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BRIEF COMMUNICATION

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

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Increasing the thermogenic activity of adipocytes holds promise as an approach to combating human obesity and related metabolic diseases. We identified induction of mouse PR domain containing 4 (Prdm4) by the small molecule butein as a means to induce expression of uncoupling protein 1 (Ucp1), increase energy expenditure, and stimulate the generation of thermogenic adipocytes. This study highlights a Prdm4-dependent pathway, modulated by small molecules, that stimulates browning of white adipose tissue.

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 the action of Ucp1 (refs. 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 those of 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 beige adipocytes, conferring resistance to obesity and improved glucose tolerance[13,14]. Stimulation of the β3-adrenergic receptor signaling, exercise[15–17], and treatment with small molecules such as β-aminoisobutyric acid, berberine, and salsalate[18–20] also promote adipocyte Ucp1 expression and improved glucose homeostasis. These observations imply that the induction of thermogenic adipocytes might 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 mouse adipocytes (**Supplementary Results**, **Supplementary Table 1**). Cells treated with butein (**1**), a small molecule derived from the plant *Rhus verniciflua* Stokes, exhibited the most robust induction of *Ucp1* mRNA expression and demonstrated anti-lipogenic activity (**Supplementary Figs. 1** and **2**). Butein treatment increased cellular mitochondrial contents and decreased numbers of large lipid droplets. Butein also increased the expression of thermogenic genes, but it suppressed the expression of pan-adipocyte-selective genes (PPARγ (*Pparg*) and *aP2*(also known as *Fabp4*))) and white-adipocyte-selective genes (including 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 Figs. 4** and **21**). Together, these data demonstrate that butein is a regulator of Ucp1 in both white and brown adipocytes.

Having established the effects of butein on *Ucp1* induction, we used butein as a tool to identify genes responsible for the thermogenic program. Temporal expression profiles showed *Ucp1* induction by butein as early as 6 h after administration. We compared gene expression profiles in C3H10T1/2 adipocytes after 6 h of treatment with butein, sulfuretin (another compound from *R. verniciflua* Stokes[21]), or resveratrol using microarray analysis. Unlike butein, neither sulfuretin nor resveratrol mediated *Ucp1* induction (**Supplementary Fig. 4a**). Therefore, 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 or resveratrol, of which we identified 127 genes (**Fig. 1**, **Supplementary Fig. 1**, and **Supplementary Data Set 1**). Next, we focused our attention on transcription factors or related genes that have been shown to influence thermogenic properties in adipocytes[22–24]. The specific induction of this subset of genes by butein, but not by sulfuretin or resveratrol, was validated by real-time 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. *Hmox1*, *Maff*, and *Atf3*, the three genes most highly induced by butein, were also included in the knockdown studies. Of these candidate genes, only inhibition of *Prdm4* 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 Figs. 7** and **21**). The other Prdm family members *Prdm2*, *Prdm3*, and *Prdm16* were not acutely regulated by butein. Furthermore, isoproterenol, sulfuretin, and resveratrol also did not affect *Prdm4* expression (**Supplementary Figs. 1c** and **8**).

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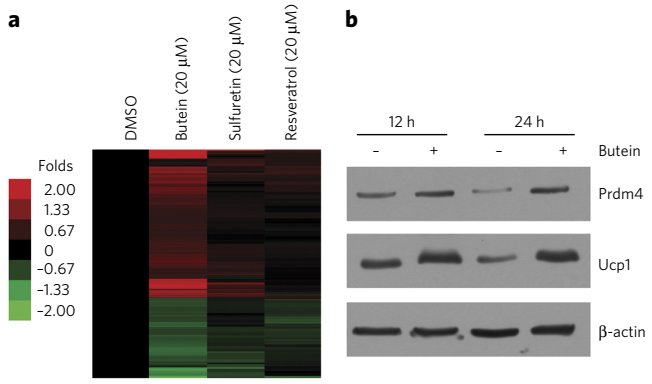

Figure 1 | Identification of Prdm4 as a butein-induced gene. (**a**) Gene-expression profiles of C3H10T1/2 differentiated adipocytes treated with butein, resveratrol or sulfuretin for 6 h. (**b**) Expression of Prdm4 and Ucp1 proteins in C3H10T1/2 adipocytes treated with butein. Uncropped images of blots are shown in **Supplementary Figure 20**.

Based on this *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 and 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-adipocyte and white-adipocyte-selective genes as 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 Figs. 10** and **21**). Silencing Prdm4 in brown adipocytes similarly inhibited the expression of thermogenic genes (**Supplementary Figs. 11** and **22**). Conversely, forced expression of Prdm4 induced *Ucp1* and mitochondrial biogenesis (**Fig. 2c** and **Supplementary Fig. 11c**) but suppressed 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 the induction of mitochondrial mass were blunted in C3H10T1/2 cells transfected with Prdm4 siRNA (**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 Prmd4 in obesity, we examined *Prdm4* mRNA levels in mice with diet-induced obesity. Levels of *Prdm4* mRNA were decreased in epididymal (eWAT) and inguinal white adipose tissue (iWAT) and BAT of obese mice. *Prdm4* expression was consistently lower in the eWAT of diabetic *db/db* mice as 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 a high-fat diet (HFD) were randomly divided and injected intraperitoneally with validated Prdm4 antisense oligonucleotides (ASO) or control ASO twice per week (25 mg per kg body weight 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 as compared to those in the control-ASO-treated mice (**Fig. 3a** and **Supplementary Fig. 15**). Body weight gain in the Prdm4 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 groups (**Supplementary Fig. 16**). Body temperature was significantly lower in the Prdm4-ASO-treated mice (36.32 °C) than in controls (36.72 °C), suggesting that Prdm4 affects thermogenic regulation *in vivo*. Consistently with this, Prdm4-ASO-treated mice had lower energy expenditure, O_2 consumption, and CO_2 production than control-ASO-treated mice (**Fig. 3c** and **Supplementary Fig. 17**). Prdm4 ASO mice showed lower expression of thermogenic genes and elevated expression 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

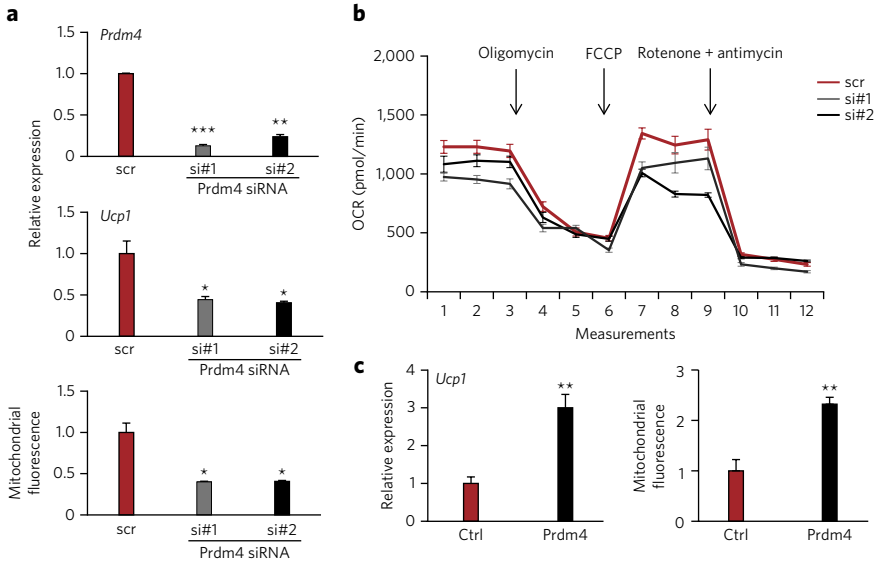

Figure 2 | Prdm4 induces Ucp1 and regulates mitochondrial respiration. (**a**) Knockdown of Prdm4 by two independent siRNAs (si#1 and si#2) reduces the expression of *Prdm4* (top) and *Ucp1* (middle) mRNA in C3H10T1/2 adipocytes as compared to that in similar cells transfected with scrambled control (scr) siRNA. Knockdown of Prdm4 also reduces mitochondrial mass (bottom). Mitochondrial staining was quantified by NIH ImageJ software. Data represent mean ± s.d. (n = 3 per group). (**b**) Knockdown of Prdm4 reduces oxygen consumption rates (OCR) as compared to that of the scr-siRNA-transfected C3H10T1/2 adipocytes. The OCR was measured in ~8-min intervals. Basal respiration, uncoupled respiration (oligomycin), maximal respiration (FCCP), and non-mitochondrial respiration (rotenone + antimycin) were determined using an XF24 Extracellular Flux Analyzer. Data represent mean ± s.d. (n = 6 per group). (**c**) Forced expression of Prdm4 induces *Ucp1* expression in C3H10T1/2 adipocytes (left). Prdm4-overexpressing cells exhibit increased mitochondrial staining compared to cells transfected with control plasmid (right). Data represent mean ± s.d. (n = 3 per group). Statistically significant differences were determined by Student's t-test (*P < 0.05; **P < 0.005; ***P < 0.0005).

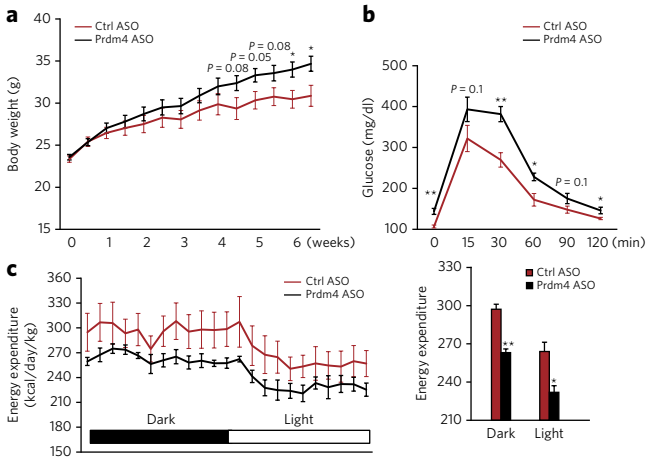

Figure 3 | Effects of Prdm4 knockdown in HFD induced obese mice. (**a**,**b**) C57BL/6 mice on a HFD were treated with Prdm4 or control (Ctrl) ASO twice per week (2.5 mg per kg body weight per dose) for 6 weeks. (**a**) Body weight gain in control- and Prdm4-ASO-injected groups. Data represent mean ± s.d. (n = 6 per group). (**b**) Results of glucose tolerance test in control- and Prdm4-ASO-injected groups. Data represent mean ± s.d. (n = 6 per group). (**c**) Mice were treated with Prdm4 or control ASO for 2.5 weeks and energy expenditure was measured before body weights started to diverge. Left, 24 h energy expenditure in Prdm4-ASO- and control-ASO-treated mice. Right, averages of dark and light periods of energy expenditure. Data represent mean ± s.d. (n = 5 per group). Statistically significant differences between the control and Prdm4 groups were determined by Student's t-test (*P < 0.05; **P < 0.005).

(**Supplementary Figs. 19** and **22**). 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 adipocyte formation 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 mice[13]. Another study demonstrates that Prdm3 and Prdm16 double-knockout mice display severely reduced expression of brown-fat-selective genes compared to wild-type mice, along with induction of white-fat-selective genes[17]. 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 identify Prdm4 as another Prdm-family member that can participate in the brown and beige fat programs.

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

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Methods

Methods and any associated references are available in the online version of the paper.

Accession codes. Microarray data sets were deposited at the Gene Expression Omnibus (accession number GSE76672).

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Acknowledgments

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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.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.

Competing financial interests

The authors declare no competing financial interests.

Additional information

Any supplementary information, chemical compound information and source data are available in the online version of the paper. Reprints and permissions information is available online at http://www.nature.com/reprints/index.html. Correspondence and requests for materials should be addressed to K.W.P.

ONLINE METHODS

Cell culture. Mouse C3H10T1/2 multipotent cells and 3T3-L1 cells were purchased from the American Type Culture Collection and cultured as previously described[26]. C3H10T1/2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (Hyclone) with 10% FBS (Hyclone). 3T3-L1 pre-adipocytes were maintained in DMEM with 10% FCS (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 μM dexamethasone (Sigma), 0.5 mM IBMX (Sigma), and 5 μg/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 μM troglitazone (Sigma). CH10T1/2 cells were refreshed with DMEM media containing 10% FBS, 1 μM troglitazone, and 5 μg/ml insulin every 2 days.

For identification of Ucp1 inducers, C3H10T1/2 adipocytes differentiated for 8 days were treated with 20 μM of bioactive small molecules (**Supplementary Table 1**) for 6 h and *Ucp1* mRNA expression was measured by real-time PCR. Butein (see **Supplementary Note**) 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. M. 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 μg/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 described[27]. 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 100 mg) were ground in liquid nitrogen and homogenized in RIPA buffer (200 μl) 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 and transferred to PVDF membranes (Bio-Rad Laboratories), and western blot analysis was performed as described[27]. The membranes were blocked for 1 h 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 chemiluminescence western blotting detection reagent (GE Healthcare).

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 RRDM4 mRNA expression levels. The selected siRNAs were then transfected using RNAi MAX (Invitrogen). The sequences for si#1 and si#2 are GAAUUACGCUCAACAGAUAUU 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 h 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 adaptation, 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 (i.p.) injection at a dose of 25 mg/kg body weight/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, O_2 consumption, and CO_2 production.

Microarray analysis. Total RNA from fully differentiated C3H10T1/2 adipocytes treated with 20 μM of butein, sulfurein or resveratrol for 6 h 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-h 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′-GAAUUACGCUCAACAGAUAUU-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 adaptation, 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 i.p. 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 h, and then the glucose levels were determined from tail-vein blood at 0, 15, 30, 60, 90, and 120 min after i.p. glucose 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 mean ± 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).

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