ARTICLES

PRDM16 controls a brown fat/skeletal muscle switch

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Brown fat can increase energy expenditure and protect against obesity through a specialized program of uncoupled respiration. Here we show by *in vivo* fate mapping that brown, but not white, fat cells arise from precursors that express *Myf5*, a gene previously thought to be expressed only in the myogenic lineage. We also demonstrate that the transcriptional regulator PRDM16 (PRD1-BF1-RIZ1 homologous domain containing 16) controls a bidirectional cell fate switch between skeletal myoblasts and brown fat cells. Loss of PRDM16 from brown fat precursors causes a loss of brown fat characteristics and promotes muscle differentiation. Conversely, ectopic expression of PRDM16 in myoblasts induces their differentiation into brown fat cells. PRDM16 stimulates brown adipogenesis by binding to PPAR-γ (peroxisome-proliferator-activated receptor-γ) and activating its transcriptional function. Finally, *Prdm16*-deficient brown fat displays an abnormal morphology, reduced thermogenic gene expression and elevated expression of muscle-specific genes. Taken together, these data indicate that PRDM16 specifies the brown fat lineage from a progenitor that expresses myoblast markers and is not involved in white adipogenesis.

The epidemic of obesity, closely associated with increases in diabetes, hypertension, hyperlipidemia, cancer and other disorders, has propelled a major interest in adipose cells and tissues. Adipose tissues contain two distinct types of fat cell, white and brown. White fat cells are specialized for the storage of chemical energy such as triglycerides, whereas brown fat cells dissipate chemical energy in the form of heat[1]. Most fat depots can be characterized as either brown or white but some brown fat cells can also be found dispersed throughout white fat depots in rodents and humans[2–4]. Similarities in cell morphology, lipid metabolism and patterns of gene expression between the two types of fat cell has led most investigators to assume that they share a common developmental origin[5–7].

Until quite recently, brown adipose tissue (BAT) was thought to be of metabolic importance only in smaller mammals and infant humans. Recent studies using positron-emission tomography scanning, however, suggest that adult humans have several discrete areas of metabolically active BAT[8]. BAT may thus have a much more important role in human metabolism than was previously appreciated. The manipulation of fat stores is an obvious therapeutic objective, but disruption of the normal differentiation or development of white adipose tissue (WAT) causes ectopic lipid storage and severe pathology (lipodystrophy) in both humans and experimental animals. Promotion of increased BAT development in humans, however, offers the possibility of increasing energy expenditure without necessarily causing dysfunction in other tissues. Indeed, experimental increases of BAT in animals have been associated with a lean and healthy phenotype[4,9–11]. In contrast, loss of BAT function is linked to obesity and metabolic disease[12].

The ability to alter BAT development in a measured way depends upon a thorough understanding of the regulatory systems that control determination in this cell type. Several transcriptional suppressors of BAT development have been identified, including pRB, p107 and RIP140 (refs 13–15). Positive transcriptional regulators include FOXC2 (ref. 9) and PRDM16 (ref. 16), but only PRDM16 has been shown to determine the fate of brown fat cells in a cell-autonomous manner. This protein, which contains several zinc fingers and a PRD1-BF1-RIZ1 homologous (PR) domain, can turn on a full set of BAT-selective genes when expressed in WAT precursors in culture or *in vivo*, while turning off the expression of several white-fat-enriched genes[16,17]. These actions of PRDM16 are, at least partly, mediated by its interaction with other transcriptional co-regulators, PGC-1α and PGC-1β, and do not require DNA binding of PRDM16. Here we show that PRDM16 can powerfully control a robust, bi-directional switch in cell fate between skeletal myoblasts and brown fat cells. Furthermore, lineage tracing studies *in vivo* show that depots of BAT but not WAT arise from *Myf5*-expressing precursors that brown fat shares with skeletal muscle.

Results

Knockdown of PRDM16 in brown fat cells induces skeletal myogenesis. To examine in detail the cellular and molecular consequence of PRDM16 depletion, we used adenoviral vectors to express a short hairpin (sh)SCR (scrambled) control or shRNA targeting PRDM16 in primary brown fat pre-adipocytes. PRDM16 protein was virtually undetectable in cultures expressing the shPRDM16 construct (Fig. 1a). By day 4 of adipogenic differentiation, cultures expressing the scrambled shRNA had undergone normal brown-fat cell differentiation (Fig. 1b). In striking contrast, shPRDM16-expressing cultures contained long tube-like cells interspersed among fat cells that were strongly marked by green fluorescent protein (GFP), which was co-expressed from the adenoviral vector. In fact, the cells that adopted this morphology were those that had preferentially

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taken up and expressed the shPRDM16 construct (Fig. 1b). Immunohistochemistry using a pan-skeletal myosin heavy chain (MyHC) antibody unequivocally identified these cells as skeletal myocytes (Fig. 1c). The myoctes were noted here but not previously[16] because these studies achieved a more complete knockdown of PRDM16; and because the presence of the GFP gene in the adenoviral vector allowed a more focused examination of the transduced cells. Gene-expression analysis showed that knockdown of PRDM16 completely ablated the expression of selective genes for brown fat cells such as *Ucp1*, *Cidea* and *Elovl3* (also known as *Cig30*) (Fig. 1d). Moreover, reduction of PRDM16 was associated with a large increase in the expression of myogenic genes, including *Myod*, myogenin (*Myg*), myosin light chain (*Mlc*), muscle creatine kinase (*Mck*) and *Myhc*. Knockdown of PRDM16 induced the expression of these genes to between 5% and 25% of their levels in C2C12 myotubes, a result generally consistent with the MyHC protein expression observed immunocytochemically in approximately 10–15% of the cells in these cultures. These results strongly suggest that PRDM16 functions in brown fat precursors to restrict skeletal muscle gene expression and development.

Brown fat and skeletal muscle but not white fat arise from *Myf5*-expressing progenitors. The induction of skeletal muscle in cultures of brown fat pre-adipocytes suggested a surprising developmental relationship between these two cell lineages. To address this question directly, we performed lineage-tracing experiments in mice. Knock-in mice expressing cre recombinase from the regulatory elements of the skeletal muscle-specific *Myf5* gene[18] were crossed with indicator mice that express yellow fluorescent protein (YFP) from the rosa26 gene locus (*R26R3-YFP*) in a cre-dependent manner. *Myf5* is a myogenic regulatory factor that is expressed in committed skeletal myogenic precursors that were previously thought to give rise exclusively to skeletal muscle. Recombination at the rosa26 locus induced by cre recombinase is heritable and irreversible, allowing the developmental progression of *Myf5*-expressing descendents to be traced by YFP expression[19] (Fig. 2a). Importantly, these *Myf5-Cre* knock-in mice have been successfully used to drive skeletal-muscle-specific cre expression in other studies[20–22].

To examine the potential contribution of *Myf5*-expressing progenitors to the adipose anlagen, immunohistochemistry with anti-GFP antibodies was used to localize expression of the YFP reporter gene in skeletal muscle, BAT and WAT from the interscapular region of 2- to 3-month-old *Myf5-Cre*:*R26R3-YFP* mice. In *Myf5-Cre* negative (control) mice that harbour the *R26R3-YFP* reporter, YFP was not expressed in any tissues (Fig. 2b). However, in *Myf5-Cre* expressing mice, YFP was readily detected in skeletal muscle and BAT but not in WAT. YFP protein was also strongly expressed in the peri-renal BAT (Supplementary Fig. 1a) but not in any classic WAT depots (not shown). Moreover, real-time PCR showed that *YFP* messenger RNA (mRNA) was induced only in BAT depots and in skeletal muscle from *Myf5-Cre*-expressing mice, and at comparable levels (Fig. 2c and Supplementary Fig. 1b). *YFP* mRNA expression was not activated in other tissues examined, including the gluteal, inguinal and epididymal WAT depots (Fig. 2c and Supplementary Fig. 1b). These results indicate that *Myf5*-expressing cells specifically gave rise to skeletal muscle and brown fat cells. Notably, *Myf5* mRNA itself was not detected in mature BAT (Supplementary Fig. 1c), indicating that it was transiently expressed at an earlier developmental stage.

In newborn mammals, BAT is apparent at distinct anatomical sites including the interscapular, perirenal and axillary depots. It is also well established that brown fat cells emerge in WAT in response to

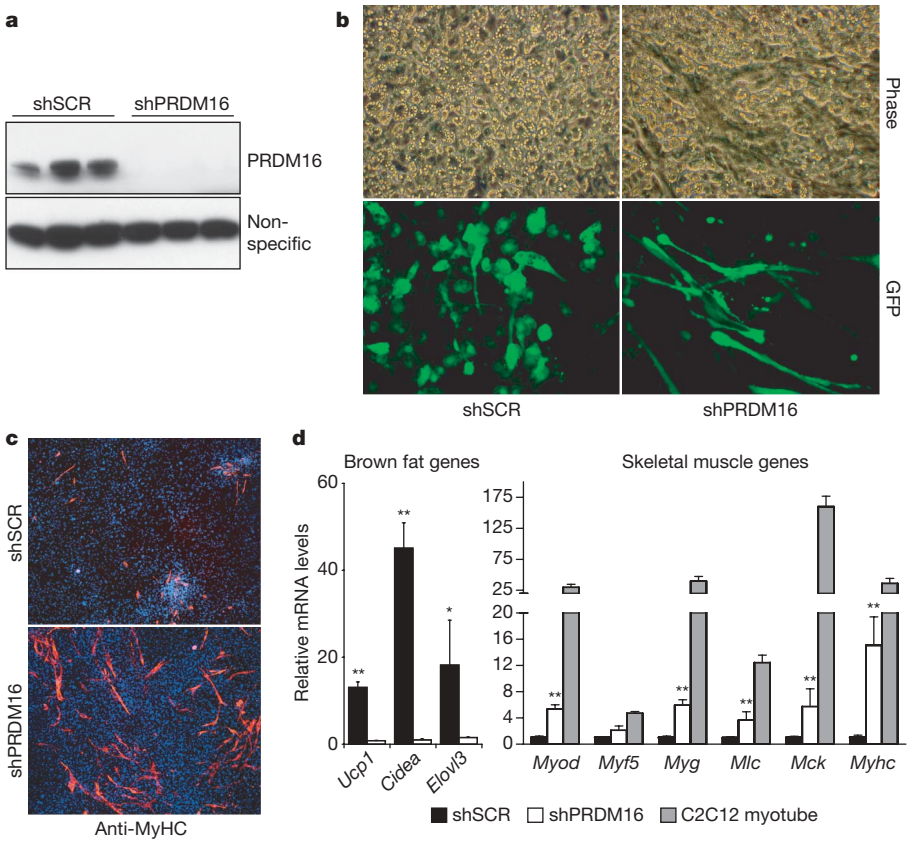

Figure 1 | Knockdown of PRDM16 in primary brown fat cells induces skeletal myogenesis. a, Western blot analysis for PRDM16 in primary brown fat cell cultures transduced with adenovirus expressing shRNA targeted to PRDM16 or a scrambled (SCR) control shRNA. **b**, These cultures were visualized by phase contrast microscopy and by GFP fluorescence. **c**, Immunocytochemistry for skeletal myosin heavy chain (MyHC) expression. **d**, Gene expression at day 4 of adipocyte differentiation including BAT-selective and skeletal-muscle-specific genes (as indicated). C2C12 myotubes were also assayed for their expression of muscle-specific genes ($n = 3$, error bars are ± s.d.; $^*P < 0.05$, $^{**}P < 0.01$).

chronic cold exposure or prolonged β-adrenergic stimulation[2,4,23]. To determine whether these β-adrenergic-induced brown fat cells in WAT are also derived from *Myf5*-expressing precursors, we treated adult *Myf5-Cre:R26R3-YFP* mice with a selective β3-adrenergic agonist, CL 316, 243, for 7 days, and examined whether the newly formed brown fat cells expressed YFP. As shown in Fig. 2d, this treatment induced the development of numerous clusters of UCP1-expressing brown fat cells in epididymal WAT. However, these brown fat cells did not express the YFP reporter gene (Fig. 2d), whereas the interscapular brown fat cells from the same mice were readily marked by YFP expression (Fig. 2d). These results suggest that the brown fat cells that develop in WAT in response to β-adrenergic signalling have an independent developmental origin from the distinct and classic BAT depots that develop before birth.

PRDM16 induces brown adipogenesis in C2C12 and primary myogenic cells. BAT depots arise from *Myf5*-expressing cells during development. To investigate whether PRDM16 is sufficient to specify the fate of brown fat in skeletal myogenic progenitors, we ectopically expressed PRDM16 in the C2C12 myoblast cell line. First, C2C12 myoblasts were transduced with control or PRDM16-expressing retroviruses, and then exposed to pro-myogenic culture conditions to induce terminal myocyte differentiation. C2C12 cells expressing the control virus differentiated efficiently into multinucleated MyHC-expressing myotubes (Supplementary Fig. 2a). PRDM16-expressing cells, however, failed to undergo myogenic differentiation (Supplementary Fig. 2a). Gene expression analysis showed that PRDM16 blocked the induction of myotube-specific genes including *Myod*, *Myg*, *Mrf4*, *Myhc* and *Mck* (Supplementary Fig. 2b). Control and PRDM16-expressing C2C12 myoblasts were also stimulated to undergo adipogenesis using adipogenic inducers. Under these conditions, the control cultures differentiated into multinucleated skeletal myotubes, whereas the PRDM16-expressing cells uniformly differentiated into lipid filled adipocytes, as shown by Oil-Red-O staining (Fig. 3a). Gene expression studies showed that PRDM16 expression increased the mRNA levels of adipocyte-specific genes, including a 20-fold increase in the PPAR-γ gene (*Pparg*) and a 250-fold elevation of *Ap2* (also known as *Fabp4*), with decreased levels of myogenic genes such as *Myod* and *Myg* (Fig. 3b). Importantly, adipocytes induced by PRDM16 expression also expressed elevated levels of specific genes for brown fat cells such as *Elovl3* and *Cidea* (more than 30,000-fold; Fig. 3c), as well as the thermogenic genes *Ucp1* and *Pgc1a* (Fig. 3d). Moreover, as in real brown fat cells, *Ucp1* and *Pgc1a* were further induced in PRDM16-derived adipocytes by elevating cyclic AMP levels through forskolin treatment (Fig. 3d). These data demonstrate that PRDM16 expression alone is sufficient to drive brown adipocyte differentiation in committed and clonal skeletal myogenic cells.

To explore the adipogenic action of PRDM16 further, we ectopically expressed PRDM16 in primary mouse myoblasts isolated from postnatal skeletal muscle. As observed in C2C12 cells, control vector (ctl)-expressing cells differentiated into multinucleated myotubes in response to an adipogenic induction cocktail. In contrast, PRDM16-transduced myoblasts differentiated at near 100% efficiency into fat-storing adipocytes (Fig. 3e). Consistent with their morphological differentiation, PRDM16-transduced cultures expressed elevated mRNA levels of pan-adipocyte specific genes, such as *Pparg*, *Ap2* and adiponectin (Fig. 3f). Moreover, adipocytes derived from PRDM16-expressing myogenic precursors strongly activated the expression of brown fat cell-specific genes including: *Elovl3*, *Cidea*, *Pgc1a* and a 1,000-fold increase in *Ucp1* levels (Fig. 3f). By western blot analysis, PRDM16-derived adipocytes expressed the brown fat cell selective UCP1 and CIDEA proteins to levels comparable with that in bona fide brown fat cells (Fig. 3g). Altogether, these results demonstrate that PRDM16 activates a complete brown fat differentiation program in muscle precursor cells.

PRDM16 binds to PPAR-γ and co-activates its transcriptional function. To explore mechanisms that could explain the pro-adipogenic actions of PRDM16, we used an unbiased approach to identify binding partners of PRDM16. To this end, the PRDM16 protein complex was immunopurified from fat cells and analysed by mass spectrometry (Supplementary Table 1). Of particular interest was the identification of PPAR-γ as a near-stoichiometric component of the PRDM16 complex (Fig. 4a). PPAR-γ was the only DNA-binding transcriptional component found in the PRDM16 complex. Independent co-expression assays in COS-7 cells again showed PPAR-γ in a complex with PRDM16 (Fig. 4b). This interaction is

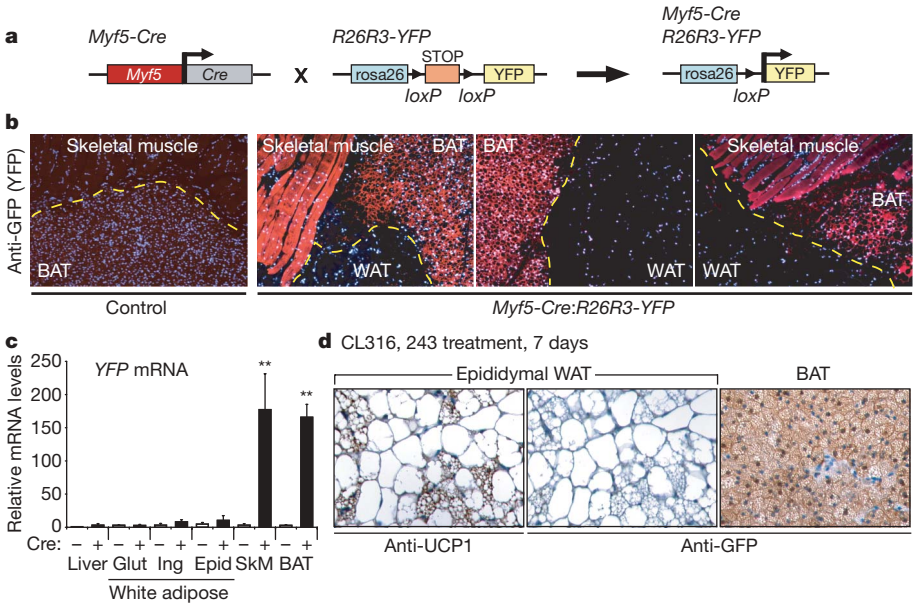

Figure 2 | Brown fat and skeletal muscle arise from *Myf5*-expressing precursors. a, *Myf5-Cre* mice were intercrossed with indicator mice that have a *YFP* gene integrated into the rosa26 locus downstream of a floxed transcriptional stop sequence (*R26R3-YFP*). Expression of cre recombinase excises the stop sequence to activate YFP expression irreversibly. **b**, Immunohistochemistry to detect YFP (GFP) expression in skeletal muscle, BAT and WAT from control (*Myf5-Cre* negative) and *Myf5-Cre:R26R3-YFP* mice. **c**, Real-time PCR of *YFP* mRNA levels in: liver; gluteal (Glut), inguinal (Ing) and epididymal (Epid) WAT; skeletal muscle (SkM) and BAT ($n = 4$ per group; error bars are ± s.e.m.; $^{**}P < 0.01$). **d**, UCP1 and GFP expression in WAT and BAT from CL316, 243-treated *Myf5-Cre:R26R3-YFP* mice.

likely to be direct, because a full-length glutathione *S*-transferase (GST)–PPAR-γ2 fusion protein purified from baculovirus infected insect cells bound to *in vitro* translated PRDM16 (Fig. 4c). PRDM16 and PPAR-γ2 interacted in a non-ligand-dependent manner, because the binding was equivalent with or without the addition of PPAR-γ ligand, rosiglitazone, to the binding reaction. Steroid receptor co-activator-1 (SRC-1), by contrast, interacted with purified GST–PPAR-γ only in the presence of rosiglitazone (Fig. 4c). Domain mapping experiments using GST immunoaffinity assays identified two regions in PRDM16 that bound to PPAR-γ, corresponding to zinc-finger-1 (ZF1) and zinc-finger-2 (ZF2) (Fig. 4d).

PPAR-γ is the master regulator of adipogenic differentiation, being both absolutely necessary and sufficient to induce adipogenesis[24–26]. Furthermore, overexpression and activation of PPAR-γ was previously shown to promote adipogenic conversion of myoblasts[27]. We therefore investigated whether PRDM16 could enhance the transcriptional activity of PPAR-γ. As shown in Fig. 4e, the expression of PRDM16 stimulated the activity of a luciferase reporter gene controlled by three tandem repeats of a PPAR binding site (3 × DR1-luciferase) by 15-fold. The reporter gene was further induced by PRDM16 in cells treated for 24 h with 1 μM rosiglitazone. In *Pparg*$^{-/-}$ cells, PRDM16 expression induced transcription of the reporter gene only in the presence of exogenously added PPAR-γ (Supplementary Fig. 3). Although the binding of PRDM16 to PPAR-γ was not ligand-dependent *in vitro*, the co-activation function of PRDM16 in cellular assays was augmented by ligand. We also examined whether PRDM16 could interact with PPAR-α, a related PPAR family member that is expressed at higher levels in brown versus white fat cells and has been implicated in UCP1 gene regulation[28]. In COS-7 cells, PRDM16 bound to PPAR-α with similar efficiency both in the absence and presence of the PPAR-α ligand, WY-14643 (Supplementary Fig. 4a). As observed with PPAR-γ, PRDM16 also activated the transcriptional function of PPAR-α in a ligand-dependent manner (Supplementary Fig. 4b). These data indicate that PRDM16 can bind and co-activate the transcriptional function of both PPAR-α and PPAR-γ.

The strong co-activation of PPAR-γ transcriptional function by PRDM16 suggests that this is a key mechanism by which PRDM16 promotes adipogenesis. In this regard, PRDM16 is unable to promote adipogenesis in *Pparg*-deficient fibroblasts (data not shown). Significantly, the adipogenic conversion of C2C12 (Supplementary Fig. 5a) and primary myoblasts by PRDM16 was largely dependent on the presence of rosiglitazone, a highly specific agonist for PPAR-γ. In C2C12 cells, PRDM16 induced the expression of adipocyte-related

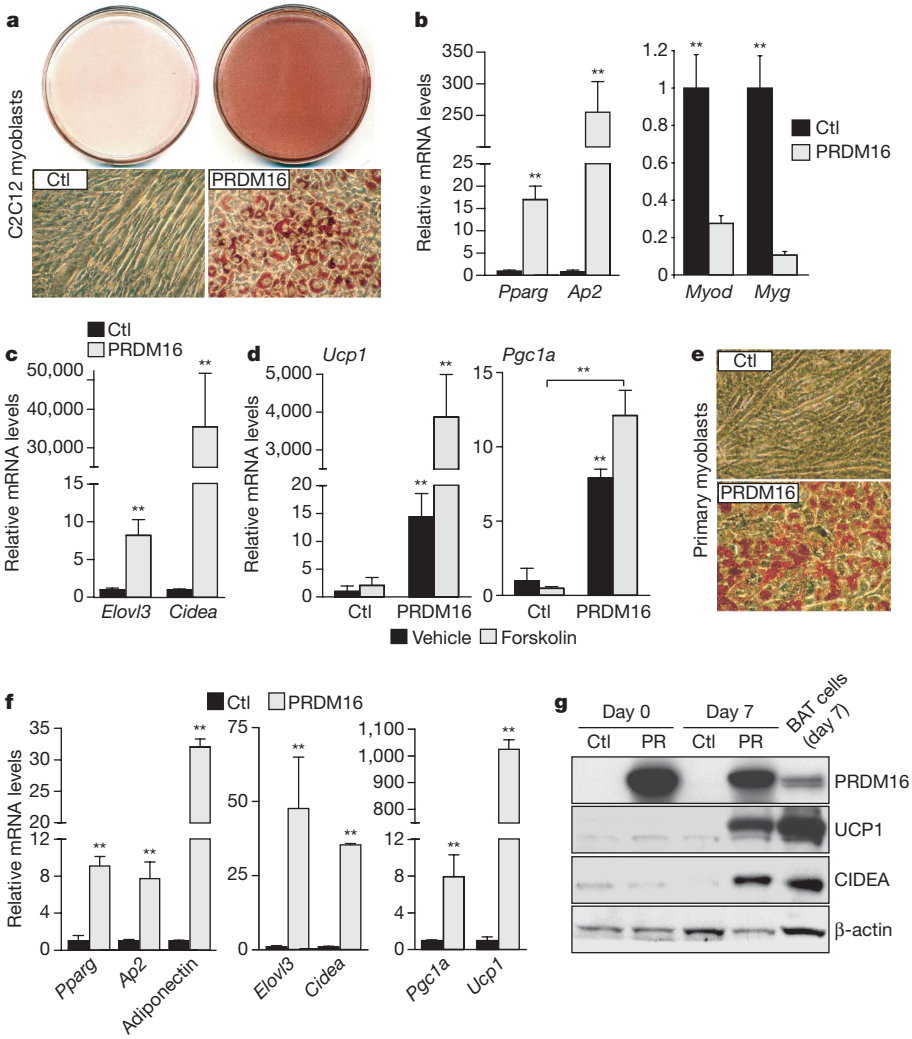

Figure 3 | PRDM16 stimulates brown adipocyte differentiation in skeletal myoblasts. a–d, C2C12 myoblasts expressing retroviral PRDM16 or vector control (Ctl) were stained with Oil-Red-O 6 days after inducing adipocyte differentiation (**a**); and analysed by real-time PCR for their expression of markers specific to: adipocytes (*Pparg*, *Ap2*) (**b**, left); skeletal muscle (*Myod*, *Myg*) (**b**, right); BAT (*Elovl3*, *Cidea*) (**c**); and thermogenesis (*Ucp1*, *Pgc1a*) (**d**). **e–g**, Ctl and PRDM16-expressing primary myoblasts were stained with Oil-Red-O 7 days after inducing adipocyte differentiation (**e**). Real-time PCR analysis of genes expressed selectively in: adipocytes (*Pparg*, *Ap2*, adiponectin) (**f**, left); BAT (*Elovl3*, *Cidea*) (**f**, centre); and during thermogenesis (*Pgc1a*, *Ucp1*) (**f**, right). **g**, Western blot analysis before (day 0) and after 7 days of differentiation. ($n = 4$; error bars are ± s.d.; $^{**}P < 0.05$).

genes like *Ap2* and adiponectin as well as *Ucp1* and *Cidea*, specific markers for brown fat cells, in a ligand-dependent manner (Supplementary Fig. 5b). These results indicate that PPAR-γ activation is required for the adipogenic function of PRDM16.

We next examined whether expression of any potent co-activator of PPAR-γ could induce adipogenesis in C2C12 myoblasts. Interestingly, PGC-1β, a strong activator of PPAR-α and PPAR-γ (ref. 29 and unpublished results) had no ability to promote adipogenesis or induce adipocyte-specific genes in C2C12 myoblasts (Supplementary Fig. 6). It was, however, able to induce several mitochondrial gene targets. These data suggest that the co-activation of PPAR-γ by PRDM16 is selectively potent in stimulating the adipocyte differentiation program in myogenic cells. Furthermore, PRDM16 was not pro-adipogenic in other non-myogenic cell types, including C3H10T1/2 cells, NIH-3T3 and several types of pre-adipocyte[16]. These data therefore suggest that the pro-adipogenic action of PRDM16 may be somewhat dependent on other factors present in myoblasts.

An important question arising from these studies was whether adipocytes derived from myoblasts through PPAR-γ function per se would always display a brown fat phenotype. To address this question, we compared the adipogenic activity of ectopically expressed PRDM16 and PPAR-γ2 in C2C12 myoblasts. In these experiments, PRDM16 stimulated adipocyte development with equal, if not better, efficiency compared with PPAR-γ2 (Supplementary Fig. 7a). Interestingly, adipocytes formed from PRDM16-expressing cells had a uniform round shape, were smaller in size and contained numerous small lipid droplets. PPAR-γ-driven adipocytes were larger, irregularly shaped and contained large lipid droplets. Both PRDM16 and PPAR-γ2 induced the expression of pan-adipocyte genes such *Ap2* and adiponectin (Supplementary Fig. 7b). However, only PRDM16 induced a complete brown fat differentiation program (Supplementary Fig. 7b). These data demonstrate that PPAR-γ can convert myogenic cells into adipocytes but PRDM16 expression additionally commits cells to the brown fat fate.

Dysregulated gene expression in *Prdm16*-deficient brown fat. PRDM16 belongs to a family of 16 PR domain-containing proteins, some of which display an overlapping expression pattern with PRDM16. The effect of global *Prdm16* deficiency on mouse and brown fat development has not been examined. Mice deficient for *Prdm16* were created by targeted insertion of a gene-trap cassette into intron 2 of the gene. The creation and general phenotypic characterization of this mouse model will be described elsewhere. Notably, mice homozygous for the gene-trap insertion ($Prdm16^{cGT/cGT}$) die at birth. We therefore analysed the phenotype of putative BAT pads from wild-type heterozygous carriers and homozygous *Prdm16* mutant (knockout) late-stage embryos (E17). First, we established that *Prdm16* mRNA was decreased in heterozygous BAT and virtually undetectable in the putative knockout BAT (Fig. 5a). By haematoxylin and eosin (H&E) staining, the putative BAT from the knockout animals had an unusual morphology, including substantially larger lipid stores (white areas in tissue) compared with wild-type BAT (Fig. 5b). No obvious muscle tissue was observed in these fat pads. Gene expression studies showed that wild-type and knockout BAT expressed similar levels of the pan-adipocyte markers *Ap2* and adiponectin (Fig. 5c). The *Prdm16*-deficient BAT, however, expressed significantly lower levels of essentially all BAT cell-selective and thermogenic genes (Fig. 5d). This included a 50% reduction of *Ucp1* mRNA levels, 75% reduction of *Elovl3*, 50% reduction of *Cidea* and a significant reduction of *Pgc1a* and mitochondrial target genes, cytochrome *c* (*Cyc*) and *Cox8b*. Strikingly, the *Prdm16*-deficient BAT also exhibited large increases in the expression of skeletal myogenic genes including four- to sixfold elevated levels of *Myod*, myogenin, *Mck* and *Myf6* (Fig. 5e). These data document a genetic requirement for PRDM16 in the appropriate development and gene expression program of BAT.

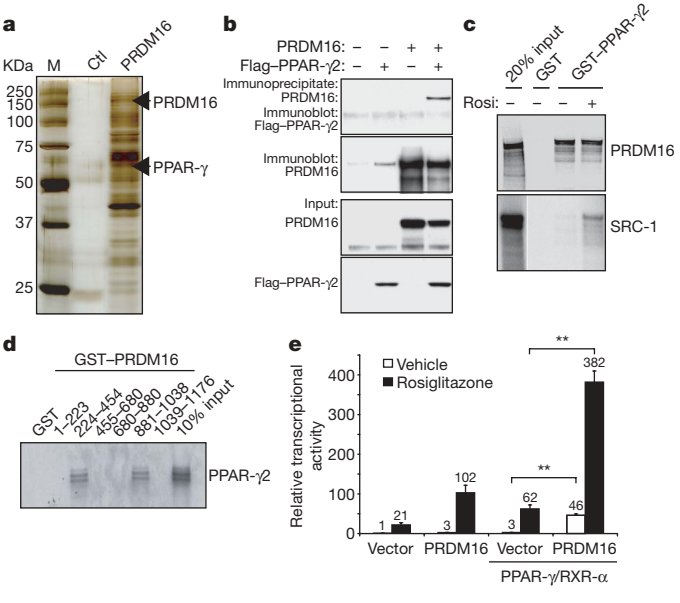

Figure 4 | PRDM16 binds and activates the transcriptional function of PPAR-γ. a, Components in the PRDM16 complex from fat cells were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and visualized by silver staining. **b**, Immunoprecipitation of PRDM16 from COS-7 cells expressing exogenous PRDM16 and/or Flag–PPAR-γ2 followed by western blot analysis to detect PPAR-γ2. **c**, GST alone or a GST fusion protein containing PPAR-γ2 was incubated with ^{35}S-labelled PRDM16 or SRC-1 protein (±1 μM rosiglitazone). **d**, GST fusion proteins containing different regions of PRDM16 were incubated with ^{35}S-labelled PPAR-γ2. **e**, Transcriptional activity of a PPAR-driven reporter gene in response to PPAR-γ/RXR-α and PRDM16 or vector expression in COS-7 cells (±1 μM rosiglitazone) ($n = 3$; error bars are ± s.d.; $^{**}P < 0.05$).

Discussion

Most models of fat development have suggested that brown and white fat cells arise from a common adipogenic precursor cell[5–7]. Given the ability of PRDM16 to drive a complete brown fat gene program[16], we had thus expected that brown fat cells lacking PRDM16 would adopt a white phenotype. However, surprisingly, loss of PRDM16 from brown fat cells caused an increase in myogenic gene expression and bona fide skeletal muscle differentiation. Furthermore, this dependence on PRDM16 was bi-directional. Increased PRDM16 expression converted both immortalized and primary skeletal muscle myoblasts into brown fat cells.

By tracking the fate of cells that express the myoblast-specific *Myf5* gene, we provide evidence for a very close developmental relationship between skeletal muscle and BAT. Significantly, our analysis reveals that WAT and BAT depots arise from independent lineages and presumably have fundamentally distinct mechanisms of determination (Supplementary Fig. 8). Cells with the morphology and molecular phenotype of brown fat can also be induced in WAT of rodents and humans by chronic cold exposure or by β-adrenergic stimulation[2,4,23,30]. In our studies, the brown fat cells that emerged in the WAT of mice treated with a β3-adrenergic agonist were not derived from *Myf5*-expressing precursors. Notably, genetic studies by Kozak and colleagues suggest that formation of brown fat cells in WAT versus BAT is controlled by distinct mechanisms[31], which is consistent with the view that these two types of brown fat cell have separate developmental origins. The identity of the cells in WAT that give rise to brown adipocytes remains unknown. It is still reasonable to speculate that a common white/brown fat progenitor exists in some/all WAT depots.

The genetic tracing experiments here do not determine whether a single $Myf5^+$ cell gives rise to both BAT and skeletal muscle, or whether separate populations of $Myf5^+$ progenitors exist. However, the interconversion between muscle and BAT induced through

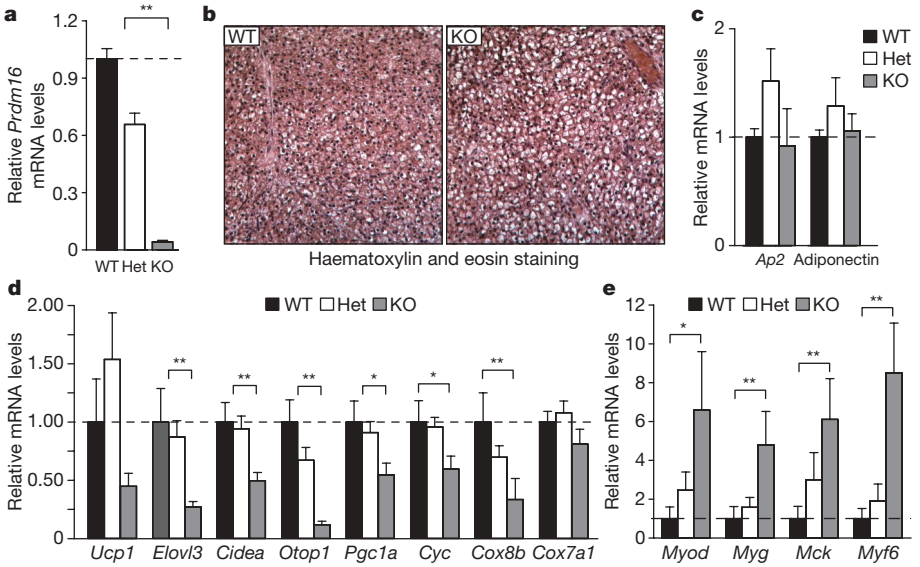

Figure 5 | Altered morphology and dysregulated gene expression in *Prdm16*-deficient brown fat. a, Real-time PCR of *Prdm16* mRNA levels in putative BAT depots from E17 wild-type (WT), heterozygous (Het) and *Prdm16* knockout (KO) mice. **b**, Haematoxylin and eosin staining of representative sections of BAT from wild-type and knockout mice. **c**–**e**, Wild-type, heterozygous and knockout BAT were examined by real-time PCR for their expression of: general adipocyte markers (*Ap2* and adiponectin) (**c**); BAT-selective genes (**d**); and skeletal muscle-specific genes (**e**) ($n = 7$–11 mice per group; error bars represent ± s.e.m.; $*P < 0.05$; $**P < 0.01$).

modulation of PRDM16 levels in culture suggests that bipotent muscle–brown fat precursors may give rise to both lineages (Supplementary Fig. 8). Future studies addressing the exact nature of the muscle–brown fat precursor cell compartment(s) are needed. Interestingly, UCP1-expressing brown adipocytes have been localized in skeletal muscle and are correlated with resistance to obesity[32]. Our results suggest that myogenic precursor cells could be the source of these 'ectopic' brown fat cells. In this regard, muscle stem cells called satellite cells can undergo adipogenesis under certain experimental conditions[33,34], though whether they assume a brown fat phenotype has not been examined.

Some recent experiments had hinted that BAT and muscle share important features. Timmons *et al.* showed that precursors of brown fat cells express a wide array of muscle-related genes[35]. Lineage-tracing experiments by Atit *et al.* revealed that embryonic BAT, along with skin and muscle, was derived from a population of engrailed-1 (*En1*)-expressing cells in the dermomyotome[36]. The contribution of *En1*-expressing cells to WAT depots was, however, not studied. *En1* is expressed early in mouse development and marks a variety of other cell lineages including specific neuronal pools. Thus a direct connection between muscle and BAT was not evident. The ontogenic relationship between BAT and skeletal muscle may explain why brown fat cells are specialized for lipid catabolism rather than storage, much like oxidative muscle. Further, BAT and skeletal muscle are both highly responsive to sympathetic nerve activity and have the capacity to perform adaptive thermogenesis.

PRDM16 appears to function in brown fat cells through its ability to co-regulate the activity of other transcription factors and co-activators (Supplementary Fig. 8). The action of PRDM16 is almost certainly dependent on its interaction with PPAR-γ because PRDM16 stimulated adipogenesis in a PPAR-γ-ligand-dependent manner, and PPAR-γ is known to be absolutely required for adipogenesis in cultured cells and in *vivo*[24,25]. Our previous study indicated that an important contribution of the ability of PRDM16 to stimulate a brown fat phenotype in white fat precursors was through its association with PGC-1α and PGC-1β. It seems extremely likely that PRDM16 interacts with other factors, as yet undefined. At a mechanistic level, it remains to be determined how PRDM16 is able to stimulate the function of the PGC-1s and PPARs.

Although the short interfering RNA-mediated depletion of PRDM16 from cultured brown fat pre-adipocytes almost completely ablated their ability to differentiate into brown fat cells, chronic loss of PRDM16 in mice caused a significant but more modest reduction in the character of brown fat cells. Skeletal muscle gene expression was readily apparent in the knockout tissue but no overt muscle fibres were observed. It is likely that one or more of the other 15 PR domain-containing family members can partly compensate for the loss of PRDM16 from BAT *in vivo*. It will be important to examine whether any of these family members has overlapping functions with PRDM16. The *Prdm16*-deficient mice die at birth, thereby precluding functional analyses of the mutant BAT in response to cold or its effect on energy balance. Tissue-selective ablation of PRDM16 will be required to address these issues.

The role of BAT in regulating energy balance and fighting obesity in rodents is well established. Humans have BAT, and recent data suggest that it might have a more significant role in the physiology of adults than was previously recognized[8]. Because PRDM16 can function as a dominant regulator of the fate of brown fat cells, it will be important to investigate whether it can be used therapeutically. For example, it may be possible to use PRDM16 to drive the formation of brown fat precursor-type cells from myoblasts or white fat precursors for autologous transplantation into fat tissues. Alternatively, identifying compounds that induce the expression of PRDM16 in white fat or muscle progenitors could be powerful in fighting obesity.

METHODS SUMMARY

DNA constructs. PRDM16 and PPAR-γ expression plasmids were described before[16,37]. Adenoviral shRNA constructs were made in the pAdtrack vector (Stratagene), using previously validated sequences[16].

Cell culture. Primary brown pre-adipocytes and myoblasts were prepared from P0–P4 Swiss-Webster mice as described before[38,39]. Adipocyte differentiation was induced by treating cells for 48 h in medium containing 10% FBS, 0.5 mM isobuylmethylxanthine, 125 nM indomethacin, 1 μM dexamethosone, 850 nM insulin, 1 nM T3 with or without 1 μM rosiglitazone (Alexis Biochemicals). After 48 h, cells were switched to medium containing 10% FBS, 850 nM insulin, 1 nM T3 with or without 1 μM rosiglitazone.

Mice. For lineage tracing studies, heterozygous *Myf5*$^{Cre/+}$ mice[18] were intercrossed with rosa26R3 (*R26R3*)-*YFP* mice[19]. *Prdm16*-deficient mice were created by homologous insertion of a gene-trap cassette and will be described elsewhere.

Immunohistochemistry. Paraffin-embedded sections were subjected to citrate-based antigen retrieval (Vector Labs) and incubated with antibodies against GFP or UCP1 (Abcam). Secondary detection was with anti-rabbit Alexa 647 (Invitrogen) or the Dako Envision+ system.

Binding studies. The PRDM16 transcriptional complex was immunopurified from adipocytes stably expressing Flag-tagged PRDM16 or a control vector. Gel-resolved proteins were excised and analysed by matrix-assisted laser desorption/ionization– reflectron time of flight mass spectrometry[40]. Co-expression assays were performed in COS-7 cells to assess binding of PRDM16 with PPAR-α and PPAR-γ. Reporter gene assays were performed in COS-7 cells or *Pparg*$^{-/-}$ fibroblasts using a luciferase reporter gene driven by three tandem copies of a PPAR response element ($3 \times$ DR1-luciferase)[41].

Real-time PCR. Complementary DNA was prepared from total RNA using the ABI reverse transcription kit. Quantitative PCR reactions contained Sybr-Green fluorescent dye (ABI). Relative mRNA expression was determined by the $\Delta\Delta$-Ct method using tata-binding protein levels as endogenous control.

Full Methods and any associated references are available in the online version of the paper at www.nature.com/nature.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Contributions P.S. and B.M.S. conceived and designed the experiments. P.S., W.Y., S. Kajimura, S.C. and H.M.C. performed the experiments. P.S. analysed the data. B.B., S. Kuang, A.S., S.D., H.E., P.T., M.A.R. and D.R.B. contributed reagents/materials/analysis tools. P.S. and B.M.S. wrote the paper.

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METHODS

Plasmids and viral vectors. Full-length mouse complementary DNA sequence for PGC-1β was cloned into pMSCV-puro retroviral vector (Stratagene). GST–PPAR-γ2 was cloned into pAcGHLT baculovirus expression plasmid (Pharmingen). Various fragments of PRDM16 were amplified by PCR and cloned into the *Eco*RI or *Bam*HI/*Xho*I sites of the bacterial expression vector pGEX-4T-2 vector (GE Healthcare).

Cell culture. COS-7, C2C12 and C3H-10T1/2 cells were obtained from the American Type Culture Collection. Immortalized brown pre-adipocytes and PPAR-$\gamma^{-/-}$ fibroblasts were described elsewhere[25,42]. For retrovirus production, phoenix packaging cells[43] were transfected by the calcium phosphate method as described[16]. For retroviral transduction, cells were treated overnight with viral supernatant supplemented with 8 μg ml^{-1} polybrene. For adenoviral infection of primary brown fat precursors, 70% confluent cell cultures were incubated with shPRDM16 or shSCR-expressing adenovirus (multiplicity of infection = 100) overnight in complete growth medium. The medium was then replaced and cells were maintained in complete growth medium for an additional 24 h before inducing adipogenic differentiation. Muscle differentiation was induced by culturing cells in 2% horse-serum/DMEM. To stimulate cyclic AMP-induced thermogenesis in cells, 10 μM forskolin was added to cultures for 4 h. Oil-Red-O staining was performed as described previously[26]. All chemicals were obtained from Sigma unless otherwise indicated.

Animals. All animal experiments were performed according to procedures approved by the Dana-Farber Cancer Institute's Institutional Animal Care and Use Committee or in accordance with the University of Ottawa regulations for animal care and handling. CL316, 243, at 1 mg kg^{-1}, was injected intraperitoneally into 8- to 10-week-old male mice daily for 7 days ($n = 5$ mice per genotype).

Immunostaining. Paraffin-embedded sections of brown fat and white fat tissues were subjected to citrate-based antigen retrieval (Vector Labs) and incubated with anti-rabbit GFP (Abcam) or anti-UCP1 (Abcam) antibodies for 30 min at room temperature. For fluorescent detection, sections were incubated with anti-rabbit antibodies labelled with Alexa fluor 647 (Invitrogen) for 30 min and counterstained with 4′,6-diamidino-2-phenylindole (DAPI). For horseradish-peroxidase-based detection, samples were processed using the Dako Envision+ system and counterstained with haematoxylin (Vector Labs). Cell cultures were fixed in 4% paraformaldehyde, followed by permeabilization in 0.3% Triton X-100. Fixed cells were incubated with anti-myosin heavy chain (MF20, Developmental Studies Hybridoma Bank (DSHB), Iowa) or anti-myogenin (F5D, DSHB) antibodies for 1 h at room temperature. Secondary detection was performed with goat anti-mouse Alexa fluor 594 (Invitrogen), and nuclei were counterstained with DAPI. Samples were visualized with a Nikon Eclipse 80i upright microscope (Nikon Imaging Center, Harvard Medical School). Digital images were acquired by a Hamamatsu Orca 100 cooled CCD (charge-coupled device) camera and Metamorph image analysis software (Molecular Devices).

Binding studies. For affinity purification of the PRDM16 transcriptional complexes, *Pparg*-deficient fibroblasts stably expressing PPAR-γ2 were infected with vectors expressing Flag-tagged PRDM16 or a control vector. These cells were grown to confluence and induced to differentiate for 4 days. Nuclei were isolated by homogenizing cells in hypotonic solution (10 mM HEPES pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$). Nuclear proteins were extracted in hypertonic solution (20 mM HEPES pH 7.9, 400 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 20% glycerol) and dialysed against binding buffer (20 mM HEPES pH 7.9, 150 mM KCl, 0.2 mM EDTA, 20% glycerol). The PRDM16 complex was purified using Flag M2 agarose (Sigma), washed in the binding buffer (150 mM KCl), and eluted with 3 × Flag peptide (Sigma). The eluted materials were separated by SDS–PAGE and visualized by silver staining or Coomassie blue dye. Gel-resolved proteins were excised, digested with trypsin and analysed by matrix-assisted laser desorption/ionization– reflectron time of flight mass spectrometry (UltraFlex TOF/TOF; Bruker) as described[40].

To examine the interaction of PRDM16 with PPAR-α and PPAR-γ2, COS-7 cells were co-transfected with expression plasmids for PRDM16 and either Flag–PPAR-α or Flag–PPAR-γ2. Whole-cell extracts were immunoprecipitated with anti-rabbit PRDM16 or anti-Flag M2 (Sigma) overnight at 4 °C. Immunoprecipitates were washed three times with washing buffer (20 mM Tris-HCl, 200 mM NaCl, 10% glycerol, 2 mM EDTA, 0.1% NP-40, 0.1 mM PMSF), separated by SDS–PAGE, transferred to PVDF membrane and blotted with anti-Flag and anti-PRDM16 antibodies. For GST immunoaffinity assays, GST–PRDM16 fusion proteins were purified from bacteria and GST–PPAR-γ2 (full-length) was purified from baculovirus-infected SF9 cells on glutathione Sepharose beads as described before[44]. ^{35}S-labelled and *in vitro* translated PRDM16, SRC1 and PPAR-γ2 were prepared using the TNT Coupled Transcription/Translation System (Promega). Equal amounts of GST fusion proteins (2 μg) were incubated overnight at 4 °C with *in vitro* translated proteins in binding buffer (20 mM HEPES pH 7.7, 300 mM KCl, 2.5 mM $MgCl_2$, 0.05% NP40, 1 mM DTT, 10% glycerol). The Sepharose beads were washed five times with binding buffer. Bound proteins were separated by SDS–PAGE and analysed by autoradiography.

Reporter gene assays. COS-7 cells or *Pparg*$^{-/-}$ fibroblasts were transfected with 50 ng PPAR-γ2 or PPAR-α and 50 ng RXR-α or empty vector together with 250 ng PRDM16 expression plasmid or vector control and 100 ng of 3 × DR1-luciferase reporter in 12-well plates using Lipofectamine 2000 (Invitrogen). Cells were harvested 48 h after transfection and assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega). Rosiglitazone or WY-14643 (1 μM) was added to some wells for 24 h before harvesting cells. Firefly luciferase reporter gene measurements were normalized to *Renilla* luciferase activity.

Expression analysis and western blotting. Total RNA was isolated from cultured cells and tissue using Trizol (Invitrogen) or RNeasy columns (Qiagen) according to the manufacturer's instructions. Real-time PCR oligonucleotide sequences are provided in Supplementary Table 2. For western blot analysis, cells or tissues were lysed in RIPA buffer (0.5% NP-40, 0.1% SDS, 150 mM NaCl, 50 mM Tris-Cl (pH 7.5)). Proteins were separated by SDS–PAGE, transferred to PVDF membrane (Millipore) and probed with anti-UCP1 (Abcam), anti-PRDM16, anti-Cidea (Chemicon), and anti-β-actin (Sigma) antibodies.

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