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Prdm4 induction by the small molecule butein promotes white adipose tissue browning

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Abstract

Increasing the thermogenic activity of adipocytes holds promise as an approach to combating human obesity and its related metabolic diseases. We identified PR domain containing 4 (Prdm4) induction by the small molecule butein as a means to induce uncoupling protein 1 expression, increase energy expenditure, and stimulate the generation of thermogenic adipocytes. This study

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Author contributions: K.W.P. and N.J.S. designed the research; S.K., S.H.C., and S.M.K. performed parts of the animal experiments; P.R., L.V., and K.R. performed cellular energy expenditure analysis; S.C. and S.H.K. performed *in vivo* metabolic studies; J.M.K. assessed the purity of the compound; N.J.S., P.R., P.T., L.V., J.H.Y., J.M.K., K.R., J.S.L., S.C.L., S.H.K., and K.W.P. were involved in data interpretation; K.W.P., N.J.S., K.R., and P.T. wrote the manuscript.

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highlights a Prdm4-dependent pathway, modulated by small molecules, that stimulates white adipose tissue browning.

Keywords

Prdm4; Ucp1; Brown adipocytes; Butein; obesity; metabolic diseases;

Adipocytes are central players in energy homeostasis that can be divided into at least two subsets^{1,2}. White adipose tissue (WAT) stores excess energy in the form of triglycerides and secretes adipokines and free fatty acids. Brown adipose tissue (BAT) is specialized to maintain body temperature by generating heat through uncoupling protein 1 (Ucp1) action^{3,4}. The presence of BAT in adult humans also influences body weight and insulin sensitivity^{2,3,5-8}. Beige adipocytes are inducible brown-like adipocytes generated within WAT through a process known as “WAT browning”. Beige adipocytes are characterized by their multilocular lipid droplets and high mitochondrial contents, similar to BAT^{9,10}; however, the origins of brown and beige adipocytes appear to be distinct^{11,12}. Transgenic expression of Prdm16 or Ucp1 in adipose tissues promotes the generation of brown-like adipocytes, conferring resistance to obesity and improved glucose tolerance^{13,14}. Stimulation of the β 3-adrenergic receptor signaling, exercise¹⁵⁻¹⁷ and treatment of small molecules such as β -aminoisobutyric acid, berberine, and salsalate^{18,19,20} also promote the expression of Ucp1 in adipocytes and improved glucose homeostasis. These observations raise the possibility that the induction of thermogenic adipocytes may offer a new approach for combating human obesity and metabolic diseases.

To identify novel small molecules that stimulate the thermogenic capacity of white adipocytes, we tested various bioactive compounds for their abilities to induce *Ucp1* expression in C3H10T1/2 adipocytes (Supplementary results, Supplementary Table 1). Cells treated with butein (**1**) exhibited the most robust induction of *Ucp1* mRNA expression and demonstrated anti-lipogenic activity (Supplementary Fig. 1 and Supplementary Fig. 2). Butein treatment increased cellular mitochondrial contents and decreased numbers of large lipid droplets. Butein also increased the expression of thermogenic genes, but suppressed the expression of pan-adipocyte (*Ppar γ* and *aP2*) and white adipocyte-selective genes, such as *resistin* (*Retn*) and *nicotinamide N-methyltransferase* (*Nnmt*) in primary adipocytes (Supplementary Fig. 3). Treatment with other anti-adipogenic compounds, including resveratrol and sulfuretin, failed to induce *Ucp1* expression. Butein also induced the expression of Ucp1 and brown adipocyte markers in T37i brown preadipocytes and primary brown adipocytes (Supplementary Fig. 4). Together, these data demonstrate that butein is a regulator of Ucp1 in both white and brown adipocytes.

Having established the effects of butein on the Ucp1 induction, we utilized butein as a tool to identify genes responsible for thermogenic program. Temporal expression profiles showed *Ucp1* induction by butein as early as 6 hours after treatment. We compared gene expression profiles in C3H10T1/2 adipocytes after 6 hour treatments with butein, sulfuretin or resveratrol using microarray analysis. Both butein and sulfuretin were isolated from *Rhus verniciflua stokes*²¹, however, sulfuretin failed to induce *Ucp1* expression. Similarly,

resveratrol did not mediate *Ucp1* induction (Supplementary Fig. 4a). Thus, we searched for candidate genes acting on *Ucp1* expression and thermogenic programs that were specifically regulated by butein (> 1.6 fold or higher) but not by sulfuretin and resveratrol. We identified 127 genes that were exclusively regulated by butein (Fig. 1, Supplementary Fig. 1, and Supplementary Data Set 1). Then, we focused our attention on transcription factors or related genes that have been shown to influence thermogenic properties in adipocytes²²⁻²⁴. The specific induction of this subset of genes by butein, but not by sulfuretin or resveratrol, was validated by realtime PCR (Supplementary Fig. 5).

We evaluated the butein-responsive transcriptional regulators identified above for their ability to affect *Ucp1* expression. Small interfering RNA (siRNA)-mediated knockdown was performed in differentiated C3H10T1/2 adipocytes, followed by measurement of *Ucp1* expression. *Hmox-1*, *Maff*, and *Atf3*, the three most highly induced genes by butein, were also included in the knockdown studies. Of these candidate genes, only *Prdm4* inhibition impaired *Ucp1* mRNA expression (Supplementary Fig. 6). Consistently, butein treatment induced *Prdm4* and *Ucp1* protein expression in C3H10T1/2 adipocytes and white and brown fat depots *in vivo* (Fig. 1 and Supplementary Fig. 7). Other *Prdm* family members *Prdm2*, *Prdm3*, and *Prdm16* were not acutely regulated by butein. Furthermore, isoproterenol, sulfuretin, and resveratrol failed to affect *Prdm4* expression (Supplementary Fig. 1c and Supplementary Fig. 8). Based on the *in vitro* and *in vivo* evidence, we selected *Prdm4* for further investigation.

To investigate the roles of *Prdm4* in preadipocytes, we transfected 3T3-L1 preadipocytes or C3H10T1/2 cells with two siRNAs targeting *Prdm4*. After induction of differentiation, the *Prdm4*-silenced cells exhibited enhanced lipid accumulation and increased expression levels of pan- and white adipocyte-selective genes compared to control cells (Supplementary Fig. 9). *Prdm4*-silenced C3H10T1/2 adipocytes also showed reduced expression of *Ucp1* and decreased mitochondrial mass (Fig. 2a). Basal oxygen consumption rates (OCR) were decreased in *Prdm4* silenced C3H10T1/2 adipocytes. Sequential treatments with compounds that modulate mitochondrial function also revealed decreases in basal, uncoupled respiration and maximal mitochondrial respiration in C3H10T1/2 preadipocytes and adipocytes (Fig. 2b and Supplementary Fig. 10). Silencing *Prdm4* in brown adipocytes similarly inhibited the expression of thermogenic genes (Supplementary Fig. 11). Conversely, forced expression of *Prdm4* induced *Ucp1* and mitochondrial biogenesis (Fig. 2c and Supplementary Fig. 11c), while suppressing pan-adipocyte and white fat-selective genes (Supplementary Fig. 12).

To test whether *Prdm4* is required for the action of butein, we treated control and *Prdm4*-knockdown cells with butein. The effect of butein on expression of *Nnmt*, *Retn*, and *Ucp1* and induction of mitochondrial mass was blunted in *Prdm4* siRNA-transfected C3H10T1/2 cells (Supplementary Fig. 13). The stimulatory effects on *Ucp1* expression and mitochondrial mass were not further enhanced by butein treatment in *Prdm4*-overexpressing cells (Supplementary Fig. 13). These data illustrate that *Prdm4* can recapitulate both butein's inhibitory action on lipogenesis and its stimulatory action on WAT browning.

To investigate the potential role of *Prdm4* in obesity, we examined *Prdm4* mRNA levels in diet induced obese mice. Levels of *Prdm4* mRNA were decreased in epididymal (eWAT),

inguinal white adipose tissue (iWAT), and BAT of obese mice. *Prdm4* expression was consistently lower in the eWAT of diabetic *db/db* mice compared to non-diabetic control mice (Supplementary Fig. 14). These observations suggest a potential link between *Prdm4* and metabolic diseases. To assess whether modulation of *Prdm4* levels *in vivo* may influence obesity and glucose homeostasis, C57BL/6 mice fed with high fat diet (HFD) were randomly divided and injected intraperitoneally with validated *Prdm4* antisense oligonucleotides (ASO) or control ASO twice per week (25mg per kg per dose) for 6 weeks. The *Prdm4* ASO reduced *Prdm4* mRNA by 38% in eWAT and 15% in the liver, but did not alter levels in skeletal muscle. Treatment of HFD fed mice with *Prdm4* ASO enhanced HFD induced body weight gain and WAT mass compared to the control ASO-treated mice (Fig. 3a and Supplementary Fig. 15). Body weight gain in the *Prdm4* ASO-treated group was associated with impaired glucose tolerance and insulin sensitivity (Fig. 3b and Supplementary Fig. 15). We also measured food intake and energy expenditure before body weights started to diverge in control ASO- and *Prdm4* ASO-treated mice (treated for 2.5 weeks). Food intake and physical activity were similar in control and *Prdm4* ASO groups (Supplementary Fig. 16). Body temperature in the *Prdm4* ASO-treated mice (36.32 °C) was significantly lower than in controls (36.72 °C), suggesting that *Prdm4* affects thermogenic regulation *in vivo*. Consistently, *Prdm4* ASO-treated mice for 2.5 weeks had lower energy expenditure, O₂ consumption, and CO₂ production than control ASO-treated mice (Fig. 3c and Supplementary Fig. 17). *Prdm4* ASO mice showed lower expression of thermogenic genes and elevated levels of *Nnmt* and *Retn* (Supplementary Fig. 18). Reduced *Ucp1* and *Prdm4* mRNA and protein levels were also observed in the eWAT of *Prdm4* ASO-treated mice (Supplementary Fig. 19). Together, these data highlight the importance of *Prdm4*-mediated cascades in thermogenic responses and metabolic diseases.

The *Prdm4*-related factor *Prdm16* is a key inducer of brown adipocytes and of beige fat formation in white fat depots^{11,13,17,25}. Adipose-specific deletion of *Prdm16* is reported to cause obesity with insulin resistance in HFD-fed mice¹³. Another study demonstrates that *Prdm3* and *Prdm16* double-knockout mice display severe reduction of brown fat-selective genes compared to wild-type mice, with induction of white fat-selective genes¹⁷. However, BAT development is still intact after the concurrent deletion of *Prdm16* and *Prdm3*, suggesting the involvement of a functionally redundant protein that has yet to be identified. In line with this speculation, our studies reveal *Prdm4* as another *Prdm* family member that can participate in the brown and beige fat programs.

In conclusion, we showed that *Prdm4* knockdown in the adipose tissue exacerbated diet-induced obesity, revealing adipose-specific effects of *Prdm4* in obesity and thermogenesis. Based on our current study, we expect that *Prdm4* regulation by natural or synthetic compounds could be an alternative therapeutic approach for obesity and metabolic diseases.

Online methods

Cell culture

Murine C3H10T1/2 multipotent cells and 3T3-L1 cells were purchased from the American Type Culture Collection and cultured as previously described²⁶. C3H10T1/2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) with 10% fetal bovine

serum (Hyclone). 3T3-L1 pre-adipocytes were maintained in DMEM with 10% fetal calf serum (Hyclone). Cells were also tested for mycoplasma contamination using MycoAlert Mycoplasma Detection Kit (Lonza). For induction of adipocyte differentiation, confluent cells were treated with adipogenic medium containing DMEM, 10% FBS, 1 μ M dexamethasone (Sigma), 0.5 mM IBMX (Sigma), and 5 μ g/ml insulin (Sigma). After incubation for 2 days, the adipogenic medium was changed to media containing DMEM, FBS and insulin. CH10T1/2 cells were further supplemented with 1 μ M troglitazone (Sigma). CH10T1/2 cells were refreshed with DMEM media containing 10% FBS, 1 μ M troglitazone, and 5 μ g/ml insulin every 2 days.

For identification of Ucp1 inducers, C3H10T1/2 adipocytes differentiated for 8 days were treated with 20 μ M of bioactive small molecules (supplementary Table 1) for 6 hours and *Ucp1* mRNA expression was measured by realtime PCR. Butein was purchased from Tokyo Chemical Industry and other compounds such as resveratrol and sulfuretin were purchased from Sigma.

Primary murine embryonic fibroblast cells and primary adipocytes were isolated and cultured as previously described^{21,26,27}. Briefly, adipose tissues were digested with collagenase and the digested cells were filtered with a cell strainer and then treated with the indicated chemicals. Dr. Marc Lombes (Paris-Sud University, France) kindly provided the T37i brown preadipocytes. To induce brown adipocyte differentiation, confluent cells were stimulated with medium containing DMEM/F-12, 10% calf serum (CS), 5 μ g/ml insulin, and 2.5 nM T3 with or without butein. The medium was refreshed every 2 days for 1 week. After induction for 7 days, the adipocytes were fixed and stained with 0.5% Oil Red O (Sigma).

RNA analysis

To measure the expression of adipocyte markers, total RNA was isolated from the cells using TRIzol reagent (Invitrogen) and reverse transcribed using RTase M-MLV (2640A, Takara). The cDNA was amplified in a thermal cycler (Takara) using the Power SYBR Premix Ex Taq (RP041A, Takara) with gene specific primer sets. Expression was normalized to 36B4. To isolate RNA from adipose tissue, homogenized TRIzol (Qiagen homogenizer, QIAGEN) samples were centrifuged and the lipid layer was removed. Total RNAs were isolated and cleaned further with phenol-chloroform extraction followed by ethanol precipitation. Total RNAs were reverse transcribed for realtime PCR analysis.

Protein analysis

Protein preparation, SDS-PAGE, and western blotting were performed as described²⁷. Briefly, adipocytes treated with butein or siRNAs were harvested and lysed in RIPA buffer (1 % NP-40, 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 10 mM NaF) containing a protease inhibitor cocktail (Roche Diagnostics). For tissue homogenates, white adipose tissues (100mg) were ground in liquid nitrogen and homogenized in RIPA buffer (200 μ l) supplemented with protease inhibitors (Roche Diagnostics). Homogenates were centrifuged for 10 min at 14,000 rpm at 4°C and supernatants collected. Protein lysates were separated on SDS-PAGE, transferred to PVDF membranes (Bio-Rad Laboratories), and western blot analysis was performed as described²⁷. The membranes were blocked for 1 hour with 5%

non-fat dry milk and incubated overnight at 4°C with primary antibodies against Prdm4 (1:2,000, ab156867, Abcam), Ucp1 (1:1,000, ab10983, Abcam), or actin (1:5,000, sc47778, Santa Cruz Biotech). The membranes were then probed with HRP-conjugated secondary antibodies (1:10,000, Ab Frontier) and developed by an enhanced chemiluminescent western blotting detection reagent (GE health care).

Overexpression and knockdown studies

PRDM4 ORF cDNA clones were purchased from Origene. The PRDM4 ORF was cloned into pcDNA3.1 and transfected into 3T3-L1 and C3H10T1/2 cells for overexpression of PRDM4. PRDM4 siRNAs were synthesized by Genolution and screened for inhibition efficiency of PRDM4 mRNA expression levels. The selected siRNAs were then transfected using RNAi MAX (Invitrogen). The sequences for si#1 and si#2 are GAAUUACGCUCAACAGAUUU and GAAAGUGAGCUGCUUUUCUUU, respectively. siRNAs transfected 3T3-L1 or C3H10T1/2 cells at 80% confluence in a concentration of 30nM. Then, the cells differentiated into adipocytes.

Metabolic studies

Cytopainter (ab112145; Abcam) was used for mitochondrial staining. Briefly, cytopainter was added to live cells and incubated for 1 hour before fixation. Then, stained cells were observed by fluorescence microscopy. Oxygen consumption rate (OCR) was determined using a Seahorse Bioscience XF24 analyzer. C3H10T1/2 cells or differentiated adipocytes were transfected with control ASO or Prdm4 ASO. Then Cells were incubated in pre-warmed unbuffered DMEM (sodium bicarbonate free, pH 7.4) for 1 h. Mitochondrial capacities were profiled by treating compounds of oligomycin (2 µg/ml), carbonyl cyanide-p-trifluoromethoxyphenylhydrazone (FCCP, 2 µM), Rotenone (1 µM) and antimycin A (1 µM).

Whole-body energy metabolism was evaluated using a oxylet systems (Panlab). Male C57BL/6N mice (7 weeks old) were purchased from Central Lab Animal Inc. After 1 week of adaption, the mice were divided into two groups. C57BL/6N mice on a HFD (Research Diets Inc.) were given PRDM4 ASO or the control ASO twice per week via intraperitoneal injection at a dose of 25 mg/kg/dose for 2.5 weeks. Body weights were not different when the energy expenditure was evaluated. Mice were placed in metabolic cages and were acclimated in the metabolic chambers for 1 day before the measuring energy expenditure, O₂ consumption, and CO₂ production.

Microarray analysis

Total RNA from fully differentiated C3H10T1/2 adipocytes treated with 20µM of butein, sulfurein or resveratrol for 6 hours were prepared using TRIzol and further purified using RNAeasy columns (QIAGEN). cDNA preparation and hybridization to Affymetrix Mouse Genome Arrays (430 version 2.0) were performed by Genochek. The data were analyzed using GeneSpring GX 7.3 software (Agilent Technologies). Microarray data sets were deposited at Gene Expression Omnibus (accession number GSE76672).

Animal studies

Male C57BL/6N mice (7 weeks old) were purchased from Central Lab Animal Inc. The mice were individually housed in a temperature-controlled room with a 12-hour light/dark cycle. For the PRDM4 ASO treatments, 15 ASOs were designed to the mouse PRDM4 mRNA sequence and screened to identify the most potent and specific ASO in adipocytes. The most potent ASO (5'-GAAUUACGCUCAACAGAUUU-3') was chosen for *in vivo* studies. A control ASO, not complementary to any known gene sequence or to PRDM4 ASO, was synthesized (Genolution) and diluted in a buffer containing 10 mmol/l Tris-HCl and 1 mmol/l EDTA (pH 7.4) before injection. After 1 week of adaption, the mice were randomly divided into two groups. The researchers were blinded to the group allocation. C57BL/6N mice on a HFD (Research Diets Inc.) were given PRDM4 ASO or the control ASO twice per week via intraperitoneal injection at a dose of 25 mg/kg/dose for 6 weeks^{28,29}. During the treatment period, the body weight and food intake were measured twice per week. For glucose tolerance tests, the mice fasted for 12 hr, and then the glucose levels were determined from tail-vein blood at 0, 15, 30, 60, 90, and 120 min after glucose intraperitoneal (i.p.) injection (2 g/kg). For insulin tolerance tests, the mice were injected i.p. with insulin (Humulin R, Eli Lilly) (0.3 U/kg). The glucose levels were determined at the times indicated above post-injection. All animal studies were carried out in accordance with the guidelines of the Animal Research Committee (SKKUIACUC-20150037) of Sungkyunkwan University.

Statistical analysis

Data are presented as means \pm s.d. Comparisons between the control and treatment groups were analyzed with unpaired Student's *t*-tests. Statistical significance was defined as $P < 0.05$ ($P < 0.05$ vs. control; ** $P < 0.005$ vs. control; $P < 0.0005$ vs. control).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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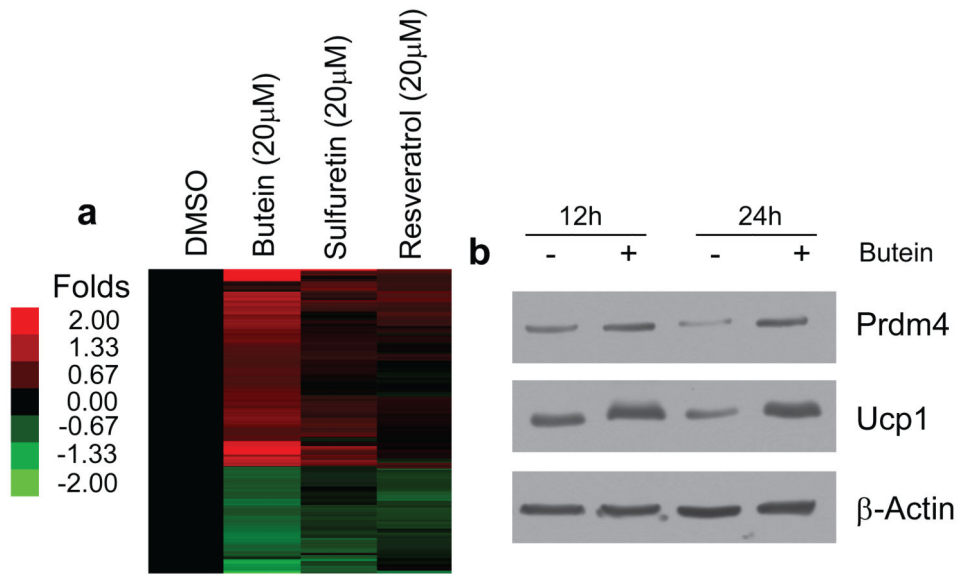


Figure 1. Identification of Prdm4 as a butein induced gene

(a) C3H10T1/2 adipocytes were treated with butein, resveratrol or sulfuretin for 6 hours and then the gene expression profiles were analyzed. (b) Butein treatments increase Prdm4 and Ucp1 protein expression in C3H10T1/2 adipocytes. Uncropped images of blots are shown in Supplementary Fig. 20.

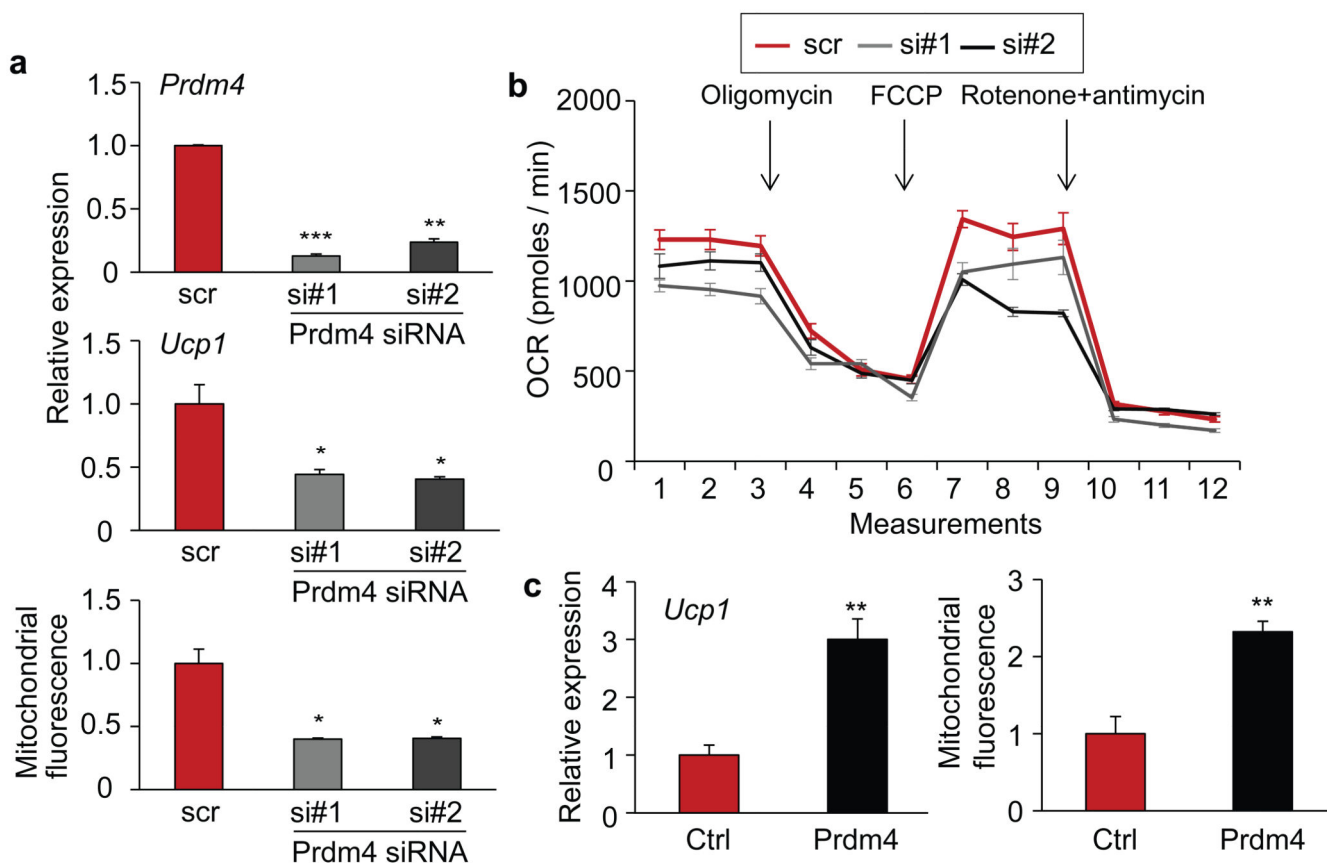


Figure 2. *Prdm4* induces *Ucp1* and regulates mitochondrial respiration

(a) Knockdown of *Prdm4* by two independent siRNAs (si#1 and si#2) reduces the expression of *Prdm4* (top) and *Ucp1* (middle) mRNA compared to the scrambled control (scr) siRNA-transfected C3H10T1/2 adipocytes. Knockdown of *Prdm4* reduces mitochondrial mass (bottom). Mitochondrial staining was quantified by NIH Image J software. Data represent means \pm s.d. (n=3). (b) Knockdown of *Prdm4* reduces the oxygen consumption rates (OCR) compared to the scrambled control siRNA-transfected differentiated C3H10T1/2 adipocytes. The OCR was measured in approximately 8 minute intervals. Basal respiration, uncoupled respiration (oligomycin), and maximal respiration (FCCP) were determined using XF24 Extracellular Flux Analyzer. Data represent means \pm s.d. (n=6). (c) Forced expression of *Prdm4* induces *Ucp1* expression in C3H10T1/2 adipocytes (left). *Prdm4*-overexpressing cells exhibit increased mitochondrial staining compared to control plasmid-transfected cells (right). Data represent means \pm s.d. (n=3). Statistically significant differences were determined by Student's *t*-test (* $P < 0.05$; ** $P < 0.005$; *** $P < 0.0005$).

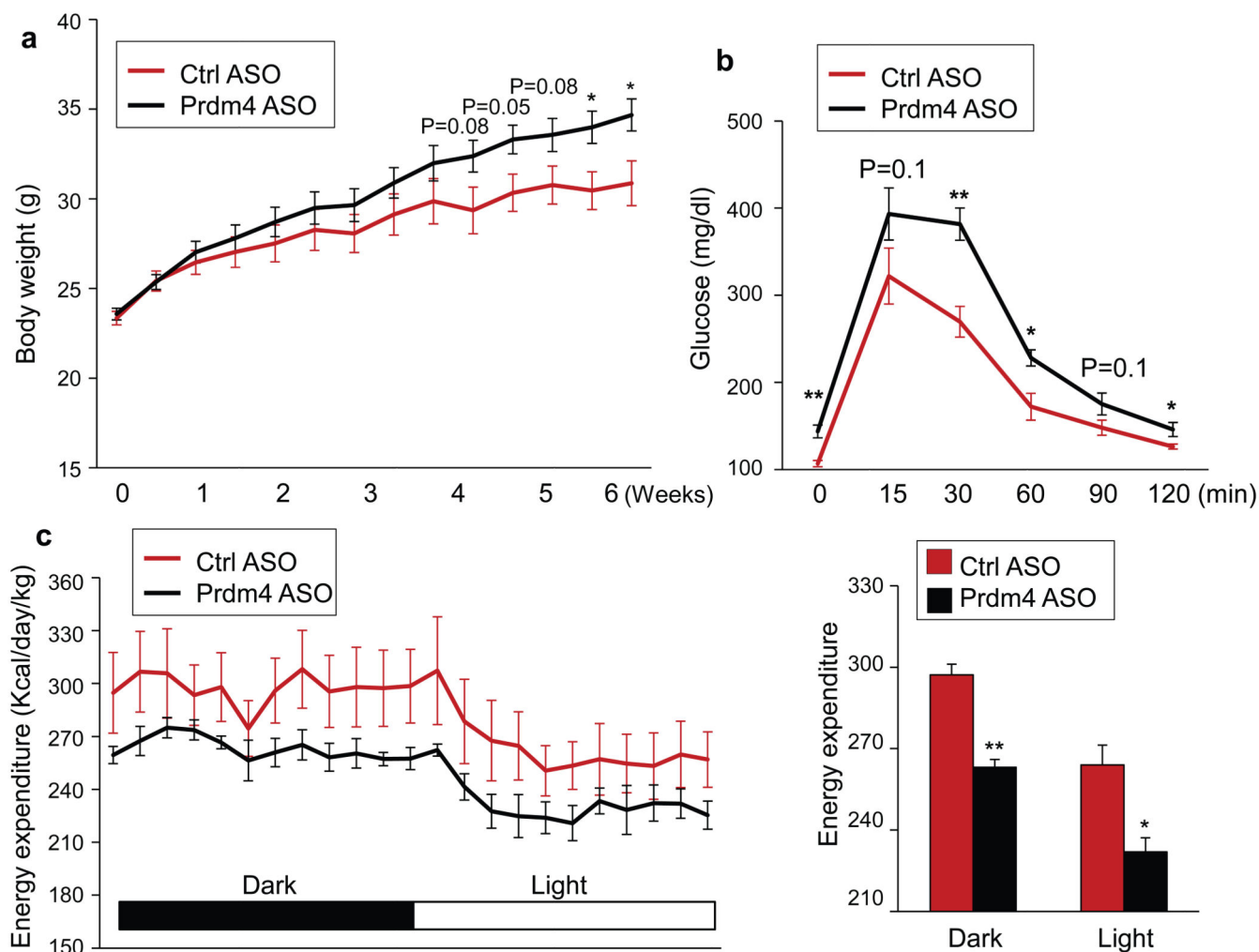


Figure 3. Effects of Prdm4 knockdown in HFD induced obese mice
(a-b) C57BL/6 mice on a HFD were treated with Prdm4 ASO or a control ASO twice per week (25mg per kg per dose) for 6 weeks. **(a)** Body weight gain in control and Prdm4 ASO-injected groups. Data represent means \pm s.d. (n=6). **(b)** Glucose tolerance test in control and Prdm4 ASO-injected groups. Data represent means \pm s.d. (n=6). **(c)** Mice were treated with Prdm4 ASO or Control ASO for 2.5 weeks and energy expenditure was measured before body weights started to diverge. 24 hour energy expenditure in Prdm4 ASO and control ASO-treated mice (left). Averages of dark and light periods of energy expenditure (right). Data represent means \pm s.d. (n=5). Statistically significant differences in the control ASO and Prdm4 ASO-injected groups were determined by Student's *t*-test (* $P < 0.05$; ** $P < 0.005$).