

Transcriptional Control of Brown Fat Determination by PRDM16

Patrick Seale,^{1,5} Shingo Kajimura,^{1,5} Wenli Yang,¹ Sherry Chin,¹ Lindsay M. Rohas,¹ Marc Uldry,¹ Geneviève Tavernier,^{2,3} Dominique Langin,^{2,3,4} and Bruce M. Spiegelman^{1,*}

¹Dana-Farber Cancer Institute and the Department of Cell Biology, Harvard Medical School, 1 Jimmy Fund Way, Boston, MA 02115, USA

²INSERM, U858, Ranguel Institute of Molecular Medicine, Obesity Research Laboratory, Toulouse, F-31432 France

³Paul Sabatier University, Louis Bugnard Institute, IFR31, Toulouse, F-31432 France

⁴CHU de Toulouse, Laboratory of Clinical Biochemistry, Biology Institute of Purpan, Toulouse, F-31059 France

⁵These authors contributed equally to this work.

*Correspondence: bruce_spiegelman@dfci.harvard.edu

DOI 10.1016/j.cmet.2007.06.001

SUMMARY

Brown fat cells are specialized to dissipate energy and can counteract obesity; however, the transcriptional basis of their determination is largely unknown. We show here that the zinc-finger protein PRDM16 is highly enriched in brown fat cells compared to white fat cells. When expressed in white fat cell progenitors, PRDM16 activates a robust brown fat phenotype including induction of *PGC-1 α* , *UCP1*, and type 2 deiodinase (*Dio2*) expression and a remarkable increase in uncoupled respiration. Transgenic expression of PRDM16 at physiological levels in white fat depots stimulates the formation of brown fat cells. Depletion of PRDM16 through shRNA expression in brown fat cells causes a near total loss of the brown characteristics. PRDM16 activates brown fat cell identity at least in part by simultaneously activating *PGC-1 α* and *PGC-1 β* through direct protein binding. These data indicate that PRDM16 can control the determination of brown fat fate.

INTRODUCTION

Adipose cells and tissue are of great interest in view of the worldwide epidemic of obesity and its associated metabolic disorders, such as insulin resistance, diabetes, dyslipidemia, and hypertension. Fat cells occur in two distinct subtypes: white fat cells, which function primarily to store energy in the form of triglycerides, and brown fat cells, which oxidize fuels and dissipate energy in the form of heat. Brown adipose tissue (BAT) is important for small mammals such as mice to defend against the cold. In humans, brown fat is abundant at birth but is rapidly replaced by white adipose tissue (WAT) and is relatively scarce in the adult as an identifiable tissue (Lean and James, 1986). On the other hand, several reports indicate that brown fat

cells are interspersed within WAT of rodents and humans (Cousin et al., 1992; Garruti and Ricquier, 1992; Guerra et al., 1998; Lean et al., 1986; Oberkofler et al., 1997; Xue et al., 2007). Identifying the mechanisms that stimulate the development and/or function of human BAT could, therefore, provide new therapeutic avenues to reduce obesity and its associated diseases.

Brown fat cells are characterized by densely packed mitochondria that contain uncoupling protein-1 (UCP1) in their inner mitochondrial membrane. UCP1, almost exclusive to brown fat cells, is a proton transporter that allows protons to leak across the mitochondrial inner membrane, thereby dissipating the electrochemical gradient normally used for ATP synthesis (reviewed by Klingenberg [1999]). The thermogenic capacity of brown fat cells, in which over 50% of cellular respiration is uncoupled from ATP synthesis, is unique among mammalian cell types (Nedergaard et al., 1977; Prusiner et al., 1968; Reed and Fain, 1968).

Brown fat cells also depend upon an extensive vascular bed and a high degree of sympathetic innervation to produce and distribute heat. Cold, sensed in the central nervous system, causes sympathetic nerves to release catecholamines, which, through the β -adrenergic receptors, increase cyclic AMP (cAMP) levels in brown fat cells (reviewed by Cannon and Nedergaard [2004]). cAMP is a critical second messenger in the function of brown fat, stimulating the levels of key thermogenic factors such as *PGC-1 α* , type 2 deiodinase (*Dio2*), and UCP1. Chronic adrenergic stimulation can also cause a proliferation of brown adipose preadipocytes and the emergence of pockets of brown fat cells in white fat depots (Bronnikov et al., 1992; Cannon and Nedergaard, 2004; Ghorbani and Himms-Hagen, 1997; Himms-Hagen et al., 2000; Nedergaard et al., 1994).

As noted above, a small number of brown adipocytes and detectable levels of *UCP1* mRNA are found in white fat of adult humans (Garruti and Ricquier, 1992; Oberkofler et al., 1997). Indeed, BAT and oxidative activity can be increased by chronic cold exposure in humans (Huttunen et al., 1981). A reemergence of BAT is also observed in adult humans with pheochromocytoma, where neuroendocrine

tumors cause excessive catecholamine secretion (Ricquier et al., 1982). Experiments in rodents have also demonstrated that chronic cold exposure or β 3-adrenergic stimulation induces the expression of UCP1 in classic white fat depots (Himms-Hagen et al., 2000). Interestingly, particular mouse strains have more brown adipose cells in their white fat depots, which correlates with a lower susceptibility to obesity and diabetes (Guerra et al., 1998). Moreover, ectopic deposits of BAT in mouse skeletal muscle protect against diet-induced obesity (Almind et al., 2007).

The extent to which BAT functions in the regulation of energy balance has been well studied in rodents. Overfeeding activates BAT thermogenesis through the stimulation of sympathetic nerves (Cannon and Nedergaard, 2004; Lupien et al., 1985; Rothwell and Stock, 1979). Increased UCP1 expression in mouse fat promotes energy expenditure, reduces adiposity, and protects animals from diet-induced and genetic forms of obesity (Cederberg et al., 2001; Ghorbani et al., 1997; Ghorbani and Himms-Hagen, 1997; Guerra et al., 1998; Kopecky et al., 1995, 1996; Tsukiyama-Kohara et al., 2001). Furthermore, specific ablation of brown fat through expression of a toxigene leads to reduced whole-body energy expenditure and increased obesity (Hamann et al., 1996; Lowell et al., 1993).

The developmental origin of fat, either brown or white, has not been fully elucidated; however, much is known about the process of adipogenic differentiation from preadipocytes. Extensive studies in cultured cell and mouse models have revealed the central role of PPAR γ in the differentiation of both brown and white adipose cells (Barak et al., 1999; Kubota et al., 1999; Nedergaard et al., 2005; Rosen et al., 1999, 2002; Tontonoz et al., 1994). Thus, PPAR γ is apparently not the primary determinant of whether a fat cell assumes a brown versus white phenotype. In fact, brown and white preadipocytes appear to be fully “committed” at that stage since fibroblastic cells cultured from brown depots differentiate as brown fat cells whereas those from white fat tissue differentiate as white adipocytes (Klaus, 1997; Klaus et al., 1995; Kozak and Kozak, 1994). The cold-induced emergence of brown adipocytes in WAT and the replacement of BAT with WAT in newborn humans and obese rodents do suggest some degree of plasticity, at least at the tissue level. However, whether mature adipocytes can “interconvert” or whether separate pools of white and brown precursors mediate “transdifferentiation” of these tissues has not been resolved.

The influence of brown fat on energy balance has thus motivated strong interest in finding a dominant regulator of brown fat cell determination. To date, a master regulator of brown fat has not been found, in terms of a single, tissue-selective factor that can positively regulate the entire program of brown fat in a cell-autonomous manner and is required for the brown fat cell phenotype. Several transcription-related factors have, however, been shown to influence the brown fat cellular phenotype. Notably, the transcriptional coactivator PGC-1 α is highly expressed in brown fat compared to white fat and can activate the

adaptive thermogenic gene program when expressed in white fat cells (Puigserver et al., 1998; Tiraby et al., 2003). However, genetic experiments in cultured brown fat cells and mice have clearly indicated that PGC-1 α , while critical for cAMP-dependent thermogenic aspects of the brown fat program, does not give cells their essential brown fat identity (Lin et al., 2004; Uldry et al., 2006).

In addition to PGC-1 α , other factors have been shown to selectively influence the function of brown versus white fat. FOXC2, when expressed by transgenesis in WAT, induces many features of BAT tissue (Cederberg et al., 2001). However, FOXC2 is not expressed preferentially in BAT versus WAT, and its role in this interconversion between tissues is probably linked to its stimulation of adrenergic signaling when it is overexpressed in vivo (Dahle et al., 2002; Gronning et al., 2006). A cell-autonomous role for FOXC2 in white-to-brown fat cell conversion has not been shown. Other factors, such as Rb (retinoblastoma), p107, and RIP140 have been shown to suppress the brown fat phenotype in white fat cells or tissue (Christian et al., 2005; Hansen et al., 2004; Leonardsson et al., 2004; Powelka et al., 2006; Scime et al., 2005). Quantitative increases in the levels of Rb, p107, or RIP140 have not, however, been demonstrated to preferentially favor the differentiation of white fat adipocytes. These data suggest that while each of these factors may participate to various degrees in the formation and/or function of brown fat cells, none of them is likely to be the central factor that determines an adipocyte to specifically commit to a brown adipose fate.

Using global expression analysis of murine transcriptional components, we found a very small number of factors that are selectively expressed in brown versus white fat cells. One of these was PRDM16, a 140 kDa PR (PRD1-BF1-RIZ1 homologous)-domain-containing protein that was first identified at a chromosomal breakpoint in myeloid leukemia (Nishikata et al., 2003). We show here that PRDM16 activates a broad program of brown fat differentiation when expressed in cultured white fat preadipocytes or in white fat depots in vivo. Uncoupled respiration accounts for the majority of the very high oxygen consumption of PRDM16-expressing cells, a classic hallmark of brown fat. Furthermore, shRNA-mediated knockdown of PRDM16 in bona fide brown fat preadipocytes allows normal differentiation into fat cells but results in a total loss of the brown fat character. Our experiments thus reveal a dominant regulatory function for PRDM16 in the brown fat lineage.

RESULTS

PRDM16 Is Expressed Selectively in Brown Adipocytes

With the goal of identifying transcriptional components that regulate the development and function of brown adipocytes, we performed a global expression screen of all genes known to be involved in transcription and those having a molecular signature suggestive of transcriptional function. White and brown fat tissue RNA samples from

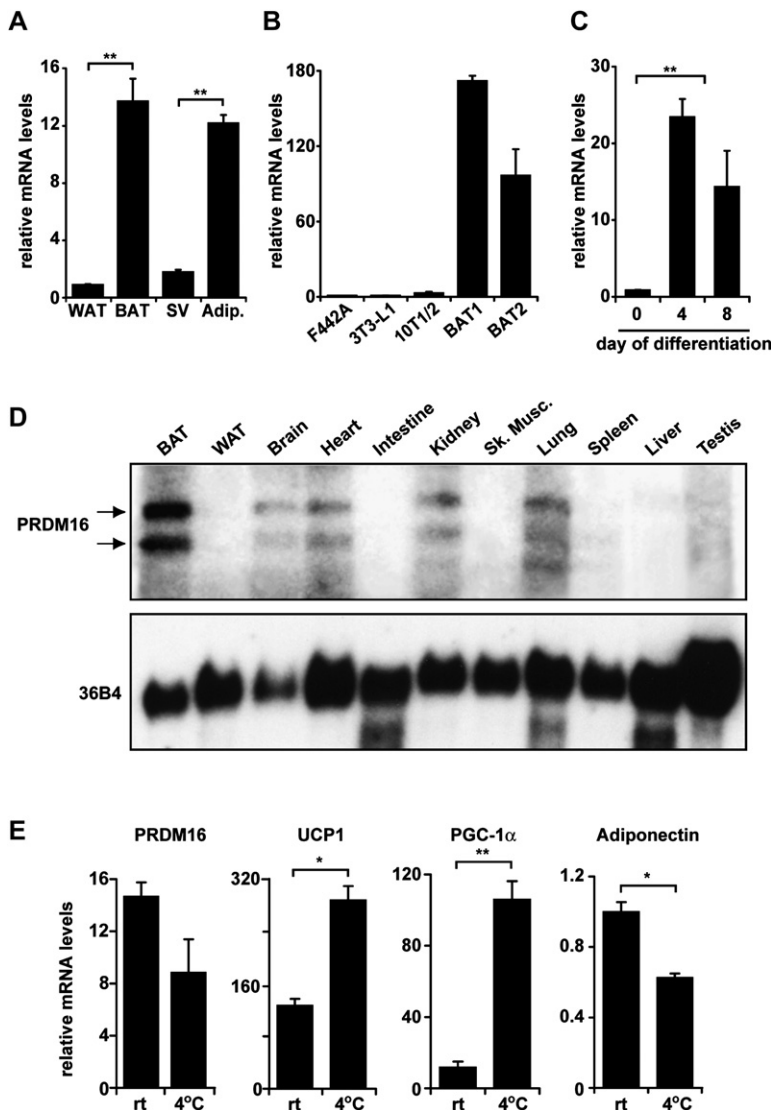


Figure 1. PRDM16 Is Expressed Selectively in Brown Fat Cells

(A) Real-time PCR analysis of *PRDM16* mRNA expression in BAT relative to WAT, and its expression in the adipocyte fraction (Adip.) compared to the stromal-vascular fraction (SV) of BAT ($n = 6$). In this and all other figures, error bars represent \pm SD unless otherwise indicated.

(B) *PRDM16* mRNA expression in adipocytes from immortalized BAT cell lines (BAT1 and BAT2) and three WAT cell lines (3T3-F442A, 3T3-L1, and C3H-10T1/2) ($n = 3$ –5 samples per cell line).

(C) *PRDM16* mRNA levels during the differentiation of immortalized BAT cell lines ($n = 3$ for each of two separate cell lines).

(D) Northern blot analysis of *PRDM16* mRNA and control *36B4* mRNA in adult mouse tissues.

(E) Expression of *PRDM16*, *UCP1*, *PGC-1 α* , and *Adiponectin* in BAT after cold exposure (4°C) of mice for 4 hr ($n = 5$ mice per group). rt = room temperature. * $p < 0.05$; ** $p < 0.01$.

C57BL/6 mice ($n = 5$) at 10–12 weeks of age were examined by RT-PCR using primer sets corresponding to all transcription-related mouse genes (Gray et al., 2004). Genes differentially regulated in these tissues were then assayed for their expression in cultured white and brown fat cells from established cell lines. Three genes, *Lhx8*, *Zic1*, and *PRDM16*, ultimately met the criteria of being preferentially expressed in brown versus white fat and having elevated (>5-fold) mRNA expression levels in immortalized brown fat cells (Uldry et al., 2006) compared to any of three immortalized white fat cell lines (3T3-L1, 3T3-F442A, and C3H-10T1/2) (data not shown). As described in detail below, ectopic expression demonstrated a function for PRDM16 in stimulating the brown fat phenotype.

Quantitative analyses of *PRDM16* expression at the mRNA level showed a 15-fold enrichment in BAT relative to WAT ($n = 6$) (Figure 1A). Fractionation of BAT showed that *PRDM16* was mainly expressed in mature brown

adipocytes as compared to the stromal-vascular fraction that contains preadipocytes and other cell types (Figure 1A). Importantly, *PRDM16* was selectively expressed in adipocytes from two immortalized brown fat cell lines (Uldry et al., 2006) as compared to its expression in white fat cells from 3T3-F442A, 3T3-L1, and C3H-10T1/2 fibroblasts (Figure 1B). Moreover, *PRDM16* mRNA expression increased 20-fold during the differentiation of brown fat cells in culture (Figure 1C). Northern blot analysis indicated that *PRDM16* was expressed at its highest levels in BAT and was virtually undetectable in WAT (Figure 1D). *PRDM16* transcripts were also detected in heart, lung, kidney, and brain. Acute cold exposure of mice (4°C for 4 hr) activated the thermogenic program of BAT without any significant regulation of *PRDM16* mRNA expression ($p = 0.13$) (Figure 1E). Similarly, *PRDM16* expression was not affected by cAMP treatment of cultured cells (data not shown). These results suggest that *PRDM16* expression in brown fat is linked to determination or differentiation, but

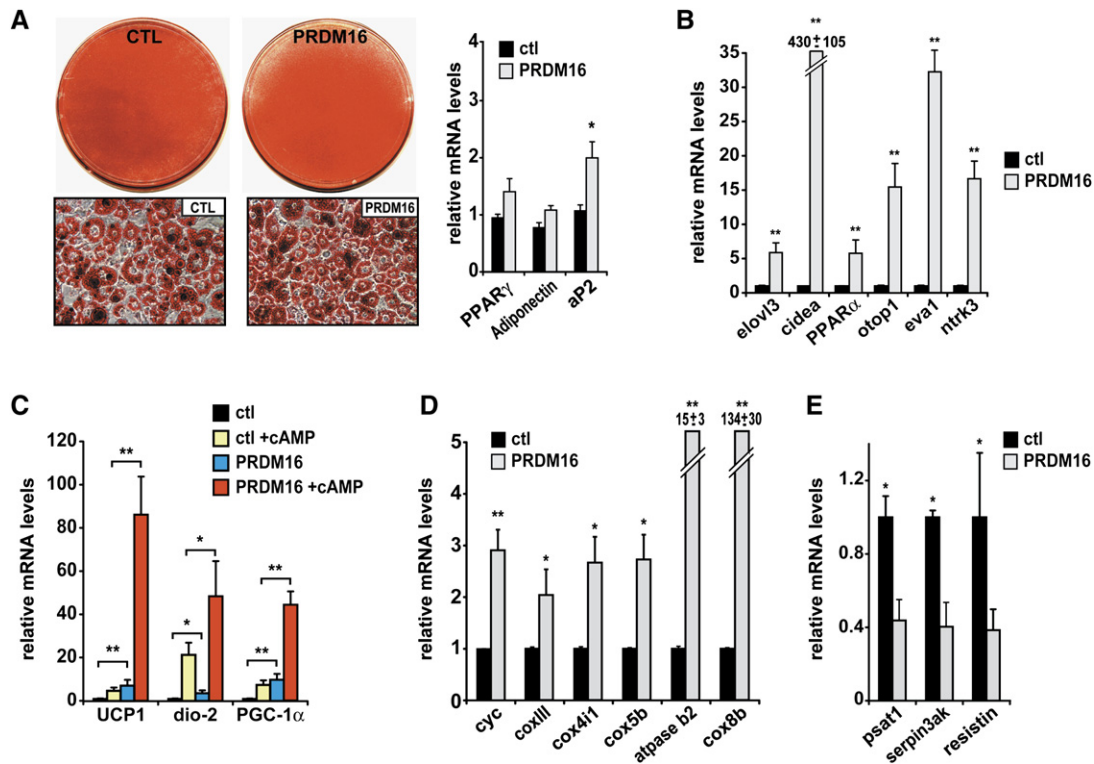


Figure 2. PRDM16 Expression Induces the Gene Program of Brown Fat Cells

Oil red O staining (A, left) of mature adipocytes (day 6) from *PPAR γ* -deficient cells expressing retroviral *PPAR γ 2* and either retroviral *PRDM16* or vector control (ctl). These adipocyte cultures were analyzed by real-time PCR for their expression of differentiation markers common to WAT and BAT (A), brown fat-selective genes (as indicated) (B), brown fat thermogenic genes (*UCP1*, type 2 deiodinase [*Dio2*], and *PGC-1 α*) with and without cAMP treatment (C), mitochondrial components (as indicated) (D), and white fat-selective markers (*Psat1*, *Serpin3ak*, and *Resistin*) (E). (n = 3.) *p < 0.05; **p < 0.01.

not to adaptive thermogenesis. Collectively, these data identify *PRDM16* as a gene that is very selectively expressed in brown versus white adipocytes but is not entirely specific to brown fat tissue.

PRDM16 Drives the Molecular Phenotype of Brown Fat Cells

To study its role in brown fat cells, we first expressed *PRDM16* in cells with no preadipose (white or brown) character whatsoever, using fibroblasts genetically deficient in *PPAR γ* (Rosen et al., 2002). A retroviral vector expressing *PRDM16* or control vector was introduced together with a *PPAR γ 2*-expressing retroviral vector into subconfluent fibroblasts and selected for stable viral integration. Cells transduced with retroviral *PRDM16* expressed mRNA levels that were 4- to 6-fold higher than its endogenous expression in cultured brown fat cells. These cell cultures were grown to confluence and stimulated to undergo adipogenic differentiation driven by the ectopic *PPAR γ 2*. At day 6 of differentiation, cultures expressing *PPAR γ 2* were composed mainly of adipocytes whether or not they also expressed *PRDM16*; ectopic *PRDM16* appeared to neither promote nor inhibit cellular differentiation per se (Figure 2A). At the molecular level, expression of genes

common to both white and brown fat lineages was either not affected (*PPAR γ* and *Adiponectin*) or moderately elevated (*aP2*) by *PRDM16* expression (Figure 2A).

To analyze the molecular phenotype of mature fat cells from *PRDM16*-expressing cultures in detail, we first performed global, unbiased expression analyses to develop a more complete set of brown versus white fat-selective genes. To this end, brown and white fat tissues from three adult C57BL/6 mice were expression profiled using Affymetrix microarrays. The mRNA levels for the 40 most highly enriched genes in both brown and white fat tissue were analyzed by qPCR in samples from three independent mice to validate the microarray data. These genes were then assayed for their expression in mature fat cells from three white (3T3-L1, 3T3-F442A, and C3H-10T1/2) and two brown fat cell lines. Altogether, we identified 19 BAT-selective and 9 WAT-selective genes whose expression correlated completely (by all pairwise comparisons) with the BAT or WAT adipocyte phenotype in tissue and in cultured cells (see Table S1 in the Supplemental Data available with this article online).

Strikingly, most brown fat-selective mRNAs were specifically induced in adipocytes from *PRDM16*-expressing cells. For example, *Elov13* (previously called *Cig30*), a

very long-chain fatty acid elongase expressed in brown but not white fat and implicated in brown fat hyperplasia (Tvrdik et al., 1997; Westerberg et al., 2006), was induced 6-fold by PRDM16 expression (Figure 2B). *Cidea*, a gene predominantly expressed in brown fat, where it is thought to regulate UCP1 activity (Zhou et al., 2003), was induced 430-fold by PRDM16 (Figure 2B). PRDM16 also elevated expression of *PPAR α* , a brown fat-selective gene and an important regulator of fatty acid oxidation (Barbera et al., 2001; Braissant et al., 1996), 5-fold (Figure 2B). In addition, several genes not previously known to be expressed selectively in brown versus white adipocytes, such as otopetrin 1 (*Otop1*), epithelial-like antigen-1 (*Eva1*), and neurotrophic tyrosine kinase receptor type 3 (*Ntrk3*), were significantly increased by PRDM16-expression (Figure 2B). Moreover, PRDM16-transduced cells expressed several of the important thermogenic genes in a cAMP-dependent manner, similar to bona fide brown fat cells in culture and in vivo. The mRNA levels of *UCP1*, *Dio2*, and *PGC-1 α* were increased by PRDM16 in unstimulated fat cells and induced to very high levels by PRDM16 in response to cAMP (Figure 2C). *UCP1*, for instance, was elevated 20-fold by cAMP in PRDM16-expressing cells, compared to only a 3-fold increase in control cells. PRDM16 expression also elevated the mRNA levels for many genes of mitochondrial oxidative phosphorylation that are known to be enriched in brown fat cells and tissue, such as cytochrome c (*Cyc*), *Cox411*, and *Cox5b* (Figure 2D). In particular, *Cox8b*, a highly brown fat-selective mitochondrial gene (see Table S1), was expressed at 134-fold higher levels in PRDM16-expressing cultures compared to control adipocyte cultures.

Interestingly, the mRNA levels of several genes that are preferentially expressed in white relative to brown fat cells were significantly repressed by PRDM16 expression, including *Psat1*, *Serpin3ak*, and *Resistin* (Figure 2E). In particular, *Resistin*, which encodes a WAT-secreted protein in mice that promotes insulin resistance (Steppan et al., 2001), was reduced by 60% at the mRNA level as a result of PRDM16 expression. Altogether, 20 of 28 (71%) of the brown/white fat-selective genes that we identified in an unbiased global expression analysis were regulated in a brown fat-specific manner by PRDM16 (Table S1). A robust regulation of brown fat-selective genes by PRDM16 was observed in other cellular models, including 3T3-F442A white preadipocytes and immortalized brown preadipocytes (Figures S1A and S1B). In particular, PRDM16 increased the mRNA levels of *UCP1* and *Cidea* by almost 200-fold and repressed *Resistin* expression by 70% in 3T3-F442A adipocytes (Figure S1A). These data demonstrate that the action of PRDM16 in activating brown fat gene expression is not unique to *PPAR γ ^{-/-}* fibroblasts and also is not dependent on exogenous *PPAR γ* expression. Moreover, PRDM16 dramatically induced the expression of the key brown fat genes *UCP1* and *PGC-1 α* , but not *Cidea* or mitochondrial components in the genetic absence of *PPAR γ* (Figure S1C). These results show that certain genes characteristic of the brown fat gene program do not absolutely require the fat cell environment

and/or *PPAR γ* function. Altogether, these data strongly suggest a role for PRDM16 as a positive regulator of the brown fat gene program.

An important question is whether PRDM16 can stimulate the transdifferentiation of mature white fat cells into brown fat. This was addressed by analyzing the effect of PRDM16 expression in stromal-vascular cells and mature adipocytes from the same white fat tissue. When PRDM16 was expressed in stromal-vascular cells prior to differentiation, this factor efficiently activated the brown fat gene program in differentiated cultures, including an induction of *UCP1* mRNA by more than 200-fold (Figure S2A). However, expression of PRDM16 using adenovirus in mature white fat cells failed to elicit a significant induction of brown fat marker genes despite robust expression of PRDM16 (Figure S2B). Importantly, PRDM16 protein expressed by adenovirus was able to induce BAT-related genes when introduced into cells before their differentiation (data not shown). Altogether, the induction of brown fat genes in adipocytes from PRDM16-expressing primary stromal-vascular cells and lack of any effect in mature cells from the same tissue strongly suggest that PRDM16 function is required before and/or during adipogenic differentiation to promote brown fat cell character.

PRDM16 Stimulates Mitochondrial Biogenesis and Uncoupled Cellular Respiration

A defining feature of brown fat cells is their abundant mitochondria and associated high rates of cellular respiration, particularly uncoupled respiration. To assay the functional consequences of PRDM16 action in fat cells, *PPAR γ ^{-/-}* cells transduced with *PRDM16* or vector control together with *PPAR γ 2* were induced to differentiate into adipocytes. Representative electron micrographs of control and PRDM16-expressing cells before and after adipogenic differentiation showed a very significant elevation of mitochondrial density by PRDM16 only in differentiated fat cells (Figure 3A). Quantitative analysis of these data revealed a 2-fold increased mitochondrial volume in PRDM16-expressing adipocytes compared to control cultures ($p < 0.001$) (Figure 3B). Oxygen consumption was measured in adipocytes using an oxygen-sensitive electrode to calculate relative rates of respiration. These experiments revealed a remarkable effect of PRDM16 on the levels of both total and uncoupled cellular respiration. Specifically, adipocytes from PRDM16-expressing cells displayed a 40% increase in total respiration and a 2-fold increase in uncoupled respiration relative to control cultures ($n = 4$) (Figure 3C). After 12 hr treatment with cAMP, PRDM16-expressing adipocytes displayed a 70% increase in total respiration relative to control cells, and >60% of their respiration was uncoupled from ATP production (Figure 3D). PRDM16 expression, therefore, induced mitochondrial biogenesis and uncoupled respiration to levels characteristic of brown fat cells (Uldry et al., 2006) but not other mammalian cell types. These results demonstrate that PRDM16 expression enables fat cells to develop the respiratory activity characteristic of brown adipocytes.

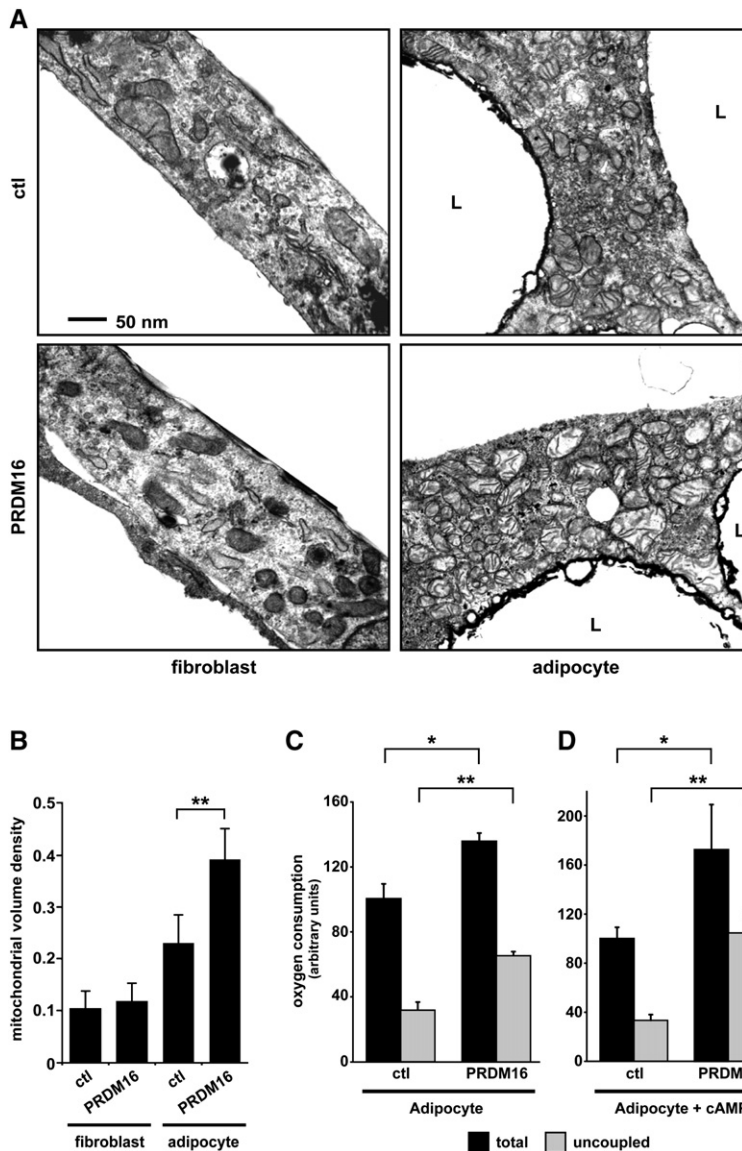


Figure 3. PRDM16 Stimulates Mitochondrial Biogenesis and Uncoupled Respiration

(A) Representative transmission electron micrographs of fibroblasts and mature adipocytes (day 6) from *PPAR γ* -deficient cells expressing retroviral *PPAR γ 2* and either retroviral *PRDM16* or vector control (ctl). L = lipid droplet.

(B) Comparison of mitochondrial volume densities from cells depicted in (A) ($n > 20$ micrographs per group).

(C and D) Total mitochondrial oxygen consumption and uncoupled respiration in mature adipocytes expressing *PRDM16* or control vector under basal conditions ($n = 4$) (C) or after stimulation with a cAMP analog for 12 hr ($n = 4$) (D). * $p < 0.05$; ** $p < 0.01$.

In Vivo Differentiation of PRDM16-Expressing Fibroblasts into “Brown” Fat

Since the environmental cues that stimulate adipocyte differentiation in cell culture and in vivo are presumably different, we sought a more physiological setting in which to study *PRDM16* action. To this end, we utilized transplantation studies in mice. Specifically, 10^7 *PPAR γ ^{-/-}* fibroblasts expressing *PPAR γ 2* and *PRDM16* or *PPAR γ 2* alone were implanted subcutaneously into nude mice. As shown originally by Green and Kehinde (1979), implanted fibroblasts were able to differentiate into fat tissue. *PRDM16*- and vector-expressing cells formed equivalently sized ectopic fat pads after 6–8 weeks (data not shown). Histological analysis showed no obvious differences in the morphology and appearance of the tissues derived from control or *PRDM16*-expressing cells, with both fat pads containing largely unilocular adipocytes

(Figure 4A). Importantly, fat pads from *PRDM16*-expressing cells displayed an average 16-fold increase in *PRDM16* mRNA compared to control fat pads (Figure 4B), well matched to the ~15-fold enrichment in brown relative to white fat tissue (Figure 1A). Immunohistochemical analysis showed that only fat tissue from *PRDM16*-expressing cells and endogenous BAT contained easily detectable levels of the brown fat-specific Cidea protein (brown staining in Figure 4A). *PRDM16* did not influence the tissue expression of the general adipogenic markers *PPAR γ* or *Adiponectin* (Figure 4C). However, the brown fat-selective genes *UCP1*, *Cidea*, *PGC-1 α* , *Elovl3*, and *PPAR α* were all induced by at least 5-fold in fat pads from *PRDM16*-transduced cells relative to control fat pads (Figure 4D). Interestingly, *PRDM16* also induced the expression of the endogenous *PRDM16* gene (*PRDM16*-3'UTR), suggesting that *PRDM16* regulates itself in a positive feedback loop

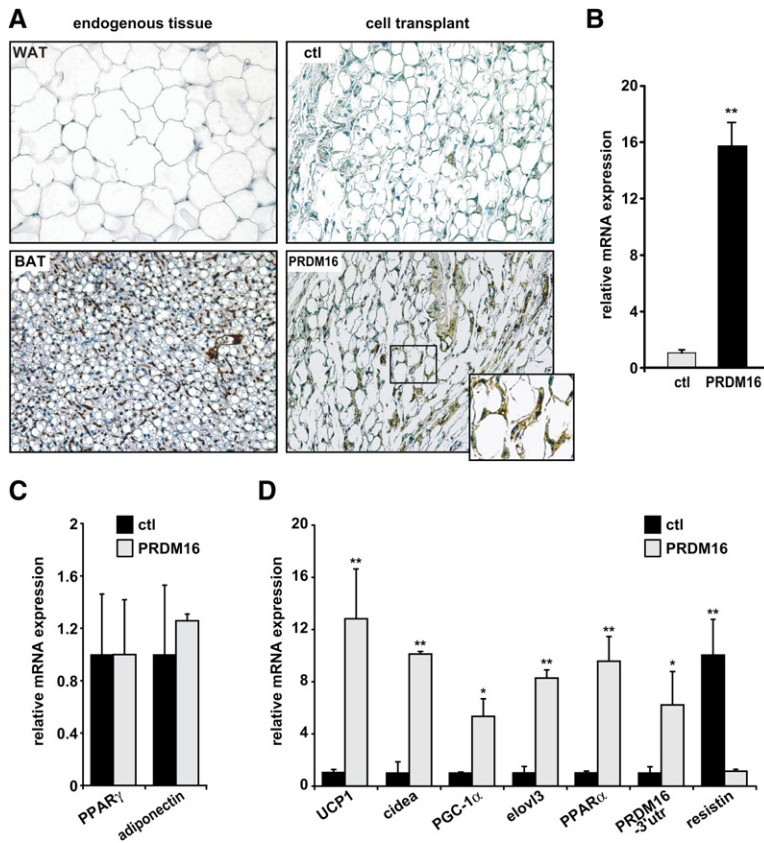


Figure 4. Differentiation of PRDM16-Expressing Cells into Brown Fat In Vivo

(A) Immunohistochemistry for Cidea protein expression in endogenous WAT and BAT and in ectopic subcutaneous fat pads formed from fibroblasts expressing PPAR γ 2 and either vector control (ctl) or PRDM16.

(B–D) Eight weeks after transplantation, ectopic fat pads were analyzed by real-time PCR for expression of PRDM16 (B); differentiation markers common to white and brown fat (PPAR γ and Adiponectin) (C); and brown fat-selective genes (UCP1, Cidea, PGC-1 α , Elovl3, PPAR α , and endogenous PRDM16 (-3'UTR)) and the white fat-selective marker Resistin (D). (n = 10 mice per cell line; error bars in [B]–[D] represent \pm SEM.) *p < 0.05; **p < 0.01.

(Figure 4D). Of note, the mRNA levels of Resistin, a white fat-selective gene, were reduced by 90% in PRDM16-expressing tissue relative to control tissue (Figure 4D). These data demonstrate that a physiological level of PRDM16 expression induces the gene expression program, but not the multilocular appearance, of BAT. This is as expected since the morphology of BAT is not cell autonomous and depends on a high degree of sympathetic innervation (Minokoshi et al., 1986; Rothwell and Stock, 1984), which presumably could not take place during the time frame of these experiments.

PRDM16 Functions as a Coregulator to Activate PGC-1 α and PGC-1 β

Since PGC-1 α has a prominent role as a regulator of brown fat thermogenic function and its mRNA is induced by the action of PRDM16, we studied PRDM16 function on this gene promoter. The expression of PRDM16 enhanced the activity of the -2 kb PGC-1 α promoter, but only in the presence of differentiation-inducing cocktail (Figure 5A). Additional experiments showed that activation of the PGC-1 α promoter by PRDM16 required a cAMP stimulus, either by adding IBMX or forskolin (data not shown). PRDM16 similarly activated the -4 kb upstream region of the UCP1 gene in forskolin-treated cells (data not shown). Since PGC-1 α is known to potently regulate its own expression in a feedback loop (Handschin et al., 2003), we asked whether PGC-1 α is required for the action

of PRDM16 on the PGC-1 α promoter. In PGC-1 α -deficient brown preadipocytes, the PGC-1 α reporter gene was only activated by PRDM16 after re-expression of PGC-1 α (Figure 5B). These data reveal a requirement for PGC-1 α protein in the activation of the -2 kb PGC-1 α promoter by PRDM16.

Previous studies showed that cells lacking PGC-1 α and PGC-1 β lose brown fat character (Uldry et al., 2006), so we investigated whether PRDM16 could enhance the transcriptional activity of PGC-1 α and PGC-1 β . To do this, PGC-1 α , PGC-1 β , PRC, SRC1, and VP16 were expressed as fusion proteins covalently bound to the Gal4 DNA-binding domain (DBD) together with either PRDM16 or control vector. The transcriptional activity of Gal4 fusions was assayed using a reporter gene driven by the Gal1-UAS sequence. In these assays, PRDM16 robustly activated both PGC-1 α and PGC-1 β but did not activate the related coactivator protein PRC (Figure 5C) or the unrelated coactivators SRC1 and VP16 (data not shown).

We then assessed whether the enhancement of PGC-1 α and PGC-1 β activity by PRDM16 was linked to a physical interaction between these proteins. Using coimmunoprecipitation assays in brown adipocytes, endogenous PGC-1 α and PGC-1 β proteins were detected in a complex with PRDM16 protein (Figure 5D). The PRDM16-PGC-1 α interaction appeared to be direct since purified GST-PGC-1 α fusion protein, but not GST alone, bound to in vitro-translated PRDM16 (Figure 5E). Domain-mapping

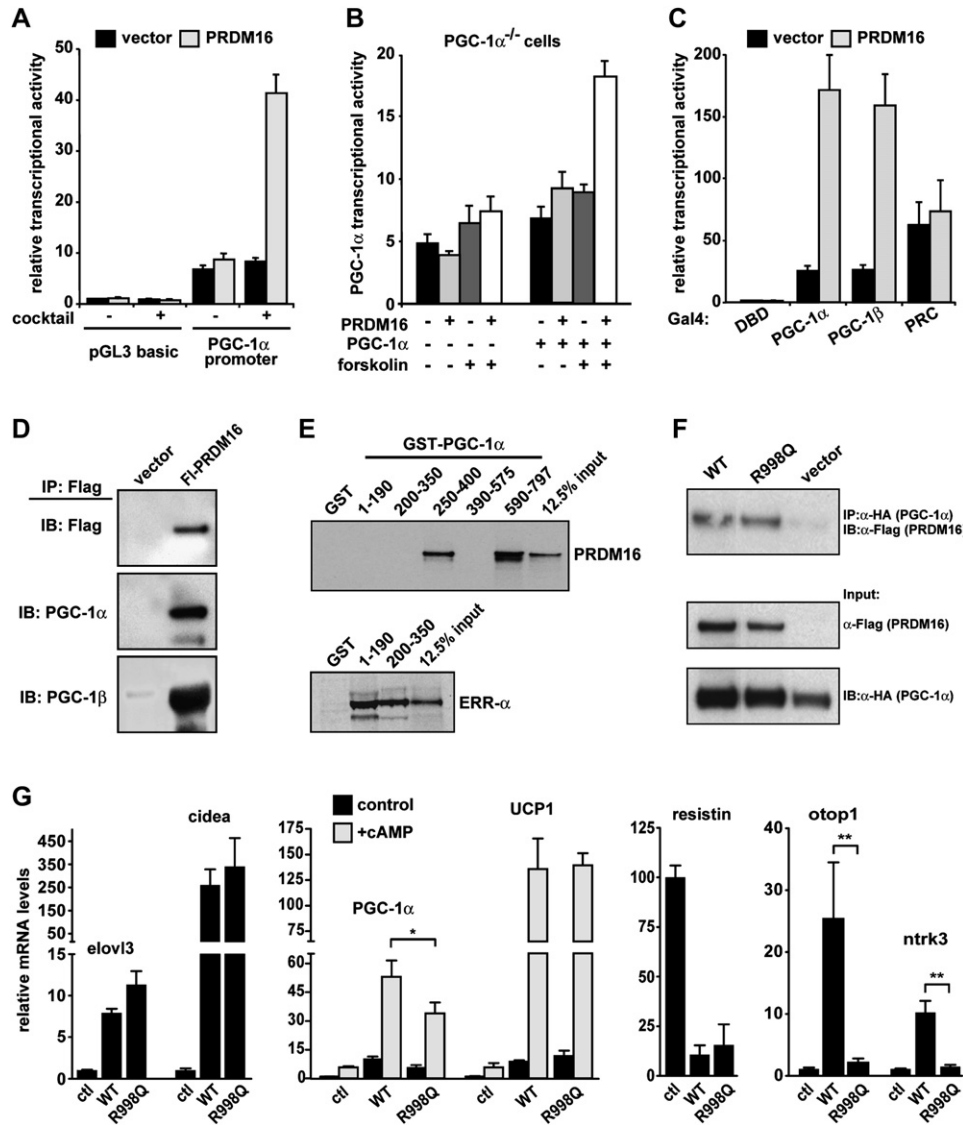


Figure 5. PRDM16 Activates PGC-1 α and PGC-1 β via Direct Binding

(A) Transcriptional activity of the -2 kb region of *PGC-1 α* in response to PRDM16 or control vector expression in brown fat preadipocytes ($n = 3$). (B) *PGC-1 α* promoter activity in response to PRDM16 or vector expression in *PGC-1 α* -deficient cells ($n = 3$). (C) Transcriptional activity of Gal4 DNA-binding domain (DBD) fusion proteins containing PGC-1 α , PGC-1 β , or PRC in response to PRDM16 or vector expression ($n = 3$). (D) FLAG-PRDM16 and its associated proteins were immunoprecipitated from brown fat preadipocytes and analyzed by western blot to detect PGC-1 α and PGC-1 β . (E) GST fusion proteins containing different regions of PGC-1 α were incubated with 35 S-labeled PRDM16 protein. ERR α was used to demonstrate binding to the 1–190 and 200–350 regions of PGC-1 α . (F) PGC-1 α and PRDM16 were coprecipitated from COS-7 cells transfected with HA-PGC-1 α and either wild-type (WT) or R998Q mutant PRDM16. The input was 2% of the cell lysate used for immunoprecipitation. (G) WT or R998Q mutant PRDM16 was expressed with PPAR γ 2 in PPAR γ $^{-/-}$ fibroblasts. After differentiation into adipocytes (day 6), real-time PCR was used to measure the mRNA expression of brown fat-selective genes (as indicated) and *Resistin*, a white fat-selective gene ($n = 3$). * $p < 0.05$; ** $p < 0.01$.

experiments identified two regions in PGC-1 α (250–400 and 590–797) that interact with PRDM16. Correct folding of PGC-1 α fragments 1–190 and 200–350 was verified by their binding to another PGC-1 α -interacting protein, ERR α . Taken together, these data strongly suggest that

PRDM16 directs at least some aspects of brown fat determination and function through a direct interaction with the PGC-1 α and PGC-1 β transcriptional coactivator proteins.

PRDM16 contains two distinct DNA-binding domains that consist of seven C2H2 repeats at the N terminus and

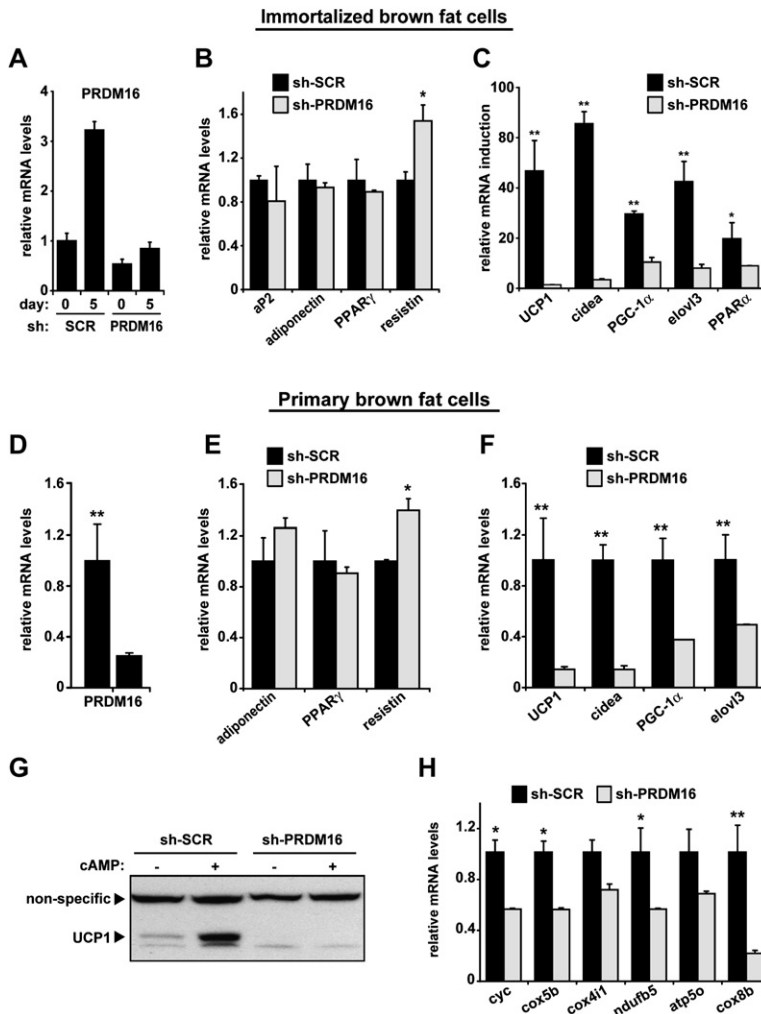


Figure 6. Knockdown of PRDM16 in Brown Fat Cells Ablates Their Brown Fat Characteristics

(A) *PRDM16* mRNA levels in immortalized brown fat cells expressing shRNA targeted to *PRDM16* or a scrambled (SCR) control shRNA before (day 0) and after their differentiation (day 5) into adipocytes.

(B) Gene expression in brown fat cells (day 5) expressing sh-*PRDM16* or sh-SCR including markers common to white and brown fat cells (*aP2*, *PPAR γ* , and *Adiponectin*) and *Resistin*, a white fat-selective gene.

(C) The differentiation-linked mRNA induction (day 0 to day 5) of brown fat-selective genes (as indicated) in sh-*PRDM16*- and sh-SCR-expressing cells.

(D–F) Gene expression in adipocytes (day 6) from sh-*PRDM16*- and sh-SCR-expressing primary brown preadipocytes including mRNA levels of *PRDM16* (D); *Adiponectin*, *PPAR γ* , and *Resistin* (E); and brown fat-selective genes (as indicated) (F).

(G) Western blot analysis of UCP1 protein levels in primary brown fat cells expressing sh-*PRDM16* or sh-SCR control with and without cAMP treatment.

(H) mRNA levels of various mitochondrial components in adipocytes from sh-*PRDM16*- and sh-SCR-expressing primary brown preadipocytes. (n = 3–5.) *p < 0.05; **p < 0.01.

three repeats at the C terminus. Since previous work has shown that PRDM16 binds directly to DNA (Nishikata et al., 2003), we asked whether PRDM16 requires direct sequence-specific DNA binding to induce the brown fat gene program. Based on the structural characteristics of zinc-finger motifs (Pavletich and Pabo, 1991), we created a series of mutants with point mutations in the conserved amino acids typically required for DNA binding. Among them, we found that an R998Q mutant allele completely loses DNA binding to the known consensus sequences of PRDM16 (Figure S3A). Importantly, the R998Q mutant allele retains the ability to interact with PGC-1 α (Figure 5F). We next examined whether the R998Q mutant protein was able to activate the brown fat gene program when expressed in *PPAR γ ^{-/-}* cells together with PPAR γ 2. Importantly, adipocytes from these cultures expressed equivalent levels of *PRDM16* mRNA (Figure S3B). Strikingly, a number of brown fat-selective genes including *Elovl3*, *Cidea*, *PGC-1 α* , and *UCP1* were induced to similar extents by R998Q mutant and wild-type PRDM16 (Figure 5G). Moreover, *Resistin*, a WAT-selective gene, was markedly repressed by both wild-type PRDM16 and the R998Q allele (Figure 5G). By contrast, wild-type

PRDM16, but not the R998Q allele, induced the expression of *Otop1* and *Ntrk3* (Figure 5G). Taken together, these results indicate that PRDM16 does not require sequence-specific DNA binding to activate many, but not all, brown fat genes and suggest that it functions in BAT determination at least in part through direct interaction with the PGC-1s and other proteins.

Knockdown of PRDM16 Ablates the Genetic Program of Brown Fat

The requirement for PRDM16 in determining the brown fat phenotype was investigated using siRNA technology to specifically knock down PRDM16 in the brown fat cell lineage. To this end, a siRNA sequence was identified that efficiently depleted *PRDM16* mRNA and protein levels in murine cells (data not shown). Expression of the corresponding shRNA by retrovirus efficiently reduced *PRDM16* expression in brown fat preadipocytes and completely blocked the differentiation-linked induction of *PRDM16* mRNA in these cells (Figure 6A). Importantly, depletion of PRDM16 in immortalized brown fat cells did not suppress morphological differentiation, nor did it impact the expression of adipocyte markers that are not specific

for brown fat, such as *aP2*, *Adiponectin*, or *PPAR γ* (Figure 6B). Strikingly, however, PRDM16 depletion resulted in a broad loss of brown fat gene expression, including severely reduced levels of *UCP1*, *Cidea*, *PGC-1 α* , *Elovl3*, and *PPAR α* relative to cells infected with a sh-scramble (SCR) control (Figure 6C). Notably, *UCP1* and *Cidea*, whose expression defines the identity of brown fat, were both decreased by 95% in PRDM16-depleted cells (Figure 6C). Furthermore, some white fat-selective mRNAs, such as *Resistin*, were increased in PRDM16-depleted cells (Figure 6B).

Loss-of-function experiments were also performed in primary brown preadipocytes immediately after their isolation. Transduction of primary brown preadipocytes with sh-PRDM16 caused an ~70% reduction of *PRDM16* mRNA at day 6 of differentiation (Figure 6D). Once again, this caused no visible decrease in morphological differentiation (data not shown) and no difference in the expression of mRNAs common to white and brown fat cells, such as *Adiponectin* and *PPAR γ* (Figure 6E). As in the immortalized cells, the expression of most, if not all, brown fat-selective genes, including *UCP1*, *Cidea*, *Elovl3*, and *PGC-1 α* , was significantly decreased in PRDM16-depleted primary brown fat cells (Figure 6F). Specifically, loss of PRDM16 caused an 85% reduction in *UCP1* and *Cidea* mRNAs and a 60% decrease in the expression of *PGC-1 α* . Furthermore, PRDM16-depleted brown fat cells exhibited a close to complete loss of UCP1 protein expression that was readily apparent in control cultures after cAMP stimulation (Figure 6G). Importantly, expression of a broad set of mitochondrial genes that are vital to brown fat function was significantly reduced by knockdown of PRDM16 in immortalized and primary brown fat cells (Figure 6H). This included a 70% reduction in the mRNA levels of *Cox8b*, a highly brown fat-selective mitochondrial gene (Figure 6H). The effect of PRDM16 depletion on the expression of the brown versus white fat-selective gene set in both cellular models is summarized in Table S1. These data establish a near absolute requirement for PRDM16 in determining the identity of brown fat cells. PRDM16, however, is not required for aspects of adipogenesis that are common to brown and white fat cells.

Transgenic PRDM16 Expression in WAT Stimulates the Formation of Brown Fat Cells

PRDM16 can drive the transcriptional program of BAT when expressed in white fat progenitors before differentiation; however, its ability to stimulate BAT cell determination in a purely in vivo context remained unclear. To address this, we sought to express PRDM16 in WAT by transgenesis. Since there are no known fat-selective promoters that express well in preadipose cells, we utilized the *aP2* promoter/enhancer, which is active during fat cell differentiation (Graves et al., 1992; Ross et al., 1990). We developed several transgenic mouse strains, and the two highest expressing strains (*aP2-T1* and *aP2-T2*) had levels of PRDM16 protein in epididymal WAT that were equivalent to or somewhat less than its expression in wild-type interscapular BAT (Figure 7A). PRDM16 is expressed

in wild-type BAT and in one transgenic WAT sample as a closely spaced doublet of 150–170 kDa; the molecular basis for this doublet is not known. Notably, transgenic PRDM16 expression induced a broad set of BAT-selective genes in epididymal white fat depots from 3- to 4-month old *aP2-PRDM16* (T1) mice (n = 7), as compared to nontransgenic littermates (n = 10) (Figure 7B). For example, the mRNA levels of *UCP1*, *Cidea*, *Cpt1b*, and *Otop1* were increased by 50-, 20-, 10-, and 20-fold, respectively, in WAT from *aP2-PRDM16* mice relative to nontransgenic mice. *PGC-1 α* , *Cox7a1*, and *Ntrk3* were also significantly elevated by transgenic expression of PRDM16. Furthermore, expression of *Resistin*, a white fat-selective gene, was slightly decreased (though not statistically significantly) in *aP2-PRDM16* transgenic fat. These data demonstrate a robust induction of the genetic program of brown fat by PRDM16 in a classic white fat depot.

WAT is poorly innervated, at least compared to BAT, and the action of PRDM16 as a determinant of BAT in cellular models was enhanced by cAMP treatment, which mimics sympathetic (adrenergic) input. We therefore treated wild-type and *aP2-PRDM16* mice (*aP2-T1* and *aP2-T2*) with CL 316243, a selective β 3-adrenergic agonist, for 6 days. In wild-type WAT, this treatment increased *PRDM16* mRNA expression by 4-fold (data not shown), coincident with the emergence of small clusters of brown fat cells (see below) and increased expression of many brown fat-selective genes (Figure 7B). However, the induction of these BAT-related genes in WAT was greatly enhanced in both strains of *aP2-PRDM16* mice (Figure 7B). In particular, *UCP1*, *PGC-1 α* , and *Cidea* were expressed at 5-fold, 4-fold, and 9-fold higher levels, respectively, in the WAT of CL 316243-treated *aP2-PRDM16* mice relative to nontransgenic mice (T1, n = 8; T2, n = 6) (Figure 7B). Importantly, BAT-selective genes were expressed in WAT of *aP2-PRDM16* animals at a significant fraction of their levels in similarly treated bona fide interscapular BAT. For example, *PGC-1 α* , *UCP1*, and *Cidea* were expressed in transgenic WAT at about 80%, 15%, and 30% of wild-type BAT levels (data not shown).

The transgenic mice were also investigated for the morphological emergence of brown fat cells. As shown in Figure 7C and as demonstrated before (Ghorbani and Himms-Hagen, 1997; Himms-Hagen et al., 1994), WAT from CL 316243-treated control animals showed few cells with a multilocular appearance that also stained positively for UCP1. In striking contrast, WAT depots from *aP2-PRDM16* mice had a genuine chimeric appearance with abundant clusters of multilocular BAT-type cells that stained intensely for UCP1 protein (Figure 7C). Importantly, all of the multilocular cells in the WAT of *aP2-PRDM16* transgenic mice expressed UCP1 protein, and all UCP1-expressing cells had a multilocular appearance, suggesting that these cells are bona fide brown fat cells. Approximately 10%–20% of all cells in epididymal WAT depots of *aP2-PRDM16* transgenic mice were BAT-type fat cells, while equivalent depots of the control animals had less than 1% BAT-type cells. These data show that PRDM16, when expressed at or below authentic BAT

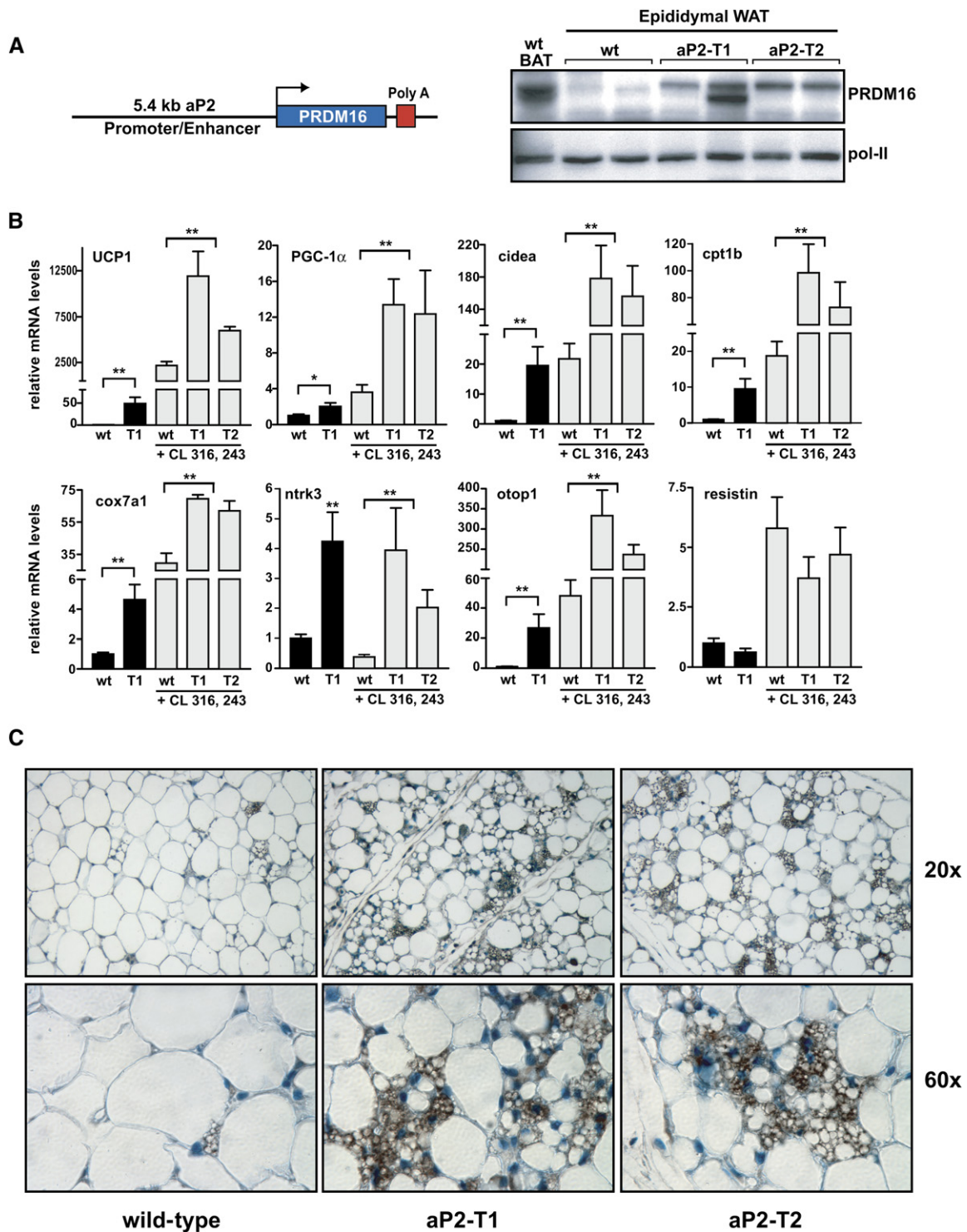


Figure 7. Transgenic Expression of PRDM16 in WAT Depots Induces the Formation of BAT Cells

(A) The fat-specific *aP2* promoter/enhancer was used to express PRDM16 in WAT depots. Western blot analysis for PRDM16 protein expression in wild-type (wt) BAT, wt WAT, and WAT from two strains of *aP2-PRDM16* transgenic mice (*aP2-T1* and *aP2-T2*) is shown. Pol II protein expression was used to control for loading.

(B) Expression of BAT-selective genes (as indicated) and *Resistin* in WAT from wild-type (wt) and *aP2-T1* transgenic mice. This gene set was also measured in WAT from wt, *aP2-T1*, and *aP2-T2* mice that had been treated with CL 316243 ($n = 7-10$ mice per group; error bars indicate \pm SEM).

(C) Immunohistochemistry for UCP1 protein (brown staining) in sections of WAT from wild-type and transgenic mice (*aP2-T1* and *aP2-T2*) after treatment with CL 316243. * $p < 0.05$; ** $p < 0.01$.

levels, can control the determination of brown adipose cells in a classic white fat depot.

DISCUSSION

A detailed understanding of the brown fat cell and its unique ability to dissipate chemical energy may offer new treatment avenues for obesity and associated diseases. In small mammals that retain distinct brown fat pads as adults, the heat produced by brown fat is an important contributor to overall energy balance (Cannon and Nedergaard, 2004; Cederberg et al., 2001; Ghorbani et al., 1997; Ghorbani and Himms-Hagen, 1997; Guerra et al., 1998; Kopecky et al., 1995, 1996; Lowell et al., 1993; Rothwell and Stock, 1979; Tsukiyama-Kohara et al., 2001). Although adult humans do not have distinct brown fat depots, they do appear to have small numbers of brown adipocytes in their white fat depots, and these brown cells proliferate under certain circumstances, such as chronic cold exposure (Garruti and Ricquier, 1992; Huttunen et al., 1981; Lean et al., 1986; Oberkofler et al., 1997). Thus, the molecular mechanisms that regulate brown fat cell determination are of significant and escalating biomedical interest.

A great deal is known about the metabolism and function of mature white and brown adipocytes; however, the developmental origins of these cell lineages have remained elusive. The majority of brown fat develops prenatally and is mature and fully functional at birth, when thermogenic requirements are particularly high (Nedergaard et al., 1986). Most WAT, on the other hand, develops postnatally in response to relative nutritional excess. It has been presumed that white and brown fat cells are closely related to each other developmentally because they express many common enzymes and both require PPAR γ for their differentiation. Interestingly, recent fate-mapping experiments in mice show that interscapular brown fat, but not white fat, arises from a population of Engrailed-1-expressing cells in the dermomyotome, a structure that also gives rise to muscle and skin (Atit et al., 2006). These data suggests that the two types of fat cells may have quite different origins.

PRDM16 is expressed very selectively in brown fat cells versus white fat cells and stimulates nearly all of the key characteristics of authentic brown fat cells when expressed at or near physiological levels. This includes enhanced mitochondrial gene expression and mitochondrial density, increased expression of PGC-1 α and UCP1, and a very large increase in the uncoupled fraction of respiration. Importantly, the expression of UCP1 and PGC-1 α induced by PRDM16 is further enhanced by cAMP, as it is in authentic brown fat cells. At a global scale, a majority of genes that are selectively expressed in brown adipocytes are positively regulated by PRDM16 (Table S1). Conversely, PRDM16 expression suppressed the mRNA levels of several genes that are selectively enriched in white fat such as *Resistin* and *Serp13ak*. Notably, PRDM16 expression does not influence the expression of those genes that are common to both brown and white fat cells. Interestingly, several BAT genes including UCP1 and PGC-1 α

were induced by PRDM16 in the genetic absence of PPAR γ (and therefore fat cell differentiation). Importantly, PRDM16 is shown here to activate a brown fat gene program in many different kinds of adipocytes as long as it is introduced before cell differentiation. These results suggest that the mechanism by which PRDM16 determines brown fat fate is at least partly separable from the adipogenic differentiation pathway common to white and brown fat cells (Figure S4). Why PRDM16 is not effective when expressed after adipogenic differentiation in these experiments is not known at present.

An effective shRNA directed against PRDM16 allowed us to ask about the requirement for this factor in the expression of brown fat-selective genes in established brown fat cell lines and in primary brown fat cells. The reduction of PRDM16 levels has no effect on morphological differentiation of these cells but causes an almost complete suppression of brown fat-selective genes, including UCP1 mRNA and protein, while leaving intact the expression of genes common to both white and brown fat cells such as PPAR γ and *aP2*. Clearly, PRDM16 is required for the expression of the brown fat phenotype in isolated cells. Examination of this feature will be important in mice ablated for PRDM16 in vivo.

A key question is how PRDM16 stimulates the development of a brown fat gene program. This protein is annotated in databases as a potential transcription factor because it possesses two zinc fingers. However, while zinc-finger proteins are often DNA-binding factors, it is also clear that zinc-finger domains can mediate protein-protein interactions (Leon and Roth, 2000). We have confirmed that PRDM16 does indeed bind to DNA in a sequence-specific manner but that this is not required for its regulation of many BAT-selective genes. On the other hand, it is clear that PRDM16 activates the expression as well as the transcriptional function of both PGC-1 α and PGC-1 β , apparently through direct physical binding. While functions of PRDM16 in other aspects of brown fat regulation are by no means ruled out, the PRDM16-stimulated activity of PGC-1 α and PGC-1 β may explain many actions of PRDM16 in brown fat determination (Figure S4).

As shown previously, PGC-1 α can activate many of the genes that comprise the thermogenic program of brown fat, such as UCP1 and *Dio2* (Puigserver et al., 1998). On the other hand, PRDM16 is shown here to have certain actions that are not consistent with a function solely through modulation of PGC-1 α expression and/or function. In fact, genetic studies have shown conclusively that mice lacking PGC-1 α retain identifiable, though abnormal, brown fat tissue (Lin et al., 2004). Similarly, isolated brown fat cells lacking PGC-1 α still express several genes characteristic of brown fat (Uldry et al., 2006). However, it is notable that shRNA-mediated suppression of PGC-1 β in cells lacking PGC-1 α caused a further loss of the brown fat phenotype (Uldry et al., 2006). Thus, it is possible that PRDM16 functions as a brown fat determination factor at least in part by robustly stimulating PGC-1 α and PGC-1 β simultaneously. PRDM16 may also increase PGC-1 co-activator function in other tissues where it is expressed,

such as heart, brain, and kidney, with important physiological consequences.

How PRDM16 achieves this activation of the PGC-1s remains to be determined. The PR domain present in a subclass of zinc-finger proteins, including PRDM16, is highly homologous to the SET domain, which is noted for its histone lysine methyltransferase activity and diverse functions in regulating chromatin structure (Huang et al., 1998; Rea et al., 2000). Other PR-domain-containing factors such as RIZ1 (retinoblastoma-interacting zinc finger-1) and Meisitz have intrinsic histone methyltransferase activity, while PRISM (PR domain in smooth muscle) binds and recruits a histone methyltransferase (Davis et al., 2006; Hayashi et al., 2005; Kim et al., 2003). Interestingly, another methyltransferase protein, PRMT1, has been shown to bind to PGC-1 α and activate it via arginine methylation (Teyssier et al., 2005). Whether the PR domain of PRDM16 has enzymatic function and whether this activity is required for stimulating brown fat gene expression remain to be established.

Components in addition to the PGC-1s that can influence the brown fat phenotype have been identified, such as FOXC2, Rb, p107, and RIP140 (Cederberg et al., 2001; Christian et al., 2005; Hansen et al., 2004; Leonardsson et al., 2004; Powelka et al., 2006; Scime et al., 2005). It will be important to investigate their genetic interactions with PRDM16. The absolute requirement for PRDM16 in the formation of brown adipocytes suggests that the mechanism of action of these other factors may involve PRDM16. Whether they act upstream or downstream of PRDM16 in the differentiation program of brown fat remains to be elucidated.

The replacement of BAT with WAT in humans and in obese mice and rats has shown that these tissues can interconvert to some extent in vivo. Similarly, prolonged exposure to cold or β -adrenergic agonists induces the appearance of brown fat cells within classic white fat depots (Himms-Hagen et al., 2000). This so-called transdifferentiation of fat by cold exposure or β -adrenergic agonists could be due to the acquisition of brown fat cell features in preformed white fat cells and/or to the differentiation of resident committed brown preadipocytes into mature brown fat cells. While this important issue is not entirely settled, the latter scenario is supported by the observation that preadipocytes contained in fat tissues are committed to the brown or white fate (Klaus, 1997; Klaus et al., 1994, 1995; Kozak and Kozak, 1994). In light of these data, it is intriguing that PRDM16 expression did not convert mature white adipocytes into brown-type cells. However, the white fat cell precursors present in whole fat tissues could be converted to a brown fat-like phenotype efficiently when PRDM16 was introduced before differentiation. Furthermore, the emergence of UCP1-positive brown fat cells in the white fat depots of transgenic mice strongly suggests that PRDM16 directs the differentiation of resident white fat progenitors into the BAT fate. The small clusters of BAT cells in wild-type WAT most likely arise from BAT progenitors that are present in small numbers within white depots. The stimulation of PRDM16 action

by β -adrenergic signaling is not surprising given the known importance of this cascade in BAT development (Cannon and Nedergaard, 2004). The identity of the mechanism by which cAMP signaling modulates the activity of PRDM16 is an important outstanding question from this study.

Taken together, inducing PRDM16 expression in preadipocytes could constitute a strategy to raise whole-body energy expenditure and prevent excess fat accumulation. This can be done, in theory, by using drugs that raise PRDM16 levels in fat cell precursors or by engineering preadipocytes ex vivo and then reinjecting them, analogous to the transplantation experiments performed here. More experimentation will be necessary to determine how this brown fat determination factor might be used to fight obesity in the context of a whole animal.

EXPERIMENTAL PROCEDURES

Plasmids and Viral Vectors

Full-length coding sequence of mouse *PRDM16* (GeneID 70673) was amplified from brown fat RNA by PCR and cloned into the XhoI/EcoRI sites of pMSCV-puro retroviral vector (Stratagene) in frame with an N-terminal FLAG tag. *PRDM16* cDNA was also cloned into the XhoI/EcoRI sites of pcDNA3.1 (Invitrogen) for expression in mammalian cells. DNA-binding mutants of *PRDM16*, including the R998Q allele, were created by site-directed mutagenesis (QuikChange, Stratagene). Adenoviral vectors for *PRDM16* expression were made in pAdTrack-CMV using the AdEasy system as described elsewhere (He et al., 1998). Adenoviral vectors for *PGC-1 α* and *GFP* (Yoon et al., 2001) and Gal4 fusion plasmids containing *PGC-1 β* (Lin et al., 2002), *PGC-1 α* , and *SRC-1* (Puigserver et al., 1999) have been described previously. The siRNA sequence identified for use in PRDM16 depletion experiments was 5'-GAAGAGCGUGAGUACAAU-3' (Dharmacon). The corresponding double-stranded DNA sequence was ligated into pSUPER-Retro (GFP-Neo) (OligoEngine) for retroviral shRNA expression. This siRNA sequence was able to specifically knock down PRDM16 protein and mRNA expression by over 75%.

Cell Culture

3T3-L1, C3H-10T1/2, and COS-7 cells were obtained from ATCC. Immortalized brown fat preadipocytes and *PPAR γ* ^{-/-} fibroblasts have been described elsewhere (Rosen et al., 2002; Uldry et al., 2006). 3T3-F442A cells were from H. Green (Green and Kehinde, 1979). Primary brown fat preadipocytes were obtained from mice by collagenase digestion as described elsewhere (Tseng et al., 2004). Primary white fat stromal-vascular and mature fat cells were fractionated according to published methods (Rodbell, 1964; Soukas et al., 2001). For retrovirus production, Phoenix packaging cells (Kinsella and Nolan, 1996) were transfected at 70% confluence by calcium phosphate coprecipitation with 10 μ g retroviral vectors; viral supernatant was harvested 48 hr later. For retroviral transduction, cells were incubated overnight with viral supernatant supplemented with 8 μ g/ml polybrene. For adenoviral infection of mature adipocytes, the fat cell fraction (see above) from epididymal WAT of 10- to 12-week-old C57BL/6 mice (n = 4) was incubated with PRDM16 (GFP) or GFP-expressing adenovirus (moi = 100) in 0.5 ml Eppendorf tubes for 2 hr in 10% FBS/DMEM. The medium was then replaced, and cells were maintained in 5% CO₂ at 37°C for an additional 48 hr prior to RNA extraction and expression analysis. GFP expressed from the adenoviral vectors was used to monitor infection efficiency, which was typically over 50%.

Adipocyte differentiation was induced by treating confluent cells for 48 hr in medium containing 10% FBS, 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 1 μ M dexamethasone, 850 nM insulin,

1 nM T_3 , 1 μ M rosiglitazone (Cayman Chemical). Two days after induction, cells were switched to maintenance medium containing 10% FBS, 850 nM insulin, 1 nM T_3 , 1 μ M rosiglitazone. To stimulate thermogenesis, cells were incubated with 0.5 mM dibutyryl cyclic AMP for 4 hr. All chemicals for cell culture were obtained from Sigma unless otherwise indicated.

Global Expression Screen of Transcription-Related Factors

RNA was isolated from brown adipose tissue (BAT) and epididymal white fat tissue (WAT) of five adult male C57BL/6 mice at 10–12 weeks of age. First-strand cDNA was prepared from RNA samples using oligo(dT) and SuperScript III (Invitrogen). cDNAs were normalized for equal loading by real-time PCR using *TBP* and *HPRT* mRNA expression levels. Thirty cycles of PCR (94°C \times 30 s, 62°C \times 45 s, 68°C \times 45 s) with primer sets described in Gray et al. (2004) were performed in 50 μ l reactions using Platinum Taq (Invitrogen). PCR products (average of 500 bp) were separated on 96-well 2% agarose E-gels (Invitrogen) containing ethidium bromide, imaged, and analyzed using E-Editor software (Invitrogen).

Reporter Gene Assays

Reporter gene assays were performed in COS-7 cells or brown fat preadipocytes. Briefly, the \sim 2 kb *PGC-1 α* reporter gene (Handschin et al., 2003) or the *UCP1* \sim 4 kb reporter (a gift from L. Kozak) was transiently transfected with PRDM16 and/or *PGC-1 α* expression plasmids in 12-well plates using FuGENE6 (Roche). Forty-eight hours after transfection, cells were harvested and reporter gene activity was measured using the Dual-Luciferase Reporter Assay System (Promega). Forskolin (100 μ M) or differentiation cocktail was added for 4 or 24 hr, respectively, prior to harvesting cells. Gal4-based reporter gene assays were performed in COS-7 cells by cotransfecting Gal4-DBD or Gal4-DBD fusion constructs with PRDM16 or control expression constructs. Firefly luciferase reporter gene measurements were normalized to Renilla luciferase activity.

Binding Studies and Electrophoretic Mobility Shift Assays

To study protein-protein interactions, brown fat preadipocytes were transduced with retroviral *PRDM16* or control vector and induced to differentiate into adipocytes. At day 6 of differentiation, whole-cell extracts were incubated with FLAG-M2 agarose (Sigma) overnight at 4°C. Immunoprecipitates were washed three times with washing buffer (20 mM Tris-HCl, 150 mM NaCl, 10% glycerol, 2 mM EDTA, 0.1% NP-40, 0.1 mM PMSF), resolved by SDS-PAGE, transferred to PVDF membrane (Millipore), and blotted with anti-*PGC-1 α* (Puigserver et al., 1999) and *PGC-1 β* (Lin et al., 2005) antibodies. For GST immunoaffinity assays, GST-*PGC-1 α* fusion proteins immobilized to glutathione Sepharose beads were purified as previously described (Wallberg et al., 2003). 35 S-labeled PRDM16 or ERR α proteins were in vitro translated using the TNT Coupled Transcription/Translation System (Promega). To examine the binding of *PGC-1 α* with the R998Q mutant allele of PRDM16, COS-7 cells were cotransfected with HA-*PGC-1 α* and FLAG-PRDM16 (wild-type and R998Q). Whole-cell extracts were immunoprecipitated with HA antibody (Roche), resolved by SDS-PAGE, and blotted with anti-FLAG antibody to detect PRDM16. For electrophoretic mobility shift assays, an oligonucleotide probe containing the PRDM16 binding site (5'-GATCCGACAAGATAA GATAAGGATCTATAAGAAGATGAGGTATG-3') (Nishikata et al., 2003) was end labeled with [γ - 32 P]ATP and incubated (5 fmol) with in vitro translated wild-type and R998Q mutant PRDM16 in binding buffer (10 mM Tris-HCl [pH 7.5], 50mM NaCl, 1mM MgCl $_2$, 0.5mM EDTA, 4% glycerol, 0.5mM DTT, 0.5 μ g poly(dI-dC)-poly(dI-dC)). Fifty or five hundred femtomoles of unlabeled probe was added to the binding reaction for competition assays. DNA-protein complexes were separated by electrophoresis on a 4% PAGE gel and then dried and exposed to X-ray film.

Oxygen Consumption Assays

PPAR γ ^{-/-} fibroblasts transduced with retroviral *PPAR γ 2* and either PRDM16 or vector control were grown to confluence and induced to differentiate into adipocytes. At day 6 of differentiation, oxygen consumption was measured in fat cells as described previously (St-Pierre et al., 2003). For cyclic-AMP-induced respiration assays, fully differentiated fat cells were incubated with 0.5 mM dibutyryl cyclic AMP for 12 hr before measuring oxygen consumption.

Electron Microscopy

Electron microscopy was performed as described previously (St-Pierre et al., 2003) on *PPAR γ* ^{-/-} cells expressing *PPAR γ 2* and PRDM16 or vector control before and after their differentiation into adipocytes (day 6). To calculate the mitochondrial volume density, a grid was laid on randomly selected micrographs ($n > 20$), and the number of points falling on mitochondria was expressed as a fraction of those landing on cell area.

Real-Time PCR Analysis and Western Blotting

Total RNA from cultured cells was isolated using QIAGEN RNeasy mini columns according to the manufacturer's instructions. Tissue RNA samples were prepared by the TRIzol method (Invitrogen). Northern blot analysis was performed as described previously (Maniatis et al., 1982). For real-time PCR analysis, RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad) and used in quantitative PCR reactions containing SYBR green fluorescent dye (ABI). Relative expression of mRNAs was determined after normalization with *TBP* levels using the $\Delta\Delta$ -Ct method. qPCR was performed using an ABI 9300 PCR machine. As a point of reference, the Ct values for both *PRDM16* and *TBP* mRNA expression in BAT were typically 24–26. Student's t test was used for comparisons and to obtain statistics. Primers used for real-time PCR are shown in Table S2. For western blot analysis, cells or tissues were lysed in RIPA buffer (0.5% NP-40, 0.1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris-HCl [pH 7.5]). Lysates were resolved by SDS-PAGE; transferred to PVDF membrane (Millipore); and probed with anti-UCP1 (Chemicon), anti-FLAG M2 (Sigma), anti-PRDM16 (rabbit polyclonal), and anti-Pol II (Santa Cruz Biotechnology).

Animals

All animal experiments were performed according to procedures approved by the Dana-Farber Cancer Institute's Institutional Animal Care and Use Committee. Mice were maintained on a standard rodent chow diet with 12 hr light and dark cycles. For acute cold-exposure studies, BAT was obtained from five 3- to 4-week-old male C57BL/6 mice that were housed at 4°C for 4 hr. For transgenic mice, the complete *PRDM16* cDNA was cloned 3' to the 5.4 kb *aP2* promoter/enhancer, and the human growth hormone polyadenylation site was inserted 3' to the cDNA. FVB mouse oocytes were injected with this construct by the Dana-Farber Cancer Institute Core Facility. CL 316243 (Sigma) at 0.5 mg/kg was injected intraperitoneally into mice daily for 6 days. Mice were euthanized for analysis of tissues 4 hr following the final injection on day 6. Transgenic and nontransgenic control littermates used for all experiments were 4- to 6-month-old males. For cell transplantation, 10⁷ fibroblasts transduced with retroviral *PPAR γ 2* and either PRDM16 or vector control were suspended in 200 μ l of 10% FBS/DMEM and implanted subcutaneously just above the sternum of nude (Nu/Nu) mice (Taconic) ($n = 10$ mice/stable cell line) using an insulin syringe attached to a 28 gauge needle. This injection site was chosen due to the almost complete absence of endogenous subcutaneous fat. For immunohistochemistry, paraffin-embedded sections were incubated with anti-Cidea (Chemicon) or anti-UCP1 (Abcam) antibodies for 30 min at room temperature, followed by detection using the ABC Vectastain Elite kit (Vector Labs) according to the manufacturer's instructions.

Transcriptional Profiling

Total RNA was isolated from the epididymal WAT and interscapular BAT of three male C57BL/6 mice at 10–12 weeks of age. Array hybridization and scanning were performed by the Dana-Farber Cancer Institute Core Facility using Affymetrix GeneChip Mouse Genome 430 2.0 arrays according to established methods (Lockhart et al., 1996). The array data were normalized using the DNA-Chip Analyzer (dChip) software (Li and Wong, 2001). The statistical significance of differences in gene expression was assessed by unpaired t test ($p < 0.05$).

Supplemental Data

Supplemental Data include two tables and four figures and can be found with this article online at <http://www.cellmetabolism.org/cgi/content/full/6/1/38/DC1/>.

ACKNOWLEDGMENTS

We thank P. Puigserver for helpful discussions and critical reading of the manuscript and C. Valle (INSERM U858) for expert technical assistance. P.S. is supported by a fellowship from the American Heart Association and was previously supported by a Canadian Institutes of Health Research fellowship. S.K. is supported by a fellowship from the Japan Society for the Promotion of Science. This work is supported by NIH grant DK31405-24 to B.M.S.; L'Agence Nationale de la Recherche grant ANR-05-PCOD-012-04 to D.L.; and European Commission FP6 project HEPADIP grant LSHM-CT-2005-018734 to D.L.

Received: February 6, 2007

Revised: May 3, 2007

Accepted: June 11, 2007

Published: July 10, 2007

REFERENCES

Almind, K., Manieri, M., Sivitz, W.I., Cinti, S., and Kahn, C.R. (2007). Ectopic brown adipose tissue in muscle provides a mechanism for differences in risk of metabolic syndrome in mice. *Proc. Natl. Acad. Sci. USA* *104*, 2366–2371.

Atit, R., Sgaier, S.K., Mohamed, O.A., Taketo, M.M., Dufort, D., Joyner, A.L., Niswander, L., and Conlon, R.A. (2006). Beta-catenin activation is necessary and sufficient to specify the dorsal dermal fate in the mouse. *Dev. Biol.* *296*, 164–176.

Barak, Y., Nelson, M.C., Ong, E.S., Jones, Y.Z., Ruiz-Lozano, P., Chien, K.R., Koder, A., and Evans, R.M. (1999). PPAR gamma is required for placental, cardiac, and adipose tissue development. *Mol. Cell* *4*, 585–595.

Barbera, M.J., Schluter, A., Pedraza, N., Iglesias, R., Villarroya, F., and Giral, M. (2001). Peroxisome proliferator-activated receptor alpha activates transcription of the brown fat uncoupling protein-1 gene. A link between regulation of the thermogenic and lipid oxidation pathways in the brown fat cell. *J. Biol. Chem.* *276*, 1486–1493.

Braissant, O., Fougère, F., Scotto, C., Dauca, M., and Wahli, W. (1996). Differential expression of peroxisome proliferator-activated receptors (PPARs): tissue distribution of PPAR-alpha, -beta, and -gamma in the adult rat. *Endocrinology* *137*, 354–366.

Bronnikov, G., Houstek, J., and Nedergaard, J. (1992). Beta-adrenergic, cAMP-mediated stimulation of proliferation of brown fat cells in primary culture. Mediation via beta 1 but not via beta 3 adrenoceptors. *J. Biol. Chem.* *267*, 2006–2013.

Cannon, B., and Nedergaard, J. (2004). Brown adipose tissue: function and physiological significance. *Physiol. Rev.* *84*, 277–359.

Cederberg, A., Gronning, L.M., Ahren, B., Tasken, K., Carlsson, P., and Enerback, S. (2001). FOXC2 is a winged helix gene that counteracts obesity, hypertriglyceridemia, and diet-induced insulin resistance. *Cell* *106*, 563–573.

Christian, M., Kiskinis, E., Debevec, D., Leonardsson, G., White, R., and Parker, M.G. (2005). RIP140-targeted repression of gene expression in adipocytes. *Mol. Cell. Biol.* *25*, 9383–9391.

Cousin, B., Cinti, S., Morroni, M., Raimbault, S., Ricquier, D., Penicaud, L., and Casteilla, L. (1992). Occurrence of brown adipocytes in rat white adipose tissue: molecular and morphological characterization. *J. Cell Sci.* *103*, 931–942.

Dahle, M.K., Gronning, L.M., Cederberg, A., Blomhoff, H.K., Miura, N., Enerback, S., Tasken, K.A., and Tasken, K. (2002). Mechanisms of FOXC2- and FOXD1-mediated regulation of the RI alpha subunit of cAMP-dependent protein kinase include release of transcriptional repression and activation by protein kinase B alpha and cAMP. *J. Biol. Chem.* *277*, 22902–22908.

Davis, C.A., Haberland, M., Arnold, M.A., Sutherland, L.B., McDonald, O.G., Richardson, J.A., Childs, G., Harris, S., Owens, G.K., and Olson, E.N. (2006). PRISM/PRDM6, a transcriptional repressor that promotes the proliferative gene program in smooth muscle cells. *Mol. Cell. Biol.* *26*, 2626–2636.

Garruti, G., and Ricquier, D. (1992). Analysis of uncoupling protein and its mRNA in adipose tissue deposits of adult humans. *Int. J. Obes. Relat. Metab. Disord.* *16*, 383–390.

Ghorbani, M., and Himms-Hagen, J. (1997). Appearance of brown adipocytes in white adipose tissue during CL 316,243-induced reversal of obesity and diabetes in Zucker fa/fa rats. *Int. J. Obes. Relat. Metab. Disord.* *21*, 465–475.

Ghorbani, M., Claus, T.H., and Himms-Hagen, J. (1997). Hypertrophy of brown adipocytes in brown and white adipose tissues and reversal of diet-induced obesity in rats treated with a beta3-adrenoceptor agonist. *Biochem. Pharmacol.* *54*, 121–131.

Graves, R.A., Tontonoz, P., Platt, K.A., Ross, S.R., and Spiegelman, B.M. (1992). Identification of a fat cell enhancer: analysis of requirements for adipose tissue-specific gene expression. *J. Cell. Biochem.* *49*, 219–224.

Gray, P.A., Fu, H., Luo, P., Zhao, Q., Yu, J., Ferrari, A., Tenzen, T., Yuk, D.I., Tsung, E.F., Cai, Z., et al. (2004). Mouse brain organization revealed through direct genome-scale TF expression analysis. *Science* *306*, 2255–2257.

Green, H., and Kehinde, O. (1979). Formation of normally differentiated subcutaneous fat pads by an established preadipose cell line. *J. Cell. Physiol.* *101*, 169–171.

Gronning, L.M., Baillie, G.S., Cederberg, A., Lynch, M.J., Houslay, M.D., Enerback, S., and Tasken, K. (2006). Reduced PDE4 expression and activity contributes to enhanced catecholamine-induced cAMP accumulation in adipocytes from FOXC2 transgenic mice. *FEBS Lett.* *580*, 4126–4130.

Guerra, C., Koza, R.A., Yamashita, H., Walsh, K., and Kozak, L.P. (1998). Emergence of brown adipocytes in white fat in mice is under genetic control. Effects on body weight and adiposity. *J. Clin. Invest.* *102*, 412–420.

Hamann, A., Flier, J.S., and Lowell, B.B. (1996). Decreased brown fat markedly enhances susceptibility to diet-induced obesity, diabetes, and hyperlipidemia. *Endocrinology* *137*, 21–29.

Handschin, C., Rhee, J., Lin, J., Tarr, P.T., and Spiegelman, B.M. (2003). An autoregulatory loop controls peroxisome proliferator-activated receptor gamma coactivator 1alpha expression in muscle. *Proc. Natl. Acad. Sci. USA* *100*, 7111–7116.

Hansen, J.B., Jorgensen, C., Petersen, R.K., Hallenborg, P., De Matteis, R., Boye, H.A., Petrovic, N., Enerback, S., Nedergaard, J., Cinti, S., et al. (2004). Retinoblastoma protein functions as a molecular switch determining white versus brown adipocyte differentiation. *Proc. Natl. Acad. Sci. USA* *101*, 4112–4117.

Hayashi, K., Yoshida, K., and Matsui, Y. (2005). A histone H3 methyltransferase controls epigenetic events required for meiotic prophase. *Nature* *438*, 374–378.

- He, T.C., Zhou, S., da Costa, L.T., Yu, J., Kinzler, K.W., and Vogelstein, B. (1998). A simplified system for generating recombinant adenoviruses. *Proc. Natl. Acad. Sci. USA* 95, 2509–2514.
- Himms-Hagen, J., Cui, J., Danforth, E., Jr., Taatjes, D.J., Lang, S.S., Waters, B.L., and Claus, T.H. (1994). Effect of CL-316,243, a thermogenic beta 3-agonist, on energy balance and brown and white adipose tissues in rats. *Am. J. Physiol.* 266, R1371–R1382.
- Himms-Hagen, J., Melnyk, A., Zingaretti, M.C., Ceresi, E., Barbatelli, G., and Cinti, S. (2000). Multilocular fat cells in WAT of CL-316243-treated rats derive directly from white adipocytes. *Am. J. Physiol. Cell Physiol.* 279, C670–C681.
- Huang, S., Shao, G., and Liu, L. (1998). The PR domain of the Rb-binding zinc finger protein RIZ1 is a protein binding interface and is related to the SET domain functioning in chromatin-mediated gene expression. *J. Biol. Chem.* 273, 15933–15939.
- Huttunen, P., Hirvonen, J., and Kinnula, V. (1981). The occurrence of brown adipose tissue in outdoor workers. *Eur. J. Appl. Physiol. Occup. Physiol.* 46, 339–345.
- Kim, K.C., Geng, L., and Huang, S. (2003). Inactivation of a histone methyltransferase by mutations in human cancers. *Cancer Res.* 63, 7619–7623.
- Kinsella, T.M., and Nolan, G.P. (1996). Episomal vectors rapidly and stably produce high-titer recombinant retrovirus. *Hum. Gene Ther.* 7, 1405–1413.
- Klaus, S. (1997). Functional differentiation of white and brown adipocytes. *Bioessays* 19, 215–223.
- Klaus, S., Choy, L., Champigny, O., Cassard-Doulcier, A.M., Ross, S., Spiegelman, B., and Ricquier, D. (1994). Characterization of the novel brown adipocyte cell line HIB 1B. Adrenergic pathways involved in regulation of uncoupling protein gene expression. *J. Cell Sci.* 107, 313–319.
- Klaus, S., Ely, M., Encke, D., and Heldmaier, G. (1995). Functional assessment of white and brown adipocyte development and energy metabolism in cell culture. Dissociation of terminal differentiation and thermogenesis in brown adipocytes. *J. Cell Sci.* 108, 3171–3180.
- Klingenberg, M. (1999). Uncoupling protein—a useful energy dissipator. *J. Bioenerg. Biomembr.* 31, 419–430.
- Kopecky, J., Clarke, G., Enerback, S., Spiegelman, B., and Kozak, L.P. (1995). Expression of the mitochondrial uncoupling protein gene from the aP2 gene promoter prevents genetic obesity. *J. Clin. Invest.* 96, 2914–2923.
- Kopecky, J., Rossmeisl, M., Hodny, Z., Srovy, I., Horakova, M., and Kolarova, P. (1996). Reduction of dietary obesity in aP2-Ucp transgenic mice: mechanism and adipose tissue morphology. *Am. J. Physiol.* 270, E776–E786.
- Kozak, U.C., and Kozak, L.P. (1994). Norepinephrine-dependent selection of brown adipocyte cell lines. *Endocrinology* 134, 906–913.
- Kubota, N., Terauchi, Y., Miki, H., Tamemoto, H., Yamauchi, T., Komeda, K., Satoh, S., Nakano, R., Ishii, C., Sugiyama, T., et al. (1999). PPAR gamma mediates high-fat diet-induced adipocyte hypertrophy and insulin resistance. *Mol. Cell* 4, 597–609.
- Lean, M.E., and James, W.P. (1986). Brown adipose tissue in man. In *Brown Adipose Tissue*, P. Trayhurn and D.G. Nicholls, eds. (London: Edward Arnold), pp. 339–365.
- Lean, M.E., James, W.P., Jennings, G., and Trayhurn, P. (1986). Brown adipose tissue in patients with pheochromocytoma. *Int. J. Obes.* 10, 219–227.
- Leon, O., and Roth, M. (2000). Zinc fingers: DNA binding and protein-protein interactions. *Biol. Res.* 33, 21–30.
- Leonardsson, G., Steel, J.H., Christian, M., Pocock, V., Milligan, S., Bell, J., So, P.W., Medina-Gomez, G., Vidal-Puig, A., White, R., and Parker, M.G. (2004). Nuclear receptor corepressor RIP140 regulates fat accumulation. *Proc. Natl. Acad. Sci. USA* 101, 8437–8442.
- Li, J., and Wong, L. (2001). Emerging patterns and gene expression data. *Genome Inform.* 12, 3–13.
- Lin, J., Puigserver, P., Donovan, J., Tarr, P., and Spiegelman, B.M. (2002). Peroxisome proliferator-activated receptor gamma coactivator 1beta (PGC-1beta), a novel PGC-1-related transcription coactivator associated with host cell factor. *J. Biol. Chem.* 277, 1645–1648.
- Lin, J., Wu, P.H., Tarr, P.T., Lindenberg, K.S., St-Pierre, J., Zhang, C.Y., Mootha, V.K., Jager, S., Vianna, C.R., Reznick, R.M., et al. (2004). Defects in adaptive energy metabolism with CNS-linked hyperactivity in PGC-1alpha null mice. *Cell* 119, 121–135.
- Lin, J., Yang, R., Tarr, P.T., Wu, P.H., Handschin, C., Li, S., Yang, W., Pei, L., Uldry, M., Tontonoz, P., et al. (2005). Hyperlipidemic effects of dietary saturated fats mediated through PGC-1beta coactivation of SREBP. *Cell* 120, 261–273.
- Lockhart, D.J., Dong, H., Byrne, M.C., Follettie, M.T., Gallo, M.V., Chee, M.S., Mittmann, M., Wang, C., Kobayashi, M., Horton, H., and Brown, E.L. (1996). Expression monitoring by hybridization to high-density oligonucleotide arrays. *Nat. Biotechnol.* 14, 1675–1680.
- Lowell, B.B., S-Susulic, V., Hamann, A., Lawitts, J.A., Himms-Hagen, J., Boyer, B.B., Kozak, L.P., and Flier, J.S. (1993). Development of obesity in transgenic mice after genetic ablation of brown adipose tissue. *Nature* 366, 740–742.
- Lupien, J.R., Glick, Z., Saito, M., and Bray, G.A. (1985). Guanosine diphosphate binding to brown adipose tissue mitochondria is increased after single meal. *Am. J. Physiol.* 249, R694–R698.
- Maniatis, T., Sambrook, J., and Fritsch, E.F. (1982). *Molecular Cloning: a Laboratory Manual* (Cold Spring Harbor, NY, USA: Cold Spring Harbor Laboratory).
- Minokoshi, Y., Saito, M., and Shimazu, T. (1986). Metabolic and morphological alterations of brown adipose tissue after sympathetic denervation in rats. *J. Auton. Nerv. Syst.* 15, 197–204.
- Nedergaard, J., Cannon, B., and Lindberg, O. (1977). Microcalorimetry of isolated mammalian cells. *Nature* 267, 518–520.
- Nedergaard, J., Connally, E., and Cannon, B. (1986). Brown adipose tissue in the mammalian neonate. In *Brown Adipose Tissue*, P. Trayhurn and D.G. Nicholls, eds. (London: Edward Arnold), pp. 1–30.
- Nedergaard, J., Bronnikov, G., Houstek, J., Golozoubova, V., and Tvrdik, P. (1994). Norepinephrine-induced proliferation of brown fat cell precursors. *Exp. Nephrol.* 2, 135.
- Nedergaard, J., Petrovic, N., Lindgren, E.M., Jacobsson, A., and Cannon, B. (2005). PPARgamma in the control of brown adipocyte differentiation. *Biochim. Biophys. Acta* 1740, 293–304.
- Nishikata, I., Sasaki, H., Iga, M., Tateno, Y., Imayoshi, S., Asou, N., Nakamura, T., and Morishita, K. (2003). A novel EVI1 gene family, MEL1, lacking a PR domain (MEL1S) is expressed mainly in t(1;3)(p36;q21)-positive AML and blocks G-CSF-induced myeloid differentiation. *Blood* 102, 3323–3332.
- Oberkofler, H., Dallinger, G., Liu, Y.M., Hell, E., Krempler, F., and Patsch, W. (1997). Uncoupling protein gene: quantification of expression levels in adipose tissues of obese and non-obese humans. *J. Lipid Res.* 38, 2125–2133.
- Pavletich, N.P., and Pabo, C.O. (1991). Zinc finger-DNA recognition: crystal structure of a Zif268-DNA complex at 2.1 Å. *Science* 252, 809–817.
- Powelka, A.M., Seth, A., Virbasius, J.V., Kiskinis, E., Nicoloso, S.M., Guilherme, A., Tang, X., Straubhaar, J., Cherniack, A.D., Parker, M.G., and Czech, M.P. (2006). Suppression of oxidative metabolism and mitochondrial biogenesis by the transcriptional corepressor RIP140 in mouse adipocytes. *J. Clin. Invest.* 116, 125–136.
- Prusiner, S.B., Cannon, B., Ching, T.M., and Lindberg, O. (1968). Oxidative metabolism in cells isolated from brown adipose tissue. 2. Catecholamine regulated respiratory control. *Eur. J. Biochem.* 7, 51–57.

- Puigserver, P., Wu, Z., Park, C.W., Graves, R., Wright, M., and Spiegelman, B.M. (1998). A cold-inducible coactivator of nuclear receptors linked to adaptive thermogenesis. *Cell* 92, 829–839.
- Puigserver, P., Adelmant, G., Wu, Z., Fan, M., Xu, J., O'Malley, B., and Spiegelman, B.M. (1999). Activation of PPARgamma coactivator-1 through transcription factor docking. *Science* 286, 1368–1371.
- Rea, S., Eisenhaber, F., O'Carroll, D., Strahl, B.D., Sun, Z.W., Schmid, M., Opravil, S., Mechtler, K., Ponting, C.P., Allis, C.D., and Jenuwein, T. (2000). Regulation of chromatin structure by site-specific histone H3 methyltransferases. *Nature* 406, 593–599.
- Reed, N., and Fain, J.N. (1968). Stimulation of respiration in brown fat cells by epinephrine, dibutyl-3',5'-adenosine monophosphate, and m-chloro(carbonyl cyanide)phenylhydrazine. *J. Biol. Chem.* 243, 2843–2848.
- Ricquier, D., Nechad, M., and Mory, G. (1982). Ultrastructural and biochemical characterization of human brown adipose tissue in pheochromocytoma. *J. Clin. Endocrinol. Metab.* 54, 803–807.
- Rodbell, M. (1964). Metabolism of isolated fat cells. I. Effects of hormones on glucose metabolism and lipolysis. *J. Biol. Chem.* 239, 375–380.
- Rosen, E.D., Sarraf, P., Troy, A.E., Bradwin, G., Moore, K., Milstone, D.S., Spiegelman, B.M., and Mortensen, R.M. (1999). PPAR gamma is required for the differentiation of adipose tissue in vivo and in vitro. *Mol. Cell* 4, 611–617.
- Rosen, E.D., Hsu, C.H., Wang, X., Sakai, S., Freeman, M.W., Gonzalez, F.J., and Spiegelman, B.M. (2002). C/EBPalpha induces adipogenesis through PPARgamma: a unified pathway. *Genes Dev.* 16, 22–26.
- Ross, S.R., Graves, R.A., Greenstein, A., Platt, K.A., Shyu, H.L., Mellovitz, B., and Spiegelman, B.M. (1990). A fat-specific enhancer is the primary determinant of gene expression for adipocyte P2 in vivo. *Proc. Natl. Acad. Sci. USA* 87, 9590–9594.
- Rothwell, N.J., and Stock, M.J. (1979). Combined effects of cafeteria and tube-feeding on energy balance in the rat. *Proc. Nutr. Soc.* 38, 5A.
- Rothwell, N.J., and Stock, M.J. (1984). Effects of denervating brown adipose tissue on the responses to cold, hyperphagia and noradrenaline treatment in the rat. *J. Physiol.* 355, 457–463.
- Scime, A., Grenier, G., Huh, M.S., Gillespie, M.A., Bevilacqua, L., Harper, M.E., and Rudnicki, M.A. (2005). Rb and p107 regulate preadipocyte differentiation into white versus brown fat through repression of PGC-1alpha. *Cell Metab.* 2, 283–295.
- Soukas, A., Socci, N.D., Saatkamp, B.D., Novelli, S., and Friedman, J.M. (2001). Distinct transcriptional profiles of adipogenesis in vivo and in vitro. *J. Biol. Chem.* 276, 34167–34174.
- Steppan, C.M., Bailey, S.T., Bhat, S., Brown, E.J., Banerjee, R.R., Wright, C.M., Patel, H.R., Ahima, R.S., and Lazar, M.A. (2001). The hormone resistin links obesity to diabetes. *Nature* 409, 307–312.
- St-Pierre, J., Lin, J., Krauss, S., Tarr, P.T., Yang, R., Newgard, C.B., and Spiegelman, B.M. (2003). Bioenergetic analysis of peroxisome proliferator-activated receptor gamma coactivators 1alpha and 1beta (PGC-1alpha and PGC-1beta) in muscle cells. *J. Biol. Chem.* 278, 26597–26603.
- Teyssier, C., Ma, H., Emter, R., Kralli, A., and Stallcup, M.R. (2005). Activation of nuclear receptor coactivator PGC-1alpha by arginine methylation. *Genes Dev.* 19, 1466–1473.
- Tiraby, C., Tavernier, G., Lefort, C., Larrouy, D., Bouillaud, F., Ricquier, D., and Langin, D. (2003). Acquisition of brown fat cell features by human white adipocytes. *J. Biol. Chem.* 278, 33370–33376.
- Tontonoz, P., Hu, E., and Spiegelman, B.M. (1994). Stimulation of adipogenesis in fibroblasts by PPAR gamma 2, a lipid-activated transcription factor. *Cell* 79, 1147–1156.
- Tseng, Y.H., Kriauciunas, K.M., Kokkotou, E., and Kahn, C.R. (2004). Differential roles of insulin receptor substrates in brown adipocyte differentiation. *Mol. Cell. Biol.* 24, 1918–1929.
- Tsukiyama-Kohara, K., Poulin, F., Kohara, M., DeMaria, C.T., Cheng, A., Wu, Z., Gingras, A.C., Katsume, A., Elchebly, M., Spiegelman, B.M., et al. (2001). Adipose tissue reduction in mice lacking the transcriptional inhibitor 4E-BP1. *Nat. Med.* 7, 1128–1132.
- Tvrdik, P., Asadi, A., Kozak, L.P., Nedergaard, J., Cannon, B., and Jacobsson, A. (1997). Cig30, a mouse member of a novel membrane protein gene family, is involved in the recruitment of brown adipose tissue. *J. Biol. Chem.* 272, 31738–31746.
- Uldry, M., Yang, W., St-Pierre, J., Lin, J., Seale, P., and Spiegelman, B.M. (2006). Complementary action of the PGC-1 coactivators in mitochondrial biogenesis and brown fat differentiation. *Cell Metab.* 3, 333–341.
- Wallberg, A.E., Yamamura, S., Malik, S., Spiegelman, B.M., and Roeder, R.G. (2003). Coordination of p300-mediated chromatin remodeling and TRAP/mediator function through coactivator PGC-1alpha. *Mol. Cell* 12, 1137–1149.
- Westerberg, R., Mansson, J.E., Golozoubova, V., Shabalina, I.G., Backlund, E.C., Tvrdik, P., Retterstol, K., Capecci, M.R., and Jacobsson, A. (2006). ELOVL3 is an important component for early onset of lipid recruitment in brown adipose tissue. *J. Biol. Chem.* 281, 4958–4968.
- Xue, B., Rim, J.S., Hogan, J.C., Coulter, A.A., Koza, R.A., and Kozak, L.P. (2007). Genetic variability affects the development of brown adipocytes in white fat but not in interscapular brown fat. *J. Lipid Res.* 48, 41–51.
- Yoon, J.C., Puigserver, P., Chen, G., Donovan, J., Wu, Z., Rhee, J., Adelmant, G., Stafford, J., Kahn, C.R., Granner, D.K., et al. (2001). Control of hepatic gluconeogenesis through the transcriptional coactivator PGC-1. *Nature* 413, 131–138.
- Zhou, Z., Yon Toh, S., Chen, Z., Guo, K., Ng, C.P., Ponniah, S., Lin, S.C., Hong, W., and Li, P. (2003). Cidea-deficient mice have lean phenotype and are resistant to obesity. *Nat. Genet.* 35, 49–56.

Accession Numbers

The microarray data set discussed herein has been deposited in the Gene Expression Omnibus repository (<http://www.ncbi.nlm.nih.gov/geo/>) with the accession number GSE8044.