam), UCP1 1:200 (ab23841, Abcam) and GFP/YFP 1:500 (ab13970, Abcam); secondary antibodies (1:200 dilution) used were Alexa Fluor 594-conjugated donkey anti-goat IgG(H+L) (A-11058, Invitrogen), , Alexa Fluor 647-

conjugated donkey anti-Rabbit IgG(H+L) (A-31573, Thermo Fisher Scientific), and Cy<sup>TM</sup>3 AffiniPure F(ab')<sub>2</sub> Fragment Donkey Anti-Chicken IgY (IgG) (H+L) 1:200 (703-166-155, Jackson ImmunoResearch). Slides were counterstained with DAPI and mounted with prolong Anti-Fade mounting medium (P36931, Invitrogen). Bright-field and fluorescence images were acquired using an inverted fluorescence phase contrast microscope (BZ-X710, Keyence).

### **Isolation of mouse primary brown adipocytes**

Brown adipocytes from the inter-scapular BAT were obtained from 10-week-old male mice. Briefly, BATs were carefully dissected and were cleaned from contaminating muscle and white adipose tissue. BATs were minced into small pieces with scissors and digested in collagenase-dispase buffer (17703034, Gibco) for 1 hour at 37 °C with shaking at 110 rpm. The cell suspension was filtered through a 200 µm nylon filter (146487, Spectrum) to remove undigested tissues followed by 3 volumes of wash buffer (DMEM/F12, Life technologies 10565018) containing 10% FBS and the filtrate was centrifuged at 200 g for 8 minutes at room temperature. The floating brown adipocytes were transferred and re-suspend with wash buffer for 3 times with the same centrifugation condition.

### **Separation of two brown adipocyte subpopulations**

For WT mice: since the two brown adipocyte subpopulations have distinct lipid droplet sizes, they have different densities. The BA-H adipocyte subpopulation, especially when mice are exposed in cold temperature, has a density slightly higher than the culture media, but lower than the SVFs. Meanwhile, the BA-L subpopulation has a density lower than the culture media and would float like the classical white adipocytes. Thus, we were able to separate the two subpopulations by mild centrifugation. Briefly, BATs from 8 mice were digested using the same digestion method described above. After filtering cells through the 200 µm nylon filter, the cell suspension was centrifuged at 100g for 5 minutes. The supernatant was collected as the BA-L subpopulation. The rest cell mixture in the pellets were pooled together into one tube and centrifuged at 100g for 5 minutes. After the centrifugation,

the pellet formed several layers of cells (with different colors), the top thick layer is the BA-H subpopulation and the top 1~2  $\mu\text{m}$  of this layer was carefully moved to a new tube for the following experiments.

For AdipoChaser-mT/mG mice: mice were cold exposed for 7 days, during the last 4 days of cold exposure, mice were switched to dox-containing chow diet. BATs were digested using the same digestion method described above. After filtering cells through the 200  $\mu\text{m}$  nylon filter, the cell suspension was centrifuged at 100g for 5min. The floating brown adipocytes were collected as BA-L subpopulation, followed by three washes in the wash buffer with the same centrifugation condition. The pellet was collected as a mixture of BA-H subpopulation and SVF. This mixture was further separated with the EasyPlate™ Magnet based method, taking advantage of the membrane-located GFP expression. The mixed cell suspension was incubated with PE-anti-GFP antibody (clone FM264G, #338004, Biolegend) and treated with the EasySep™ reagents (17656, Stem Cell Technologies) according to the manufacturer's instructions. The GFP positive brown adipocytes were then separated using the EasyPlate™ EasySep™ Magnet (18102, Stem Cell Technologies).

### **Respiration measurements**

Cellular Oxygen consumption rate (OCR) was measured with Agilent Seahorse XF24 extracellular flux analyzer (Agilent Technologies). The two brown adipocyte subpopulations from 10-week-old male WT and AdipoChaser-mT/mG mice were separated and collected with the method described above. After washing with the XF24 assay medium, the brown adipocyte was resuspended in Matrigel (356231, Corning) and seeded in a XF24 islet capture microplate (101122-100, Agilent Technologies) at a density of 6,000 cells per well and covered with the islet capture screen. The same number of primary white adipocytes (isolated from the inguinal white adipose depot from the same mice) was used as a control. Primary SVF from BAT was used as another control, these cells were first cultured in a dish, and then the cells that attached were collected for the assay (50,000 SVF cells per well). After plating the cells, 500ul XF assay medium, containing 1mM pyruvate, 2mM glutamine, 10mM glucose, was added to each well and the oxygen consumption was measured using XF cell mito stress test kit (103015-100, Agilent Technologies) according to the manufacturer's instructions. Basal respiration was measured three times

followed three oxygen consumption measurement after the injection of 8uM oligomycin, 10uM FCCP, 5uM rotenone/antimycin.

### **Mitochondrial membrane potential**

Mitochondrial membrane potentials of primary brown adipocytes were measured through MitoTracker Deep Red (far red-fluorescent dye) staining. Briefly, brown adipocytes from 10-week-old AdipoChaser-mT/mG mice were freshly collected and incubated with 100nM MitoTracker™ Deep Red FM (M22426, Invitrogen) for 30 min at 37 °C. After washing twice with washing medium, the stained brown adipocytes were pipetted onto a glass slide with a coverslip on the top. Tomato (abs/em ~554/581 nm) and GFP (abs/em ~488/509 nm) fluorescence of brown adipocyte and MitoTracker Deep Red (abs/em ~644/665 nm) of active mitochondria were then monitored using a fluorescent microscope (BZ-X710, Keyence).

### **Three-dimensional imaging of BAT**

For the three-dimensional fluorescence-imaging of BAT, we utilized passive CLARITY technique (PACT) as described (50) with minor modification. Briefly, 10-week-old *Ucp1*-GFP mice were anesthetized and transcardially perfused with PBS containing 0.5% NaNO<sub>2</sub> (237213, Sigma) and 10 U/ml heparin (H3149, Sigma), followed by ice-cold 4% PFA. Inter-scapular BAT was dissected and post-fixed in 4% PFA at 4 °C overnight. The fixed BAT was sliced into 1-mm-thick slices and incubated in hydrogel monomer solution (4% acrylamide [#1610140, Bio-Rad] and 0.25% VA-044 [G20864, FUJIFILM] in PBS) at 4 °C overnight. The hydrogel solution was degassed in the nonvacuum desiccator for 30 min and then polymerized for 5 hours at 37 °C. The excess hydrogel was removed from the tissue, and the tissue was incubated in clearing solution (8% SDS in borate buffer [28341, Thermo Scientific] PH8.5) at 37 °C with gentle agitation until visible clearing of the tissue was observed. The cleared tissue was gently washed in PBS at room temperature for 2 hours 3 times and overnight with agitation until bubbles are gone. The tissue was then immersed in rabbit anti-TH polyclonal antibody 1:200 (ab152, Abcam) and goat anti-GFP (FITC) polyclonal antibody 1:200 (ab6662, Abcam) solution containing 0.1% Triton X-100 in PBS at room temperature for 2 days. Tissue was washed with PBS for 1 hour at room temperature for 3 times and

incubated with donkey anti-rabbit IgG H&L-Alexa Fluor 647 secondary antibody 1:200 (A31573, Invitrogen) solution containing 0.1% Triton X-100 in PBS at room temperature for 2 days. The stained tissue was washed 3 times with PBS for 1 hour, submerged in refractive index matching solution RIMS (30 ml of 20 mM phosphate buffer PH 7.5, 40 g Histodenz, 500  $\mu$ l 10% Sodium Azide solution) and incubated at room temperature until it reaches the desired transparency. The tissue was then placed in glass slides with 0.5-1.0 mm thick iSpacer, and the iSpacer was overfilled with RIMS and sealed with a coverslip. Three-dimensional images of the cleared BAT were acquired with tile scanning using a confocal laser scanning microscope (LSM 880, Zeiss) with 10X and 20X magnifications and the Zeiss Zen black software.

### **Single-cell RNA-sequencing and analysis**

Brown adipocytes from 10-week-old C57BL/6J male mice were freshly collected and resuspended in PBS containing 0.04% BSA at a concentration of 700~1,200 cells/ $\mu$ l. Cell number and viability were measured using a TC20 Automated Cell Counter (BioRad). Single-cell RNA libraries were prepared according to the Chromium™ Single Cell 3' Reagent Kits v2 User Guide (10x Genomics). Approximately 10,000 cells were loaded on a Chromium single cell Controller instrument (10x Genomics) to generate single cell gel beads in emulsion (GEMs). The barcoded sequencing libraries were constructed using the Chromium Single-Cell 3' Library Kit (10x Genomics) for enzymatic fragmentation, end-repair, A-tailing, adaptor ligation, ligation cleanup, sample index PCR, and PCR cleanup. Libraries were sequenced with a Hiseq 2500 instrument (Illumina) with a depth of 50 k-100 k reads per cell. Raw sequencing data were processed using the 10x Genomics Cell Ranger pipeline (version 2.0) to generate FASTQ files and aligned to mm10 genome to gene expression count. The subsequent data analysis was performed using “Seurat” package and R scripts. Cells with mitochondrial read rate > 50% and < 200 detectable genes were considered as low-quality and filtered out. Normalized and scaled data were clustered using the top significant principal components of highly variable genes. The t-distributed stochastic neighbor embedding (t-SNE) algorithm was used to visualize the resulting clusters. Cluster-specific markers were identified to generate heatmap and feature plots in the identified cell clusters. Genes were

compared between different clusters using Bioconductor package “Limma” on normalized data. Gene Set Enrichment Analysis (GSEA) v3 was performed using genes ranked by the fold changes between different clusters to evaluate the significant activation of the C2 KEGG gene sets in MSigDb (<http://software.broadinstitute.org/gsea/msigdb/collections.jsp>). For cell type classification, we identified the top 20 genes that are significantly higher expressed in each cluster compared to other clusters, then we used the CTen Cell Type Enrichment Analysis and uploaded all the identified gene symbols. All BA-L, BA-H, and WA population are identified as adipocytes. More specifically, the BA-H population is identified as brown adipocytes, the WA population is identified as white adipocytes, and BA-L population was not identified with a specific adipocyte type. Since the BA-L population is clustered very close to the BA-H population, and they have much higher UCP1 expression than the WA subpopulation, we define these cells as brown adipocyte with low thermogenic activity.

### **Quantitative real-time RT-PCR**

Total RNA from BAT tissues was isolated using the Trizol reagent (15596026, Thermo Fisher Scientific). First-strand cDNA was synthesized with iScript cDNA synthesis kit (1708891, Bio-Rad) according to the manufacturer’s instructions. Real-time quantitative PCR was done with the SYBR Green PCR system (A25742, Applied Biosystems), using *Tbp* as an internal control for normalization. All primer sequences are: *Ucp1* (sense: TCTCAGCCGGCTTAATGACTG; anti-sense: GGCTTGCATTCTGACCTTCAC), *Adipoq* (sense: GTTGCAAGCTCTCCTGTTCC; antisense: ATCCAACCTGCACAAGTT), *Pparg1a* (sense: ACAACGCGGACAGAATTGAG; antisense: TTCTCAAGAGCAGCGAAAGC), *Cidea* (sense: TCCTATGCTGCACAGATGACG; antisense: TGCTCTTCTGTATCGCCCAGT), *Tbp* (sense: CAGCCTTCCACCTTATGCTC; antisense: CCGTAAGGCATCATTGGACT)

### **Fatty acid uptake assay**

Primary brown adipocytes were plated at 50,000 cells/100  $\mu$ L/well in a 96 well black wall/clear bottom poly-D lysine plate. Fatty Acid Uptake Kit (MAK156, Sigma) was used to measure the rate of fatty acid intake. Briefly,

100  $\mu$ l of fatty acid mixture was added into the well, and incubated for 10 min, the fluorescence signal was measured with the SPARK® multimode microplate reader (TECAN) at  $\lambda_{ex} = 485/\lambda_{em} = 515$  nm.

## References

- 48 Sun, K. *et al.* Dichotomous effects of VEGF-A on adipose tissue dysfunction. *Proceedings of the National Academy of Sciences* **109**, 5874-5879, doi:10.1073/pnas.1200447109 (2012).
- 49 Sun, K. *et al.* Brown adipose tissue derived VEGF-A modulates cold tolerance and energy expenditure. *Mol Metab* **3**, 474-483, doi:10.1016/j.molmet.2014.03.010 (2014).
- 50 Treweek, J. B. *et al.* Whole-body tissue stabilization and selective extractions via tissue-hydrogel hybrids for high-resolution intact circuit mapping and phenotyping. *Nature protocols* **10**, 1860-1896, doi:10.1038/nprot.2015.122 (2015).