Supplemental Data

Supplemental Experimental Procedures

Cell culture

Mouse 3T3-L1 preadipocytes (ATCC) were cultured and maintained in DMEM (Hyclone, GE Healthcare) supplemented with 10% newborn calf serum containing penicillin (100U/ml)-streptomycin (100µg/ml). White adipocyte differentiation was induced by treating cells for 48 h in 10% FBS–DMEM containing 0.5 mM isobutylmethylxanthine, 1 µM dexamethasone, 167 nM insulin, followed by 48 h of 10% FBS–DMEM with 167 nM insulin. After this induction period, cells were maintained in 10% FBS–DMEM until harvest at Day 8. For induction into beige adipocytes, differentiated 3T3-L1 cells were treated with 10 µM Forskolin for 4-6 hrs. HEK293T and HeLa cells (ATCC) were maintained in DMEM supplemented with 10% FBS-DMEM containing penicillin (100U/ml)-streptomycin (100µg/ml). Cultures were maintained at 37°C and 5% CO2.

Animal Studies

All experiments involving animals were approved by the Institutional Animal Care and Use Committee or in accordance with approved institutional protocols of Agency for Science, Technology and Research (A*STAR). Mice with conditional HOXC10$^{flox/flox}$ alleles were generated by insertion of loxP targeting sequences flanking exon 2 of the HOXC10 gene. This line was generated by Shanghai Biomodel Organism Co., Ltd., China. Adiponectin-Cre and Myf5-Cre mice were obtained from The Jackson Laboratory. Mice were maintained at room temperature unless otherwise specified, with a 12 hr light-dark cycle and free access to food and water. For cold exposure studies, eight to ten-week old C57BL/6J (wild-type) mice were individually housed and exposed to 4°C for 7 days. For chronic β-3 adrenergic agonist

administration, saline or β3-adrenergic receptor (AR) agonist CL316,243 compound (1mg/kg) was intraperitoneally administered daily for 7 days.

Plasmid constructs

The expression of full-length cDNA for human HOXC10 was constructed as described previously (22). The mammalian expression of human ubiquitin with HA-tag was a gift from Dr Edward Yeh (Addgene plasmid #18712). The full-length cDNA sequence for human KTCD2, KCTD5 and KCTD17 was cloned into pLenti-HIKO lentiviral vector with a V5 (KCTD2), Myc (KCTD5) or HA (KCTD17) tag sequence.

Immunoprecipitation and Immunoblotting

HEK293T and HeLa cells transfected with Flag-hHOXC10 were washed in PBS and lysed in lysis buffer containing 10 mM Tris pH 7.5, 150 mM NaCl, 50 mM NaF, complete protease and phosphatase cocktail inhibitors (Roche). Lysates were cleared by centrifugation and immunoprecipitated with anti-FLAG affinity gel (Sigma) overnight at 4˚C. Lysates were resolved by SDS-PAGE, and transferred onto PVDF membranes (Invitrogen) following standard procedures. Blots were incubated with antibodies against FLAG (Sigma, F1807), HOXC10 (Abcam, ab153904), Myc (Cell Signaling Technology, 2276), V5 (Abcam, Ab27671), HA (BioLegend, 901502) and KCTD2/5/17 (Proteintech 15553-1-AP). KCTD2/5/17 antibody is raised against KCTD5 but also cross-reacts with KCTD2 and KCTD17.

Mass Spectrometry Analysis

3T3-L1 cells expressing Flag-hHOXC10 were maintained and differentiated using either heavy or light SILAC media. Cells were treated with DMSO or 10μM forskolin for 2.5 hours and

total cell lysate was harvested separately. After protein quantification, 6mg of total cell lysates were mixed in a 1:1 ratio (H:L) and immunoprecipitated using anti-Flag beads or mouse IgG, followed by elution with FLAG peptide. Proteins pulled-down will be sent for mass spectrometry-based quantitative proteomics. Eluted protein samples were separated using one-dimensional 4–12% NuPage Novex Bis–Tris Gel (Life technologies), stained with the Colloidal Blue Staining Kit (Invitrogen) followed by in-gel trypsin digestion using published procedures (# Shevchenko A, Tomas H, Havlis J, Olsen JV, Mann M (2006) In-gel digestion for mass spectrometric characterization of proteins and proteomes. Nat Protoc 1: 2856–2860). Samples were analyzed on an Orbitrap XL (Thermo Fisher. Survey full scan MS spectra (m/z 310–1400) were acquired with a resolution of r = 60,000, an AGC target of 1e6 and a maximum injection time of 700ms. The ten most intense peptide ions in each survey scan with an ion intensity of > 2000 counts and a charge state ≥ 2 were isolated sequentially to a target value of 1e4 and fragmented in the linear ion trap by collisionally-induced dissociation using a normalized collision energy of 35%. A dynamic exclusion was applied using a maximum exclusion list of 500 with one repeat count and exclusion duration of 30 s. Peptide and protein quantification was performed with MaxQuant v 1.3.0.5 using uniprot 2014 mouse fasta with trypsin/P selection and maximum cleavage of 2. Cysteine carbamidomethylation was searched as a fixed modification, and protein N-term acetylation and oxidized methionine were searched as variable modifications. Labelled arginine and lysine were specified as fixed modification. Proteins were considered identified when supported by at least one unique peptide with a minimum length of seven amino acids. Peptide FDR and protein FDR were set at 0.01.

Cycloheximide Chase Assay

Following differentiation, 3T3-L1 cells were treated with DMSO, forskolin or protein kinase A (PKA) inhibitor (H89) in the presence of cycloheximide (Sigma). Cells were harvested at

indicated time points, and total protein concentrations were determined by Bradford assay. Proteins were separated by SDS-PAGE, immunoblotted with anti-FLAG and anti-HOXC10, and analyzed by Odyssey infrared imaging system (LICOR Biosciences).

Proteasomal activity

We used the Proteasome Activity Fluorometric Assay (UBP Bio) according to the manufacturer's instructions to measure chymotrypsin-like, trypsin-like and caspase-like activities. In each assay, for each sample, MG132 was included as background control to determine the specific proteasomal activity Δ(slope) over time.

***In vitro* ubiquitination assay**

HeLa cells were transfected with Flag-hHOXC10 and HA-Ubiquitin plasmids for 24 hours prior to treatment with MG132 (Merck) for 8 h before harvested. Cells were washed in PBS and lysed in lysis buffer described above, with an addition of 10 mM N-ethylmalemide and 10 mM Iodoacetic acid (Sigma). At least 1 mg of total protein was used for the *in vivo* ubiquitination assay. The precipitates were subjected to immunoblotting described above via wet transfer. Proteins were being transferred at 30V for 3hours. Blots were incubated with antibodies against ubiquitin (Santa Cruz, sc-8017), FLAG (Sigma, F1807), HOXC10 (Abcam, ab153904) and KCTD2/5/17 (Proteintech 15553-1-AP).

Supplemental Figure 1: Phenotypic analysis of AHKO mice fed regular chow diet. (A) Growth curve illustrating weekly total body weight and cumulative body weight change and (B) total body length and cumulative body length change in AHKO mice compared to wild type controls starting from 6 weeks of age, kept at room temperature and fed chow diet, n=14. (C) *In vivo* body composition by EchoMRI of total fat and total lean mass and (D) isolated adipose tissue depots and liver of control and AHKO mice at 20 weeks of age. (E) Relative mRNA levels of Ucp-1-independent thermogenic markers, n=10. (F) Plasma insulin levels and (G) Fed and fasting blood glucose levels in control and AHKO mice. (H) Average daily food consumption in control and AHKO mice.

Supplemental Figure 2: Phenotypic and metabolic analysis of Myf5 HKO mice fed regular chow diet. (A) Total body weight and cumulative body weight change starting from 6 weeks of age, kept at room temperature. (B) *In vivo* body composition by EchoMRI of total fat and total lean mass in control and Myf5 HKO mice at 18 weeks of age. Common adipogenic and thermogenic browning genes in (C) brown adipose tissue and (D) subcutaneous WAT. (E) Body temperature was measured following 7-day cold challenge at 10weeks of age. (F) Glucose tolerance test and (G) area under curve of control and Myf5 HKO mice between 15-16 weeks of age. (H) Mean average heat production, (I) respiratory exchange ratio (RER) and (J) activity level determined at 14 weeks of age, by indirect calorimetry in metabolic chambers during the 3-day measurement (after an initial 48 hours of acclimation period). Dark and light phase cumulative means within dark or light phase and each time point were compared by student's t-test between Myf5 HKO mice and their littermate male control mice. Data are presented as mean ± SEM. *$p<0.05$ (n=4).

Supplemental Figure 3: Phenotypic and metabolic analysis of Myf5 HKO mice on high fat diet intervention for 15 weeks. (A) Total body weight and cumulative body weight change starting from 6 weeks of age, kept at room temperature. (B) *In vivo* body composition by EchoMRI of total fat and total lean mass in control and Myf5 HKO mice at 20 weeks of age. (C) Representation of photograph and (D) H&E staining of BAT of control and Myf5 HKO mice. Scale bar = 20μm. (E) Relative mRNA levels of common adipocyte genes and brown/beige-selective thermogenic markers in BAT. (F) Representation of photograph and (G) H&E staining of subcutaneous WAT of control and Myf5 KO mice. Scale bar = 20μm. (H) Relative mRNA levels of common adipocyte genes and brown-selective thermogenic markers in subcutaneous WAT. (I) Body temperature was measured following 7-day cold challenge at 10weeks of age. (J) Glucose tolerance test and (K) area under curve of control and Myf5 HKO mice between 15-16 weeks of age. (L) Mean average heat production, (M) respiratory exchange ratio (RER) and (N) activity level determined at 14 weeks of age, by indirect calorimetry in metabolic chambers during the 3-day measurement (after an initial 48 hours of acclimation period). Dark and light phase cumulative means within dark or light phase and each time point were compared by student's t-test between Myf5 HKO mice and their littermate male control mice. Data are presented as mean ± SEM. *$p<0.05$ (n=8).

Supplemental Figure 4: Effects of β-Adrenergic Receptor Activation in BAT of AHKO mice. Representative H&E staining and corresponding relative mRNA levels of brown fat selective markers in (A-B) gonadal WAT (gWAT) and (C-D) BAT from control and AHKO mice at 20weeks of age fed on chow diet. Scale bar = 100μm. n=10. (E) Representative H&E staining and (F) Relative mRNA levels of brown fat selective markers of BAT of control and AHKO mice treated with CL316,243 to induce browning. Scale bar = 100μm. n=4. Data are presented as mean ± SEM. Student's t test, *$p<0.05$, **$p<0.01$, ***$p<0.001$. n=4.

Supplemental Figure 5: *HOXC10* mRNA expression is increased in subcutaneous WAT following cold exposure and β3-Adrenergic Activation. (A) *HOXC10* and corresponding *Ucp-1* mRNA levels in subcutaneous WAT isolated from wild type C57BL6/J mice held at room temperature (RT) or exposed to 4°C. (B) *HOXC10* and corresponding *Ucp-1* mRNA levels in subcutaneous WAT isolated from wild type C57BL6/J mice treated daily with CL-316,243 (1 mg/kg) or vehicle (saline) for 7days (n=5mice per group). Data are presented as mean ± SEM. Student's t test, *p<0.05, **p<0.01, ***p<0.001.

Supplemental Table 1: SILAC experiment to identify potential HOXC10 interacting proteins. L3: Total cell lysates from differentiated adipocytes expressing flag-tagged HOXC10 (light and heavy labelled) were used for IP with anti-Flag antibody (heavy sample, H) and mouse IgG (light sample, L). Flag peptide elution were used for both samples. L5: Differentiated adipocytes expressing flag-tagged HOXC10 (light and heavy labelled) were treated with 10μM forskolin (heavy sample, H) or DMSO (light sample, L) for 2.5h. Total cell lysates were used for IP with anti-Flag antibody and Flag peptide elution was performed on both samples. The E3 ligases KCTD2, KCTD5 and KCTD17 (highlighted in yellow) were found to potentially interact with HOXC10 (high ratio H/L, L3), however the ratio H/L count of KTCD5 (1) indicates a potentially low confident ID.