Metabolism Clinical and Experimental 101 (2019) 153977

Contents lists available at ScienceDirect

Metabolism Clinical and Experimental

journal homepage: www.metabolismjournal.com

Basic Science

Preadipocyte factor 1 regulates adipose tissue browning via TNF-α-converting enzyme-mediated cleavage

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ARTICLE INFO

Article history:
Received 26 July 2019
Accepted 11 September 2019

Keywords:
Adipocyte
Beige fat
Energy homeostasis
Preadipocyte factor 1
Thermogenesis

ABSTRACT

Background: Increasing adaptive thermogenesis in adipose tissue may be a potential therapeutic target for overcoming obesity and obesity-related disorders. Preadipocyte factor 1 (Pref-1), a preadipocyte secreted protein, plays an inhibitory role in adipogenic differentiation. However, the role of Pref-1 in adipose tissue browning remains unknown. We investigated whether Pref-1 regulates thermogenic program and beige fat biogenesis.
Methods: Pref-1 expression levels were examined in inguinal white adipose tissue (iWAT) and differentiated 3T3-L1 adipocytes in thermogenic conditions induced by cold exposure or a beta-adrenergic stimulus (CL316,243). Overexpression and knockdown studies were performed both in vivo and in vitro to clarify the role of Pref-1 in iWAT browning.
Results: Cold exposure or CL316,243 induced a thermogenic program in adipose tissue of C57BL/6N mice and in 3T3-L1 adipocytes. Notably, Pref-1 levels were down-regulated in iWAT and adipocytes under these conditions. Overexpressing Pref-1 showed reduced thermogenic gene expressions in response to CL316,243 treatment, whereas depletion of Pref-1 augmented thermogenic program in 3T3-L1 adipocytes. Correspondingly, treating C57BL/6N mice with Pref-1 resulted in reduced expression of thermogenic and beige fat markers, a reduced rate of oxygen consumption, blunting of UCP1 expression and beige fat formation in iWAT in response to cold exposure or CL316,243 injection compared to the untreated mice. The opposite phenotype was observed in mice with inducible fat-specific knock-out of Pref-1. Mechanistically, these effects were regulated by modulation of TNF-α-converting enzyme activity and Pref-1 cleavage.
Conclusion: Our findings establish a novel role of Pref-1 that regulates adaptive thermogenesis. This offers a unique target for improving energy homeostasis and treating obesity.

1. Introduction

Obesity is associated with serious comorbidities, such as diabetes, cardiovascular disease, chronic kidney disease, certain cancers and mortality. Despite its alarming increase and threat to global health [1], measures that are effective at reducing adiposity are limited. Lifestyle intervention is difficult to maintain, and the use of anti-obesity drugs is often related to safety problems [2,3]. Since the identification of functional brown adipose tissue (BAT) in adult humans [4–6], biogenesis or induction of brown or beige adipocytes has been considered a promising target to improve energy homeostasis and overcome obesity. Notably, manipulation of beige adipocytes was sufficient to alter energy expenditure and glucose homeostasis in mouse models [7,8]. Although various stimuli, including environmental factors, hormones, cytokines and metabolites, have been suggested to have a browning effect [9–11], further understating of this issue is necessary.

Preadipocyte factor 1 [Pref-1; also called Delta-like protein 1 (Dlk1)] is a molecular gatekeeper of adipogenesis that is mainly expressed in preadipocytes [12,13]. It is synthesized as a transmembrane protein that contains six EGF-like repeats at the extracellular domain. Its soluble form, produced by TNF-α-converting enzyme (TACE)-induced proteolytic cleavage, is biologically active and acts in an autocrine/paracrine manner. Pref-1 expression inhibits adipocyte differentiation, and downregulation of Pref-1 is required during differentiation, affecting growth and development of adipose tissue. Pref-1 null mice display

Abbreviations: BAT, brown adipose tissue; BMP, bone morphogenetic protein; Dlk1, Delta-like protein 1; eWAT, epididymal white adipose tissue; FCCP, trifluoromethoxy carbonylcyanide phenylhydrazone; GTT, glucose tolerance test; IBMX, 3-isobutyl-1-methylzanthine; ITT, insulin tolerance test; iWAT, inguinal white adipose tissue; OCR, oxygen consumption rate; PBS, phosphate-buffered saline; Pref-1, Preadipocyte factor 1; RT, room temperature; SVF, stromal vascular fraction; TACE, TNF-α-converting enzyme; WAT, white adipose tissue.

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https://doi.org/10.1016/j.metabol.2019.153977
0026-0495/

accelerated adiposity and increased serum lipid metabolites [14]. Conversely, mice overexpressing Pref-1 show a decrease in adipose tissue mass, reduced expression of adipocyte markers, and reduced levels of adipocyte-secreted hormones. These mice also suffer from lower insulin sensitivity, hypertriglyceridemia, and impaired glucose tolerance due to reduced adipose tissue development [15].

Pref-1 may also be involved in brown adipocyte differentiation. The adipogenic program could be initiated by bone morphogenetic protein (BMP) 7-induced suppression of Pref-1 in brown preadipocytes with insulin resistance [16]. Under the control of C/EBPδ, upregulation of Pref-1 impaired thermogenic gene expression in BAT [17]. However, the role of Pref-1 in the browning of white adipose tissue remains to be elucidated. Therefore, we aimed to explore whether Pref-1 regulates a thermogenic program and beige fat biogenesis by performing overexpression and knockdown studies in both in vivo and in vitro model system. Here we show that Pref-1 regulates adipose tissue browning via TACE-mediated cleavage in response to adrenergic stimuli.

2. Materials and methods

2.1. Animal experiments

The Animal Care Committee of The Catholic University of Korea approved the experimental protocol, and all procedures performed followed the ethical guidelines for animal studies. Male C57BL/6N mice were purchased from Orient Bio Inc. (Sungnam, Korea). *aP2-CreERT2* transgenic mice were kindly provided by Prof. Hail Kim (Korea Advanced Institute of Science and Technology, Daejeon, Korea) and *Dlk1 flox* mice (STOCK *Dlk1*$^{tm1.1Jvs}$/J) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). Since *Dlk1* is paternally inherited, female *aP2-CreERT2* mice were crossed with male *Dlk1 flox* mice. To generate mice with inducible inactivation of the *Pref-1* gene in adipocytes (*aP2-Dlk1* KO), tamoxifen [2 mg/100 μl of corn oil (Sigma-Aldrich, ST. Louis, MO, USA)] was intraperitoneally (IP) injected for 5 consecutive days in 8-week-old mice. For genotyping, PCR was performed using the Allele-In-One Tail PCR Direct PCR system (Allele Biotechnology, San Diego, CA, USA) with the following conditions; *aP2-CreERT2*; 95 °C for 20 s, 56 °C for 30 s, and 72 °C for 45 s; *Dlk1 flox*; 94 °C for 1 min, 59 °C for 1 min, and 72 °C for 30 s. PCR primers are described in Supplementary Table 1.

For cold exposure studies, each mouse (male, 10 weeks of age) was housed in an individual cage in a cold room (4 °C) for the indicated time. For pharmacological induction of thermogenesis using a β3-adrenergic receptor agonist, mice were subjected to an IP injection of CL316,243 [1 mg/kg body weight/day (Sigma-Aldrich)] or vehicle (saline) for 3 consecutive days. For thermoneutral condition studies, mice were housed in a 30 °C room starting at 5 weeks of age. To test the effect of Pref-1 overexpression on white adipose tissue (WAT) thermogenesis, mice were IP injected daily with 4 μg/kg of recombinant mouse Pref-1-Fc (AdipoGen Inc., Incheon, Korea) or vehicle [phosphate-buffered saline, PBS (Thermo Fisher Scientific, Waltham, MA, USA)] for 10 days.

2.2. Measurement of body temperature

Core body temperature was measured using a digital thermometer (Tecpel CO. LTD., New Taipei City, Taiwan) with an attached rectal probe. Rectal temperature was recorded at the indicated time points.

2.3. Cell culture and transfection

3T3-L1 preadipocytes were kindly provided by Prof. Jae Bum Kim (Seoul National University, Seoul, Korea). Cells were cultured and differentiated according to published methods [18]. Human Pref-1-expressing vector [pSPORT6-hDLK1 (Open Biosystems, Huntsville, AL, USA)] and Pref-1 siRNA [siDLK (Santa Cruz Biotechnology, Santa Cruz, CA, USA)] were used to regulate Pref-1 levels in the cells. On day 6 after induction of differentiation, Pref-1-expressing vector or siPref-1 were delivered to cells via a lipid-mediated Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, USA) according to published methods [19]. pCMV and SignalSilence control siRNA (Cell Signaling Technology, Beverly, MA, USA) were used as transfection controls. For induction of the thermogenic program, cells were treated with 0.5 μM of CL316,243 for the indicated times after 24 h of transfection.

2.4. Primary adipocyte and SVF isolation

Primary adipocytes and stromal vascular fraction (SVF) cells were prepared according to published methods [20]. Briefly, inguinal WAT (iWAT) and epididymal WAT (eWAT) were washed with PBS, minced and incubated in collagenase type I (Thermo Fisher Scientific). Freshly isolated and cultured SVF cells were differentiated with the standard induction medium containing 3-isobutyl-1-methylzanthine (IBMX, Sigma-Aldrich), dexamethasone (Sigma-Aldrich) and insulin (Roche, Indianapolis, IN, USA).

2.5. RNA extraction and RT-qPCR

Total RNA was extracted from tissues and cells using the TRIzol reagent (Invitrogen). Total RNA (1 μg) was used in the RT reaction with the PrimeScript™ 1st strand cDNA synthesis kit (Takara Bio Inc., Tokyo, Japan), according to the manufacturer's instructions. All qPCR reactions were performed with PowerSYBR® Green PCR Mater Mix (Applied Biosystems, Foster City, CA, USA). PCR primer sequences are described in Supplementary Table 1.

2.6. Western blot analysis

Western blot analysis was performed as previously described [19]. In brief, total protein lysates were separated by SDS-PAGE and transferred onto an Immobilon polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). After blocking, these membranes were incubated with following primary antibodies and HRP-conjugated secondary antibodies: anti-UCP1 (1:2000, ab10983, Abcam, Cambridge, MA, USA), anti-Pref-1 (1:500, AF8277, R&D systems, Minneapolis, MN, USA), anti-total OXPHOS (1:4000, ab110413, Abcam), anti-Cytochrome C (1:4000, ab110325, Abcam), anti-HSP90 (1:2000, sc7947, Santa Cruz Biotechnology) or anti-β-actin (1:5000, A5441, Sigma-Aldrich). Proteins were visualized using an enhanced chemiluminescence kit in accordance with the manufacturer's recommendations.

2.7. Histology and immunohistochemistry

Tissues were fixed with 10% formalin solution (Sigma-Aldrich) for 16 h, processed using a standard paraffin-embedding protocol and sectioned as described previously [21]. Rehydrated sections were stained with hematoxylin and eosin (Sigma-Aldrich) or incubated with anti-UCP1 antibody. Anti-rabbit biotinylated antibodies and normal serum were purchased from Vector Laboratory (Burlingame, CA, USA). Images were acquired on an optical microscope [DMI 6000B, (Leica, Wetzlar, Germany)] and analyzed with LAS-AF software.

2.8. Oxygen consumption rate (OCR) measurement

Mitochondrial function in tissues and cells was measured using the mitochondrial stress test (Seahorse Bioscience, North Billerica, MA, USA) in accordance with manufacturer's instructions. Fully-differentiated cells were rinsed 3 times with assay medium supplemented with glucose (25 mM) and pyruvate (1 mM) and incubated in a 37 °C, non-CO_2 incubator for 1.5 h. For analysis of iWAT OCR, small (~10 mg) pieces of iWAT were washed with assay medium, and placed in the center of each well of an XF24 Islet capture microplate using an islet capture insert tool. Mitochondrial inhibitors were added at the

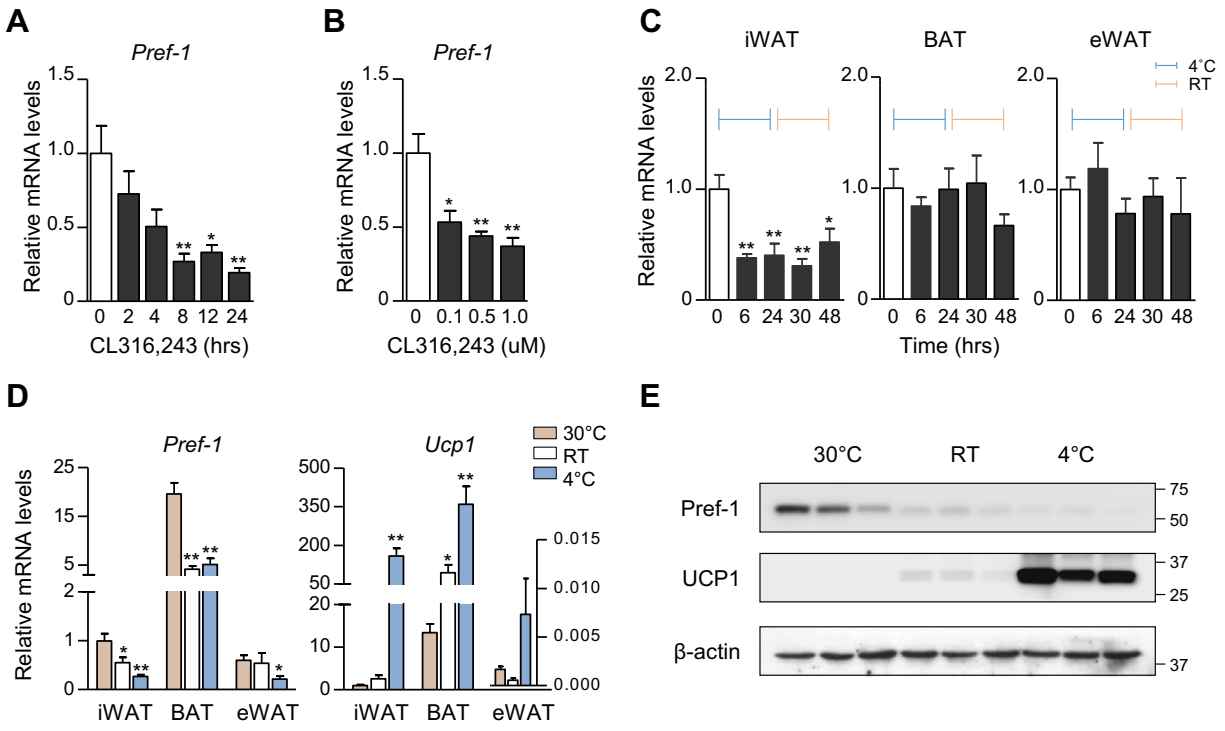

Fig. 1. Decrease in Pref-1 expression levels in response to thermogenic stimuli. Relative expression of *Pref-1* mRNA levels in differentiated 3T3-L1 cells treated with 0.5 μM of CL316,243 for indicated times (A) and indicated doses for 24 h (B) ($n = 4$). (C) *Pref-1* mRNA expression levels in inguinal white adipose tissue (iWAT), brown adipose tissue (BAT) and epididymal white adipose tissue (eWAT) from mice during cold exposure and warm acclimation ($n = 5$). mRNA (D) and protein (E) expression levels of Pref-1 and UCP1 in iWAT from mice maintained at thermoneutrality (30 °C), room temperature (RT, 23 °C) or exposed to cold temperature (4 °C) for 24 h ($n = 6$). Data are presented as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with untreated or thermoneutral controls.

following concentrations; 1.0 μM oligomycin, 0.5 mM trifluoromethoxy carbonylcyanide phenylhydrazone (FCCP), and a 0.5 mM mix of rotenone and antimycin A (Seahorse Bioscience). To induce the thermogenic program, 0.5 μM CL316,243 and 10 μM isoproterenol were added. A clark-type oxygen electrode (Mitocell S200, Strathkelvin Instruments, Scotland) was also used to measure tissue OCR. Minced iWAT was incubated in 500 μl of respiration buffer (4.5 g/l glucose, 2% BSA, 1 mM pyruvate in PBS), and OCR was quantified.

2.9. IP-glucose and insulin tolerance test

For the glucose tolerance test (GTT), a 50% glucose solution was injected IP (2 g/kg body weight) after overnight fasting. For the insulin tolerance test (ITT), human insulin was injected IP (1.0 U/kg body weight) after 5 h of food withdrawal. Blood samples were drawn from the tail vein at the indicated time after administering either glucose or insulin. Glucose levels were measured using a glucometer (Arkray, Minneapolis, MN, USA).

2.10. TACE activity assay

Fully-differentiated 3T3-L1 cells were treated with 0.5 μM CL316,243 for 60 min to determine if TACE activity was altered in the thermogenic condition. TACE activity was measured using a SensoLyte® 520 TACE (α-secretase) activity assay kit (Anaspec Inc., Fremont, CA, USA).

2.11. TACE activity modulation

To determine whether adipocyte thermogenesis is controlled through TACE-mediated cleavage of Pref-1, TACE enzymatic activity was enhanced or inhibited by recombinant mouse TACE/ADAM17 [0.5 μg/ml, (R&D systems)] or GM6001 [10 μM, (Calbiochem, San Diego, CA, USA)], respectively. At 24 h after overexpression of Pref-1, 3T3-L1 cells were rinsed and incubated with serum-free medium. Cells were pre-treated with recombinant mouse TACE/ADAM17 or GM6001 for 10 min before stimulating thermogenesis.

2.12. ELISA

Soluble Pref-1 levels were measured to assess if Pref-1 secretion is involved in adipocyte thermogenesis using the mouse protein Delta Homolog 1 (Dlk1) ELISA kit following the manufacturer's protocol (Cusabio, Wuhan, China).

2.13. Statistical analysis

All data were analyzed using Prism GraphPad Software (San Diego, CA, USA). Data are presented as the means ± SE. An unpaired or paired Student's *t*-test was performed as appropriate. A *P* value <0.05 was considered statistically significant.

3. Results

3.1. Pref-1 expression is affected by thermogenic stimuli

Pref-1 expression during adipocyte differentiation was confirmed using SVF isolated from mouse adipose tissue and the 3T3-L1 cell line. Pref-1 expression was significantly lower in differentiated adipocytes compared with preadipocytes, as has been previously shown (Supplemental Fig. 1A–B). Additionally, primary adipocytes had reduced expression of Pref-1 when compared with SVF in both iWAT and eWAT.

To test whether Pref-1 expression is affected by thermogenic stimuli, 3T3-L1 adipocytes were treated with CL316,243 at day 6 of differentiation for 24 h. As expected, thermogenic genes (*Ucp1*, *Pgc1α*, *Dio2*, *Elovl3*, *Cidea*, *Cox8b*) and beige fat marker (*Cd137*, *Tbx1*, *Tmem26*) expression markedly increased (Supplemental Fig. 2A–B), whereas Pref-1 levels were markedly reduced in a time- and dose-dependent manner (Fig. 1A–B). Decrease in Pref-1 expression levels were not due to

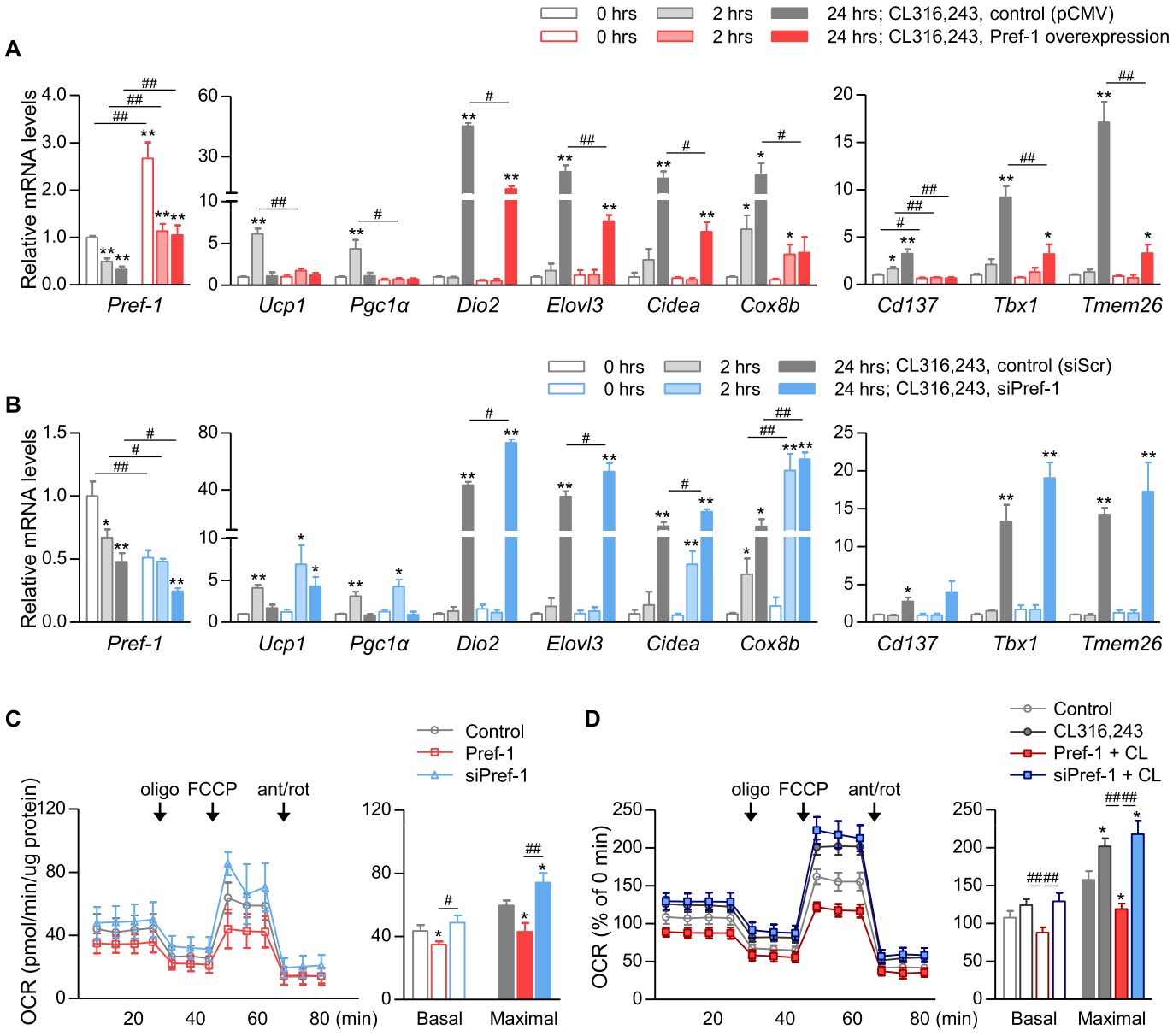

Fig. 2. Changes in adipocyte thermogenesis by Pref-1 modulation. Relative expression levels of *Pref-1*, thermogenic genes and beige fat markers in Pref-1 overexpressing (A) or knock-down (B) 3T3-L1 cells treated with 0.5 μM of CL316,243 (CL) for indicated times ($n = 4$). (C) Oxygen consumption rates (OCR) in Pref-1-overexpressing or knock-down 3T3-L1 cells. Basal and maximal respiration was measured as described in the experimental procedures ($n = 6$). (D) Effect of Pref-1 on CL-induced cellular OCR ($n = 8$). Oligo, oligomycin; FCCP, trifluoromethoxy carbonylcyanide phenylhydrazone; ant/rot, antimycin A/rotenone. Data are presented as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with untreated control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between groups.

differentiation, as this was not observed during the same period in untreated cells (Supplemental Fig. 1C). We expanded these observations to an in vivo model system. C57BL/6N mice housed at room temperature (RT) were subjected to 4 °C cold exposure for 24 h and then moved back to RT for 24 h (Supplemental Fig. 3A). Core body temperature decreased to approximately 36 °C after cold exposure and recovered rapidly after mice were returned to RT (Supplemental Fig. 3B). The expression of thermogenic genes and beige fat markers in iWAT was markedly increased by cold exposure and were decreased by returning the mice to RT (Supplemental Fig. 3C). Histological findings (beige fat formation and UCP1 staining) were compatible with these changes (Supplemental Fig. 3D). Interestingly, Pref-1 expression was decreased in iWAT by exposure to cold temperatures (Fig. 1C). In contrast, Pref-1 expression in iWAT of mice housed in thermoneutral conditions (30 °C) for 3 weeks was significantly higher than Pref-1 expression levels in mice housed at RT (Fig. 1D–E, Supplemental Fig. 4A). Temperature-dependent changes in Pref-1 and UCP1 expression were prominent in iWAT, but less clear in eWAT. These data suggest that Pref-1 expression is suppressed by thermogenic stimuli both in vitro and in vivo.

3.2. Thermogenic gene expression and OCR are affected by modulating Pref-1 in 3T3-L1 cells

We performed overexpression or knock-down studies in 3T3-L1 cells to further examine how Pref-1 affects thermogenesis. Transfection of a Pref-1-overexpression vector resulted in approximately 3-fold higher expression of *Pref-1*. Pref-1 overexpressing cells showed blunted expression of thermogenic and beige fat genes in response to CL316,243 treatment compared to controls (Fig. 2A). In contrast, opposite findings were observed by Pref-1 knock-down (Fig. 2B). CL316,243-induced expression of *Dio2*, *Elovl3*, *Cidea* and *Cox8b* was amplified by siPref-1

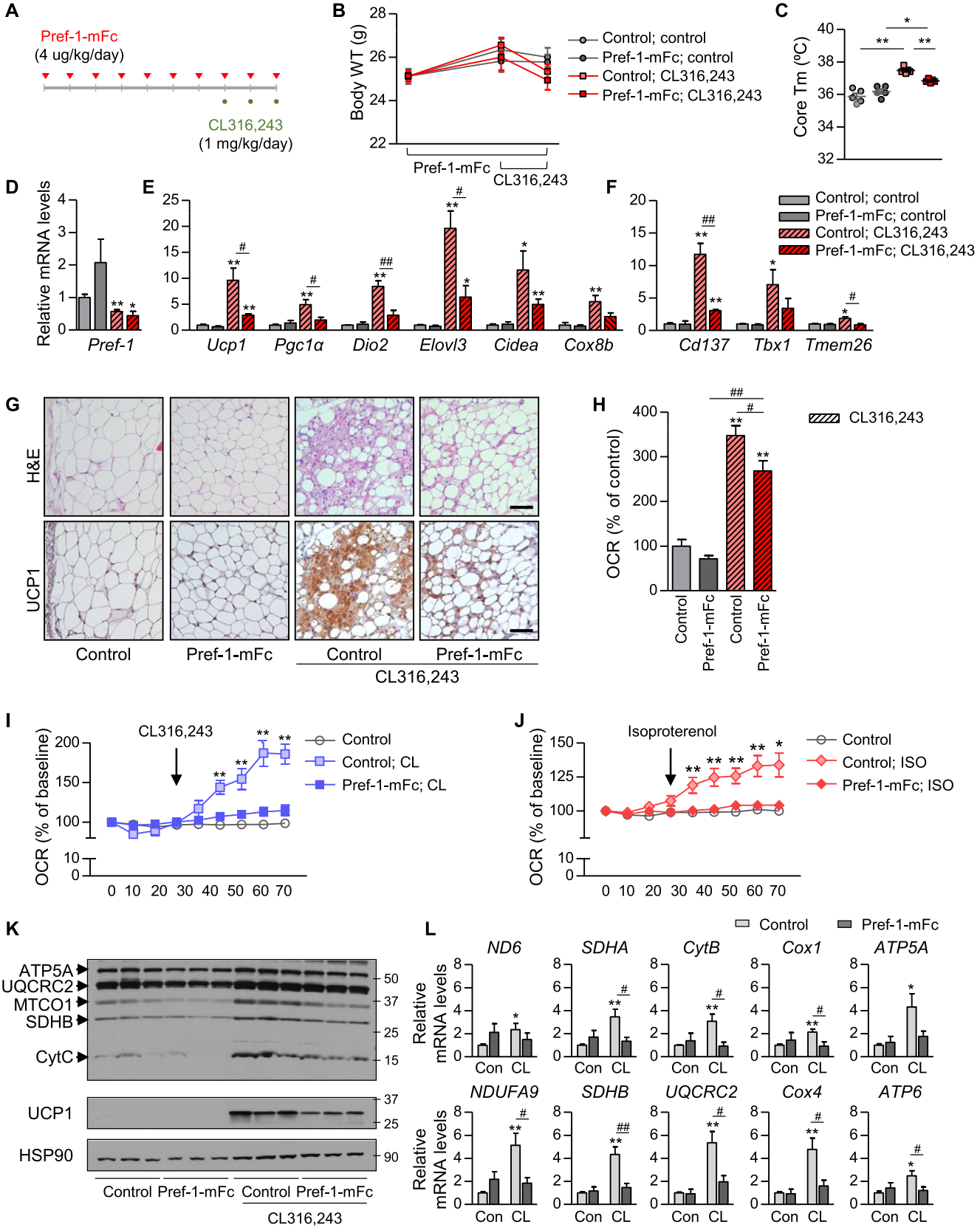

Fig. 3. Pref-1 treatment inhibits thermogenic activity and browning of inguinal white adipose tissue (iWAT) in CL316,243 (CL)-treated mice. (A) Schematic representation of the experiment. Body weight (B), core body temperature (C), relative expression levels of *Pref-1* (D), thermogenic genes (E) and beige fat markers (F) in iWAT of vehicle or Pref-1-mFc treated mice with or without CL treatment ($n = 5$). Representative histologic findings (G) (scale bar, 50 um) and tissue oxygen consumption rates (OCR) (H) of iWAT under the same conditions ($n = 5$). OCR of iWAT from wild type mice before and after 0.5 μM CL (I) or 10 μM isoproterenol (ISO, J) treatment ($n = 7$). Tissues were pre-incubated with vehicle or Pref-1-mFc in respiration buffer for 1.5 h before OCR measurement. Western blot of OXPHOS complex and UCP1 (K) ($n = 3$) and relative expression levels of OXPHOS genes (L) ($n = 5$) in iWAT of vehicle or Pref-1-mFc treated mice with or without CL treatment. Data are presented as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with untreated control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between groups.

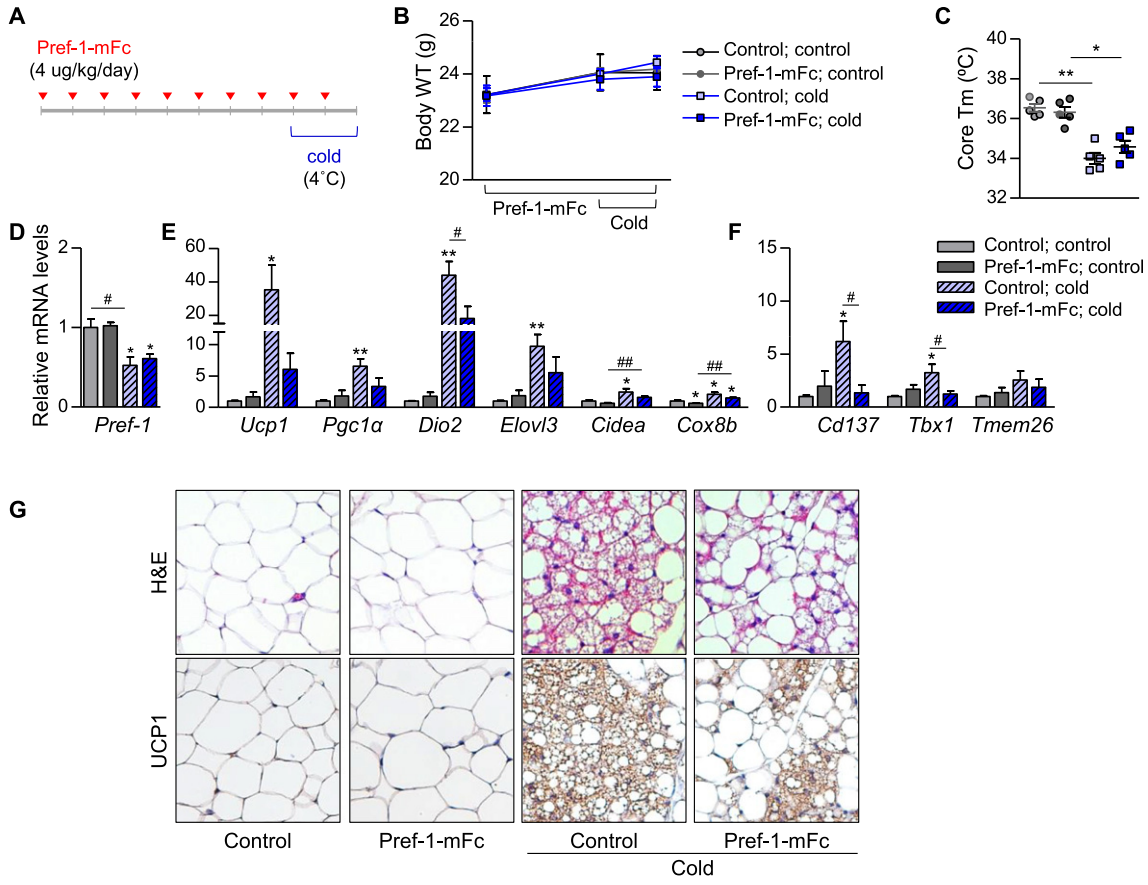

Fig. 4. Pref-1 treatment inhibits thermogenic activity and browning of inguinal white adipose tissue (iWAT) in cold-exposed mice. (A) Schematic representation of the experiment. Body weight (B), core body temperature (C), relative expression levels of *Pref-1* (D), thermogenic genes (E) and beige fat markers (F) in iWAT of vehicle or Pref-1-mFc treated mice with or without 48 h of cold exposure ($n = 5$). (G) Representative histologic findings of iWAT under the same conditions (scale bar, 50 μm). Data are presented as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with unexposed control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between groups.

transfection. The basal and maximal OCR were inversely correlated with the levels of Pref-1 expression, where OCR was decreased by Pref-1 overexpression and increased by Pref-1 knock-down (Fig. 2C). The OCR increase achieved by CL316,243 treatment was absent in Pref-1-overexpressing cells (Fig. 2D). These data suggest that modulation of Pref-1 expression in an in vitro system results in changes in thermogenic gene expression and energy expenditure.

3.3. Pref-1 treatment impairs thermogenesis and browning of iWAT in mice

Next, we investigated the effect of Pref-1 treatment in vivo. Eight week-old C57BL/6N mice were injected IP with 4 μg/kg/day of Pref-1 protein or PBS (control) for 10 days and were given 1 mg/kg/day of CL316,243 for the last 3 days (Fig. 3A). Western blot of serum indicated significantly higher concentration of Pref-1 in Pref-1-Fc treated mice (Supplemental Fig. 5). CL316,243 injection induced weight loss in both control and Pref-1 groups, but no difference between groups was observed by this short-term treatment (Fig. 3B). CL316,243-induced rise in core temperature was significantly lower by Pref-1 pretreatment when compared with the PBS-treated group (Fig. 3C). *Pref-1* expression was suppressed by CL316,243 in both groups (Fig. 3D). Similar to the findings observed in 3T3-L1 adipocytes, the increased expressions of thermogenic and beige fat genes in response to CL316,243 treatment were significantly blunted in the iWAT of the Pref-1 group compared to the controls (Fig. 3E–F). Consistently, browning of iWAT was impaired, and ex vivo OCR was reduced in the Pref-1-treated group (Fig. 3G–H). In addition, acute effects of adrenergic stimuli were examined using iWAT explants. Increased OCR by CL316,243 or isoproterenol treatment was not observed in tissues incubated with Pref-1-containing media (Fig. 3I–J). Western blot analysis of UCP1 and OXPHOS complex subunits also confirmed the inhibitory role of Pref-1 in adipose tissue browning (Fig. 3K, Supplemental Fig. 4B). Analysis of gene expression patterns of OXPHOS complex subunits supported functional changes (Fig. 3L). These effects of Pref-1 were not prominent in eWAT or BAT (Supplemental Fig. 6A–C).

We also tested whether cold exposure, instead of pharmacological stimulus, would exert a similar effect. Eight week-old C57BL/6N mice were injected IP with Pref-1 protein or PBS for 10 days and exposed to 4 °C cold for the last 48 h (Fig. 4A). There were no differences in the body weight or core temperature after cold exposure between two groups (Fig. 4B–C). *Pref-1* expression was suppressed in both groups (Fig. 4D). Similar to the findings observed with CL316,243 treatment, the increased expression of thermogenic and beige fat genes in response to cold exposure was significantly blunted in the iWAT from the Pref-1 group compared to the controls (Fig. 4E–F). Histologically, iWAT browning was impaired in the Pref-1-treated group (Fig. 4G). Collectively, these data suggest that Pref-1 pretreatment attenuates adipose tissue browning and thermogenesis in mice with beta-adrenergic stimulation.

3.4. Pref-1 knock-out amplifies thermogenesis and browning of iWAT in mice

We generated adipose tissue-specific inducible Pref-1 knock-out mice to examine the effect of Pref-1 depletion, and compared the CL316,243-induced thermogenic potential with a control group (Fig. 5A). Because Pref-1 expression level is higher in thermoneutral conditions compared to RT (Fig. 1D–E), these mice were housed at 30 °C from 5 weeks of age for clearer detection of the effect of Pref-1

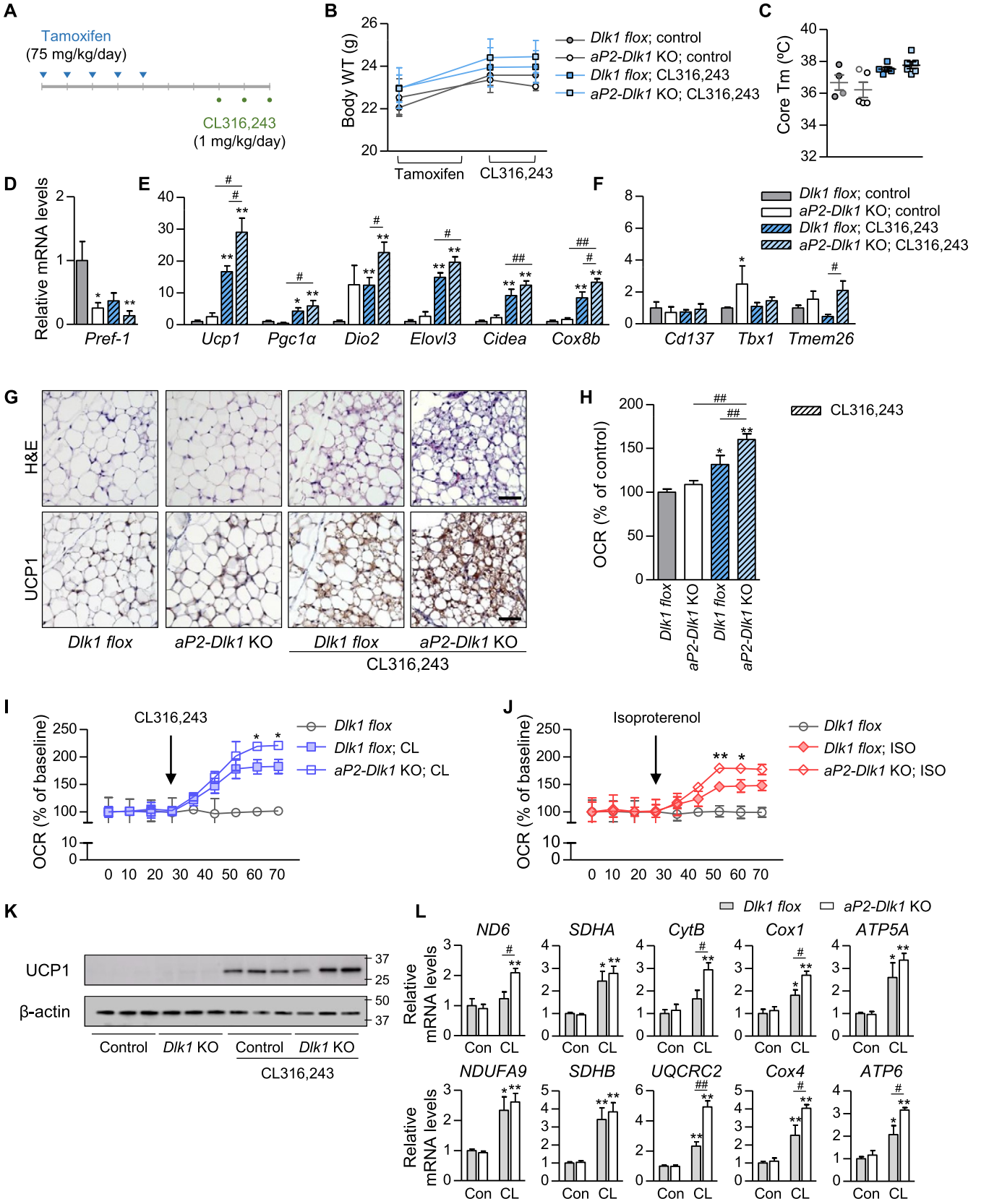

Fig. 5. Pref-1 knock-out in adipocytes promotes thermogenic activity and browning of inguinal white adipose tissue (iWAT) in CL316,243 (CL)-treated mice. Mice were raised in a thermoneutral environment from 5 wks of age. (A) Schematic representation of the experiment. Body weight (B), core body temperature (C), relative expression levels of *Pref-1* (D), thermogenic genes (E) and beige fat markers (F) in iWAT of *Dlk1 flox* or *aP2-Dlk1* KO mice with or without CL treatment ($n = 4$–6). Representative histologic findings (G) (scale bar, 50 μm) and tissue oxygen consumption rates (OCR) (H) of iWAT under the same conditions ($n = 4$–6). OCR of iWAT from control or *aP2-Dlk1* KO mice before and after 0.5 μM CL (I) or 10 μM isoproterenol (ISO, J) treatment ($n = 3$–7). Western blot of UCP1 (K) ($n = 3$) and relative expression levels of OXPHOS genes (L) ($n = 4$–6) in iWAT of *Dlk1 flox* or *aP2-Dlk1* KO mice with or without CL treatment. Data are presented as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with untreated control, *DLk1 flox*;CL (I) or *Dlk1 flox*;ISO (J). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between groups.

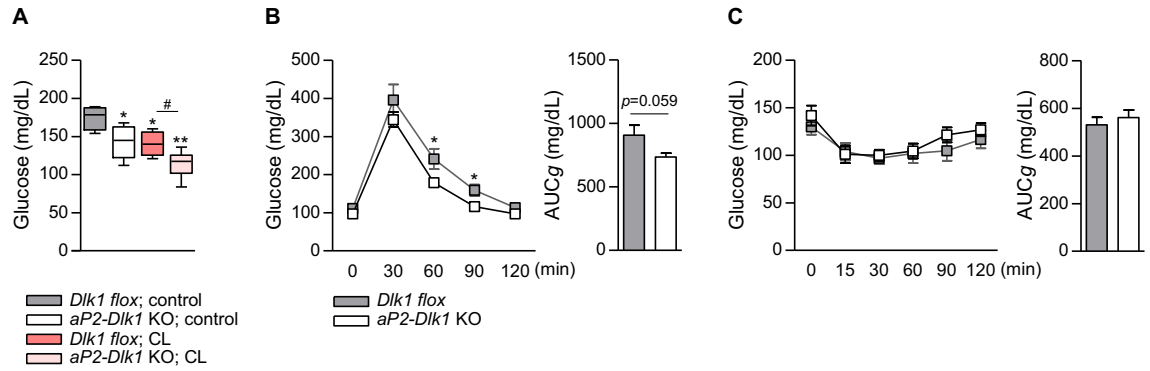

Fig. 6. Effect of adipose tissue-specific Pref-1 knock-out on glucose and insulin tolerance. (A) Blood glucose levels in fed state of *Dlk1 flox* and *aP2-Dlk1* KO mice with or without CL316,243 (CL) treatment ($n = 4$–6). (B) Intraperitoneal glucose tolerance test in mice with overnight fasting ($n = 4$). (C) Insulin tolerance test in mice after 5 h fasting ($n = 4$–6). Data are presented as the mean ± SE. $^{*}P < 0.05$ compared with the control group (two-tailed Student's *t*-test).

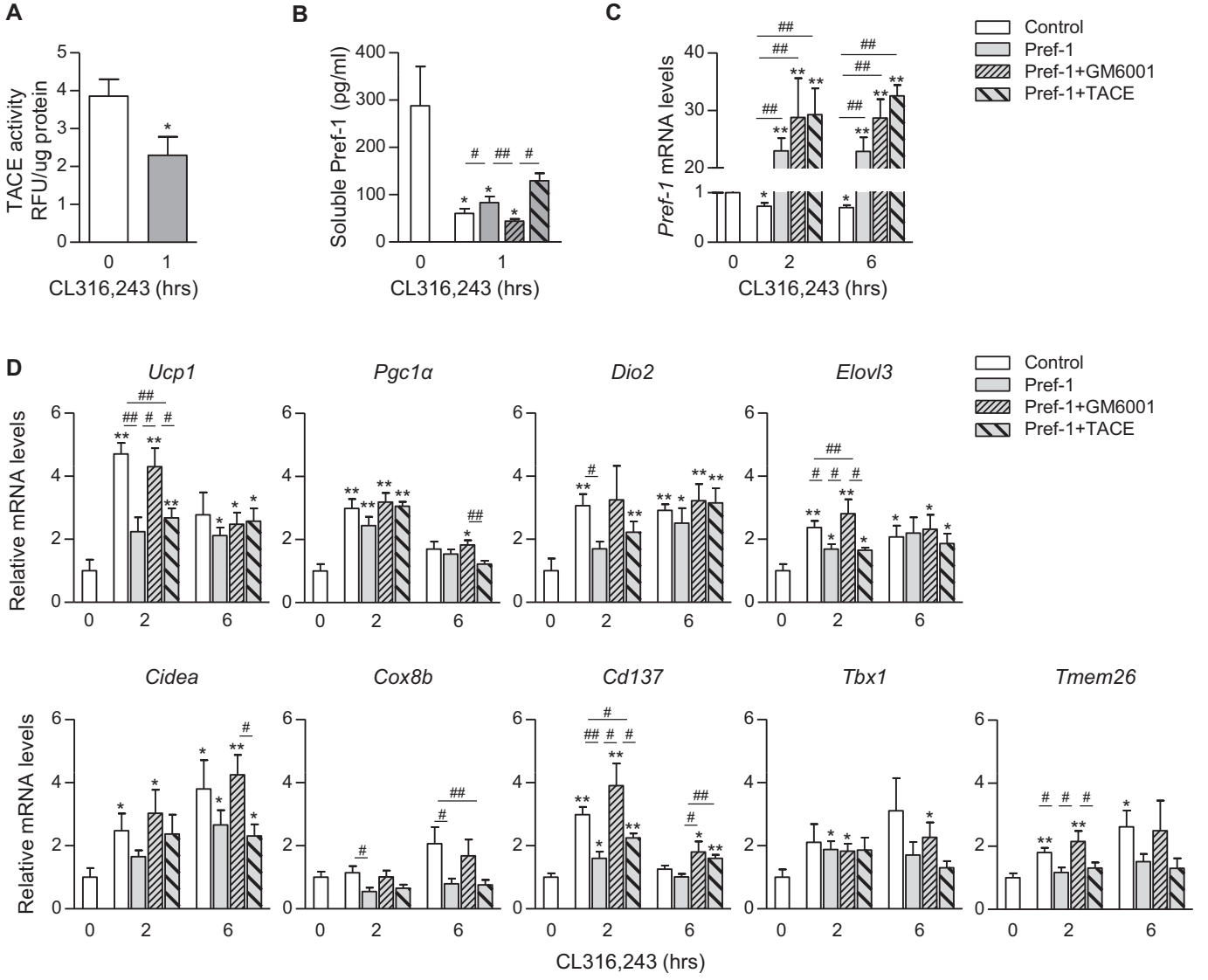

Fig. 7. Regulation of adipocyte thermogenesis by TACE-mediated Pref-1 cleavage. (A) TACE activity in 3T3-L1 cells after 0.5 μM CL316,243 (CL) treatment ($n = 6$). (B) Secreted Pref-1 level into 3T3-L1 cell culture medium after 1 h exposure to 0.5 μM CL ($n = 6$). Relative mRNA expression of *Pref-1* (C), thermogenic genes and beige fat markers (D) in 3T3-L1 cells by CL-induced thermogenesis ($n = 6$). Cells were transfected with empty vector or *Pref-1* plasmid one day before CL treatment. Selective pharmacological stimulation or inhibition of TACE activity were performed by recombinant mouse TACE or GM6001, respectively, before induction of thermogenesis. Data are presented as the mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$ compared with untreated control. $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ between groups.

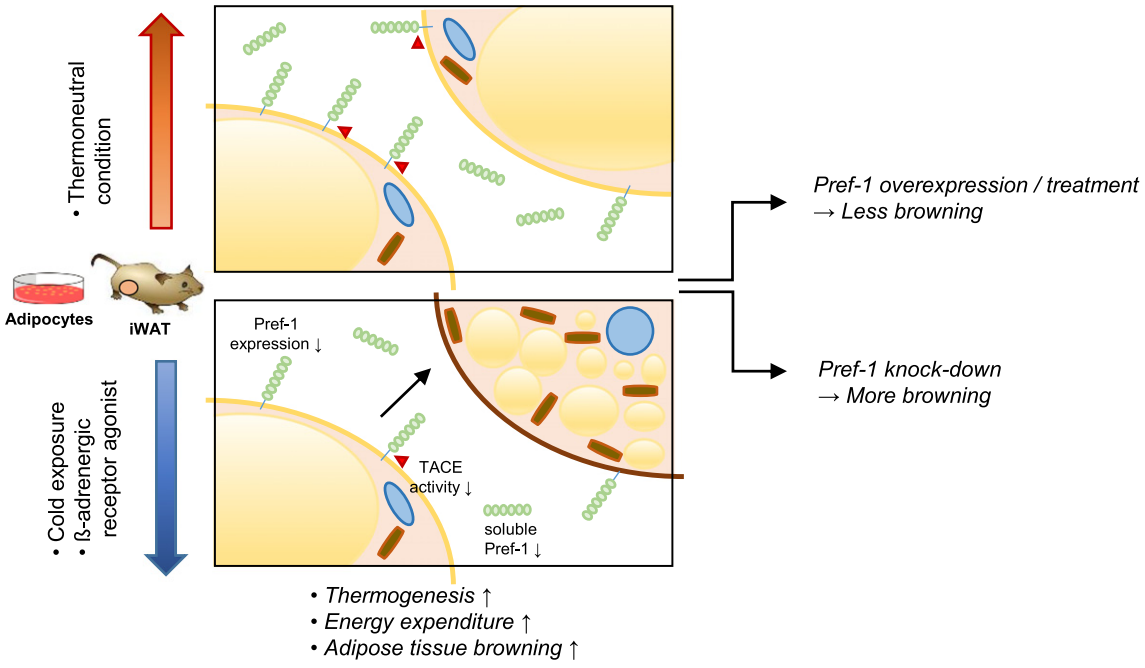

Fig. 8. Proposed model for a role of Pref-1 in adipose tissue browning.

knock-out. There were no differences in the body weight or core temperature between groups after CL316,243 treatment (Fig. 5B–C). *Pref-1* expression was further suppressed by CL316,243 treatment in *aP2-Dlk1* KO mice (Fig. 5D). In contrast to the findings observed by Pref-1 treatment, increased expression of thermogenic genes in response to CL316,243 treatment was significantly augmented in the iWAT of *aP2-Dlk1* KO mice compared to *Dlk1 flox* mice, whereas the effect on beige fat markers was not prominent (Fig. 5E–F). Consistently, beige fat formation was increased, and ex vivo OCR was higher in *aP2-Dlk1* KO mice compared to *Dlk1 flox* mice (Fig. 5G–H). Additionally, acute treatment with CL316,243 or isoproterenol in iWAT explants resulted in an increased OCR in *aP2-Dlk1* KO mice compared to *Dlk1 flox* mice (Fig. 5I–J). Western blot analysis of UCP1 and gene expression patterns of OXPHOS complex subunits supported functional changes (Fig. 5K–L, Supplemental Fig. 4C). These effects of Pref-1 knock-out were not prominent in BAT (Supplemental Fig. 7A–C). Because lower random glucose levels were noted in *aP2-Dlk1* KO mice compared to *Dlk1 flox* mice both before and after CL316,243 treatment (Fig. 6A), we also performed GTT and ITT. Slightly improved glucose tolerance was observed with borderline significance in *aP2-Dlk1* KO mice compared to *Dlk1 flox* mice, whereas no difference in insulin sensitivity was observed within a few days of inducing genetic knock-out (Fig. 6B–C). Collectively, these data suggest that Pref-1 knock-out amplifies adipose tissue browning and thermogenesis in mice with beta-adrenergic stimulation.

3.5. Thermogenic gene expression is dependent on TACE-mediated Pref-1 cleavage

We measured TACE activity in 3T3-L1 cells after short-term treatment with CL316,243 to explore a possible mechanism that explains how thermogenic stimuli might control Pref-1 expression. We found that TACE activity is remarkably suppressed in this condition, resulting in markedly reduced levels of soluble Pref-1, which is produced by TACE-induced proteolytic cleavage, in the culture medium (Fig. 7A–B). Furthermore, treatment of GM6001, a TACE inhibitor, decreased levels of soluble Pref-1, whereas increasing TACE levels showed the opposite effect in 3T3-L1 cells overexpressing Pref-1 (Fig. 7B). Cellular *Pref-1* expression levels were unchanged by either GM6001 or TACE treatment (Fig. 7C). CL316,243-induced increase in the expression of thermogenic genes and beige fat markers was generally preserved by GM6001 whereas these were blunted by TACE treatment in 3T3-L1 cells (Fig. 7D). These data suggest that CL316,243-induced expression of thermogenic and beige fat genes is modulated by TACE activity and subsequent changes in soluble Pref-1 levels, which is directly influenced by adrenergic stimuli.

4. Discussion

Activation of brown or beige adipocytes has received great attention as a novel approach to improve energy homeostasis and overcome obesity. In this study, we identified Pref-1 as a novel regulator of WAT browning. Levels of Pref-1 expression were inversely correlated to levels of UCP1 in iWAT from mice. The major finding of our study is that modulation of Pref-1 level affects cold or CL316,243-induced thermogenic activity, including brown or beige fat gene expression, iWAT browning and mitochondrial function. Mechanistically, TACE activity and production of soluble Pref-1 were affected by beta-adrenergic stimuli, leading to alterations in the autocrine or paracrine actions of Pref-1 (Fig. 8).

Pref-1 plays a critical role in regulating differentiation in white adipocytes [12,13]. It acts primarily by maintaining a preadipocyte state, and its expression is downregulated during the differentiation process. A similar role for Pref-1 was also observed in brown adipocytes. BMP7 promotes differentiation of brown preadipocytes and triggers commitment of mesenchymal progenitor cells to a brown adipocyte lineage. Overexpression of BMP7 in mice leads to increased energy expenditure and thermogenesis [22]. In a study using insulin receptor substrate 1-deficient brown adipocytes, Pref-1 was identified as a direct target of BMP7, showing that BMP7-mediated Pref-1 suppression restored brown adipogenesis and expression of thermogenic markers in cells with insulin resistance [16]. In C/EBPα-null mice, a rodent model of impaired fetal BAT differentiation, Pref-1 was overexpressed and UCP1 was underexpressed. Pref-1 gene transcription was under the control of transcription factor C/EBPδ, and overexpression of C/EBPδ was observed in C/EBPα-null BAT [17]. These data suggest a possible role of Pref-1 in controlling energy expenditure through nonshivering thermogenesis.

Our data show that Pref-1 and UCP1 expression is dependent on the ambient temperature in every adipose tissue depot we studied, although less prominent in eWAT or BAT. The browning effect in response to thermogenic stimuli was also more prominent in iWAT compared to eWAT, as expected. Notably, the regulation of adipose tissue browning by Pref-1 modulation was only observed in iWAT, but not in eWAT. Whether this is due to depot-specific action of Pref-1 or lower thermogenic potential of eWAT warrants further investigation.

Since induced browning of adipose tissue and its reversible nature have been observed, the source of these beige adipocytes (transdifferentiation from white adipocytes vs. de novo differentiation of white or beige precursor cells) was under debate [9,23–26]. Since beige adipocytes are composed of heterogenous cell populations, it is also plausible that multiple mechanisms are involved. In our experiments using differentiated 3T3-L1 adipocytes, modulation of Pref-1 expression level influenced the CL316,243-induced activation of thermogenic genes and OCR. However, the CL316,243-induced increase in brown or beige fat markers was negligible in undifferentiated preadipocytes, possibly due to significantly higher expression of Pref-1 at this stage. Additionally, knock-down of Pref-1 in undifferentiated preadipocytes did not induce significant changes in thermogenic gene expression (data not shown). These observations suggest that the observed role of Pref-1 is exerted by regulating transdifferentiation of mature white adipocytes into cells expressing beige cell characteristics.

Pref-1 has been shown to directly interact with fibronectin, and this interaction via the integrin α5ß1 signaling pathway activates MEK/ERK, which increases Sox9 and inhibits adipocyte differentiation [27]. However, because the specific receptor of Pref-1 is yet unknown, identifying this receptor would help achieve detailed exploration of the signaling pathway involved in the metabolic action of Pref-1. Although the molecular mechanism of Pref-1-mediated control of adipose tissue browning is unclear at this point, a novel finding of this study is that TACE activity and the resulting soluble Pref-1 level are under the influence of adrenergic stimuli. Activation of the sympathetic nervous system is a key component in initiating adaptive thermogenesis in adipose tissue [28]. Beyond its classical mechanism of activating a cAMP-protein kinase A pathway, several other mechanisms have been suggested, such as regulating AMP-activated protein kinase or controlling norepinephrine uptake by sympathetic neuron-associated macrophages [29,30]. Our study adds another plausible mechanism.

Based on the known role of Pref-1, it could be assumed that adipose tissue or serum Pref-1 level might be associated with a metabolic phenotype in humans. A study comparing adipose tissue expression of Pref-1 in metabolically healthy and unhealthy obese subjects undergoing bariatric surgery showed that metabolically healthy obese individuals had lower levels of Pref-1 in adipose tissues, along with a more favorable inflammatory profile [31]. Another study demonstrated higher serum Pref-1 level with obesity, which was negatively associated with insulin sensitivity, and that these levels decreased after bariatric surgery [32]. In contrast, some studies identified a negative correlation of Pref-1 with type 2 diabetes in obese individuals, or found that subjects with lower levels of circulating Pref-1 are at an increased risk of developing diabetes [33,34]. Inconsistent results among studies might have been caused by different clinical characteristics of study participants or by the methods used for measuring Pref-1. We found subtle improvement of glucose tolerance with borderline significance in adipose tissue-specific Pref-1 knock-out mice. The longer effects of Pref-1 deletion, or response to metabolic stress, such as a high fat diet, should be explored in the future as the duration of knockout in our experiments was very short. It would also be interesting to know whether Pref-1 expression is associated with the amount of brown or beige fat and thermogenic potential in humans.

In conclusion, we demonstrated that Pref-1 expression level is dependent on ambient temperature and that adaptive thermogenesis is controlled by Pref-1 in iWAT and 3T3-L1 adipocytes. These findings open a new possibility of promoting beige fat biogenesis and improving energy homeostasis by regulating Pref-1 for the treatment of obesity and metabolic diseases.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.metabol.2019.153977.

Author contributions

MR and SHL designed and performed the experiments. JWK and MWL provided technical assistance. JWK, MWL, and KHY analyzed data and contributed to discussion. MR and SHL wrote the manuscript with input from all the authors.

Funding

This work was supported by grants from the National Research Foundation of Korea (NRF-2015R1C1A1A02037164, NRF-2018R1D1A1B07043223) and Korean Diabetes Association (2016S-2) to SHL.

Declaration of competing interest

The authors declare that they have no competing interests.

References

[1] Trends in adult body-mass index in 200 countries from 1975 to 2014: a pooled analysis of 1698 population-based measurement studies with 192 million participants, Lancet 2016;387:1377–96.
[2] Adan RA. Mechanisms underlying current and future anti-obesity drugs. Trends Neurosci 2013;36:133–40.
[3] Wing RR, Bolin P, Brancati FL, Bray GA, Clark JM, Coday M, et al. Cardiovascular effects of intensive lifestyle intervention in type 2 diabetes. N Engl J Med 2013;369:145–54.
[4] Cypess AM, Lehman S, Williams G, Tal I, Rodman D, Goldfine AB, et al. Identification and importance of brown adipose tissue in adult humans. N Engl J Med 2009;360:1509–17.
[5] van Marken Lichtenbelt WD, Vanhommerig JW, Smulders NM, Drossaerts JM, Kemerink GJ, Bouvy ND, et al. Cold-activated brown adipose tissue in healthy men. N Engl J Med 2009;360:1500–8.
[6] Virtanen KA, Lidell ME, Orava J, Heglind M, Westergren R, Niemi T, et al. Functional brown adipose tissue in healthy adults. N Engl J Med 2009;360:1518–25.
[7] Cohen P, Levy JD, Zhang Y, Frontini A, Kolodin DP, Svensson KJ, et al. Ablation of PRDM16 and beige adipose causes metabolic dysfunction and a subcutaneous to visceral fat switch. Cell 2014;156:304–16.
[8] Lee MW, Odegaard JI, Mukundan L, Qiu Y, Molofsky AB, Nussbaum JC, et al. Activated type 2 innate lymphoid cells regulate beige fat biogenesis. Cell 2015;160:74–87.
[9] Sidossis L, Kajimura S. Brown and beige fat in humans: thermogenic adipocytes that control energy and glucose homeostasis. J Clin Invest 2015;125:478–86.
[10] Bartelt A, Heeren J. Adipose tissue browning and metabolic health. Nat Rev Endocrinol 2014;10:24–36.
[11] Fang D, Shi X, Lu T, Ruan H, Gao Y. The glycoprotein follistatin-like 1 promotes brown adipose thermogenesis. Metabolism 2019;98:16–26.
[12] Hudak CS, Sul HS. Pref-1, a gatekeeper of adipogenesis. Front Endocrinol (Lausanne) 2013;4:79.
[13] Sul HS. Minireview: Pref-1: role in adipogenesis and mesenchymal cell fate. Mol Endocrinol 2009;23:1717–25.
[14] Moon YS, Smas CM, Lee K, Villena JA, Kim KH, Yun EJ, et al. Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. Mol Cell Biol 2002;22:5585–92.
[15] Lee K, Villena JA, Moon YS, Kim KH, Lee S, Kang C, et al. Inhibition of adipogenesis and development of glucose intolerance by soluble preadipocyte factor-1 (Pref-1). J Clin Invest 2003;111:453–61.
[16] Zhang H, Schulz TJ, Espinoza DO, Huang TL, Emanuelli B, Kristiansen K, et al. Cross talk between insulin and bone morphogenetic protein signaling systems in brown adipogenesis. Mol Cell Biol 2010;30:4224–33.
[17] Armengol J, Villena JA, Hondares E, Carmona MC, Sul HS, Iglesias R, et al. Pref-1 in brown adipose tissue: specific involvement in brown adipocyte differentiation and regulatory role of C/EBPdelta. Biochem J 2012;443:799–810.
[18] Ham M, Choe SS, Shin KC, Choi G, Kim JW, Noh JR, et al. Glucose-6-phosphate dehydrogenase deficiency improves insulin resistance with reduced adipose tissue inflammation in obesity. Diabetes 2016;65:2624–38.
[19] Rhee M, Lee SH, Kim JW, Ham DS, Park HS, Yang HK, et al. Preadipocyte factor 1 induces pancreatic ductal cell differentiation into insulin-producing cells. Sci Rep 2016;6:23960.
[20] Lee YK, Sohn JH, Han JS, Park YJ, Jeon YG, Ji Y, et al. Perilipin 3 deficiency stimulates thermogenic beige adipocytes through PPARalpha activation. Diabetes 2018;67:791–804.

[21] Kim JW, Park SY, You YH, Ham DS, Park HS, Lee SH, et al. Targeting PGC-1alpha to overcome the harmful effects of glucocorticoids in porcine neonatal pancreas cell clusters. Transplantation 2014;97:273–9.
[22] Tseng YH, Kokkotou E, Schulz TJ, Huang TL, Winnay JN, Taniguchi CM, et al. New role of bone morphogenetic protein 7 in brown adipogenesis and energy expenditure. Nature 2008;454:1000–4.
[23] Rosenwald M, Perdikari A, Rulicke T, Wolfrum C. Bi-directional interconversion of brite and white adipocytes. Nat Cell Biol 2013;15:659–67.
[24] Lee YH, Petkova AP, Mottillo EP, Granneman JG. In vivo identification of bipotential adipocyte progenitors recruited by beta3-adrenoceptor activation and high-fat feeding. Cell Metab 2012;15:480–91.
[25] Barbatelli G, Murano I, Madsen L, Hao Q, Jimenez M, Kristiansen K, et al. The emergence of cold-induced brown adipocytes in mouse white fat depots is determined predominantly by white to brown adipocyte transdifferentiation. Am J Physiol Endocrinol Metab 2010;298:E1244–53.
[26] Wang QA, Tao C, Gupta RK, Scherer PE. Tracking adipogenesis during white adipose tissue development, expansion and regeneration. Nat Med 2013;19:1338–44.
[27] Wang Y, Zhao L, Smas C, Sul HS. Pref-1 interacts with fibronectin to inhibit adipocyte differentiation. Mol Cell Biol 2010;30:3480–92.
[28] Lowell BB, Spiegelman BM. Towards a molecular understanding of adaptive thermogenesis. Nature 2000;404:652–60.
[29] Pulinilkunnil T, He H, Kong D, Asakura K, Peroni OD, Lee A, et al. Adrenergic regulation of AMP-activated protein kinase in brown adipose tissue in vivo. J Biol Chem 2011;286:8798–809.
[30] Pirzgalska RM, Seixas E, Seidman JS, Link VM, Sanchez NM, Mahu I, et al. Sympathetic neuron-associated macrophages contribute to obesity by importing and metabolizing norepinephrine. Nat Med 2017;23:1309–18.
[31] O'Connell J, Lynch L, Hogan A, Cawood TJ, O'Shea D. Preadipocyte factor-1 is associated with metabolic profile in severe obesity. J Clin Endocrinol Metab 2011;96:E680–4.
[32] Chacon MR, Miranda M, Jensen CH, Fernandez-Real JM, Vilarrasa N, Gutierrez C, et al. Human serum levels of fetal antigen 1 (FA1/Dlk1) increase with obesity, are negatively associated with insulin sensitivity and modulate inflammation in vitro. Int J Obes (Lond) 2008;32:1122–9.
[33] Flehmig G, Scholz M, Kloting N, Fasshauer M, Tonjes A, Stumvoll M, et al. Identification of adipokine clusters related to parameters of fat mass, insulin sensitivity and inflammation. PLoS One 2014;9:e99785.
[34] Lee SH, Rhee M, Yang HK, Ha HS, Lee JH, Kwon HS, et al. Serum preadipocyte factor 1 concentrations and risk of developing diabetes: a nested case-control study. Diabet Med 2016;33:631–8.