doi:10.1038/nature08262

LETTERS

Initiation of myoblast to brown fat switch by a PRDM16–C/EBP-β transcriptional complex

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Brown adipose cells are specialized to dissipate chemical energy in the form of heat, as a physiological defence against cold and obesity[1]. PRDM16 (PR domain containing 16) is a 140 kDa zinc finger protein that robustly induces brown fat determination and differentiation[2]. Recent data suggests that brown fat cells arise *in vivo* from a *Myf5*-positive, myoblastic lineage by the action of PRDM16 (ref. 3); however, the molecular mechanisms responsible for this developmental switch is unclear. Here we show that PRDM16 forms a transcriptional complex with the active form of C/EBP-β (also known as LAP), acting as a critical molecular unit that controls the cell fate switch from myoblastic precursors to brown fat cells. Forced expression of PRDM16 and C/EBP-β is sufficient to induce a fully functional brown fat program in naive fibroblastic cells, including skin fibroblasts from mouse and man. Transplantation of fibroblasts expressing these two factors into mice gives rise to an ectopic fat pad with the morphological and biochemical characteristics of brown fat. Like endogenous brown fat, this synthetic brown fat tissue acts as a sink for glucose uptake, as determined by positron emission tomography with fluorodeoxyglucose. These data indicate that the PRDM16–C/EBP-β complex initiates brown fat formation from myoblastic precursors, and may provide opportunities for the development of new therapeutics for obesity and type-2 diabetes.

Because of the importance of brown adipose tissue (BAT) as a natural defence against hypothermia and obesity[1], and its demonstrated presence in adult humans[4–7], understanding its formation in mechanistic detail may open new avenues to the development of new therapeutics for metabolic diseases such as obesity and type-2 diabetes. Several transcriptional regulators have been identified that positively or negatively control BAT development including RB1 (ref. 8), p107 (also known as RBL1)[9], RIP140 (NRIP1)[10] and FOXC2 (ref. 11). Most recently, we have shown that PRDM16, a 140-kDa zinc finger protein, functions as a bidirectional switch in brown fat cell fate by stimulating the development of brown fat cells from white preadipocytes[12,13] and from *Myf5*-positive myoblastic precursors[3] *in vitro* and *in vivo*. At a molecular level, PRDM16 works as a transcriptional co-regulatory protein by co-activating PPARγ (peroxisome proliferator-activated receptor γ), which is considered the 'master' gene of fat cell differentiation[14,15], and this is almost certainly an important event in the conversion of myoblasts to brown adipocytes[3]. However, both isoforms of PPARγ are expressed at very low levels in primary and immortalized myoblasts, whereas they are abundantly expressed in white and brown preadipocytes (Supplementary Fig. 1). Hence, it is very likely that PRDM16 initiates the process of myoblast to brown fat conversion by complexing with other DNA-binding factors, well before the co-activation of PPARγ.

We therefore devised a strategy to address this, as illustrated in Fig. 1a. In brief, we performed proteomic analyses of transcriptional complexes formed with wild-type PRDM16 or different mutant alleles that were differentiation-competent or -incompetent. Transcription factors that co-purified preferentially with differentiation-competent PRDM16 proteins were identified; their expression in white and brown fat was then analysed and compared to that of PRDM16. Subsequently, we examined their function in the process of myoblast to brown fat conversion through PRDM16.

As shown in Fig. 1b, wild type PRDM16 and a mutant protein lacking the PR (PRD1-BF1-RIZ1 homologous) domain (ΔPR; amino acids 91–223) that shares homology to the SET chromatin remodelling domain[16,17], induced brown fat cell differentiation from myoblasts. In

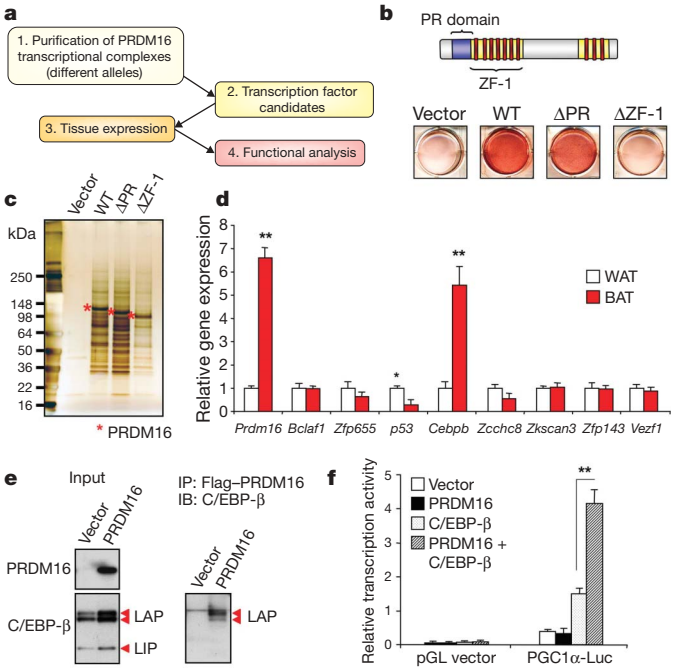

Figure 1 | Identification of C/EBP-β as a critical binding partner in the PRDM16 transcriptional complex. **a**, Strategy to identify key PRDM16 binding partners. **b**, C2C12 myoblasts expressing indicated viral vectors were stained with Oil Red O 6 days after inducing adipocyte differentiation. **c**, PRDM16 transcriptional complex was immunopurified from brown fat cells expressing full-length or deletion mutants of PRDM16. **d**, Gene expression of known or predicted transcription factors identified in the PRDM16 complex in BAT and WAT. $n = 6$. **e**, Endogenous C/EBP-β was detected in the PRDM16 complex by western blotting. Input is shown to the left. IB, immunoblot; IP, immunoprecipitate. **f**, Transcriptional activity of the *Pgc1a* promoter in response to PRDM16 and/or C/EBP-β. $n = 3$; all error bars are s.e.m.; $^*P < 0.05$, $^{**}P < 0.01$.

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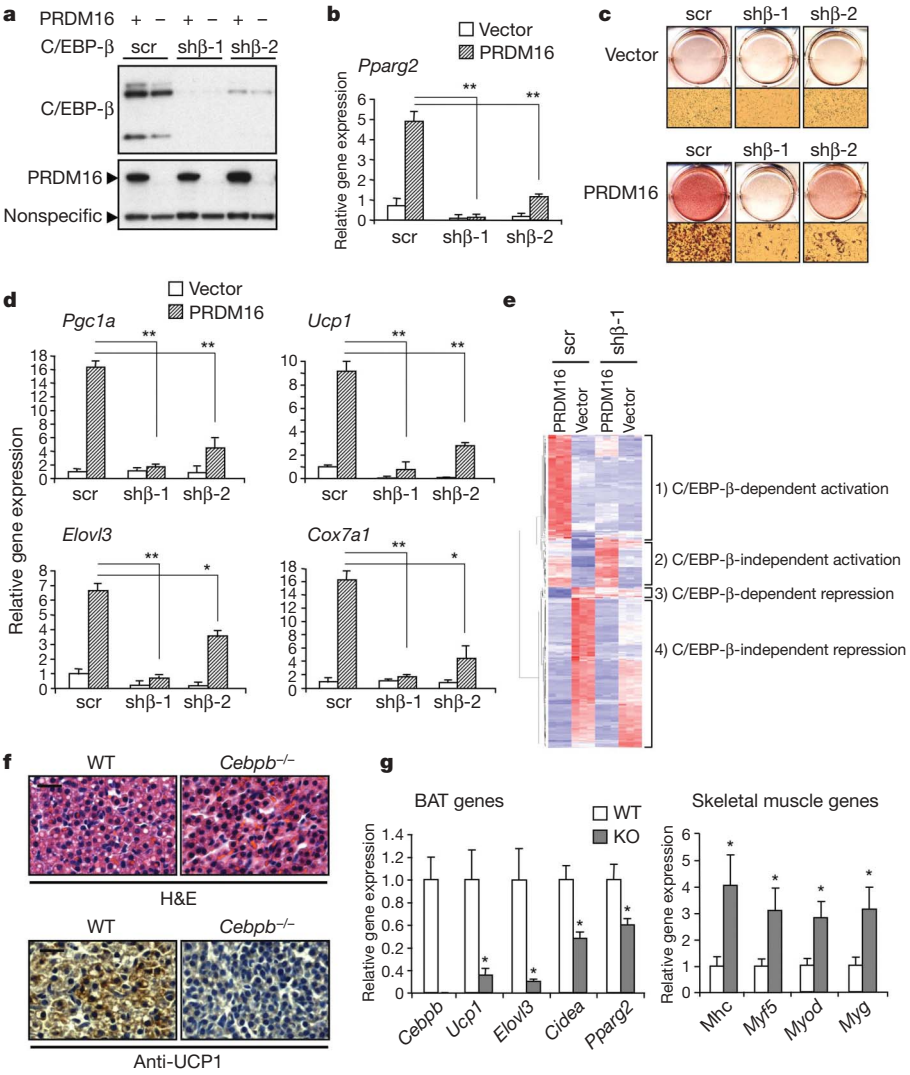

Figure 2 | C/EBP-β is required for initiation of the myoblast to brown fat conversion by PRDM16. **a**, Western blot analysis for C/EBP-β and PRDM16 in C2C12 myoblasts expressing scr, shβ-1 or shβ-2, with PRDM16 or vector. **b**, *Pparg2* gene expression. $n = 3$. **c**, The cells were stained with Oil Red O 6 days after inducing adipocyte differentiation. **d**, BAT-selective gene expression. $n = 4$. **e**, Microarray analysis of undifferentiated C2C12 myoblasts expressing scr or shβ-1 with PRDM16 or vector. $n = 3$. **f**, Top, haematoxylin and eosin (H&E) staining of BAT from wild-type (WT) and C/EBP-β knockout (KO) mice. Bottom, immunohistochemistry to detect UCP1 expression. Scale bars, 20 μm. **g**, mRNA expression of BAT and skeletal-muscle-selective genes in BAT from E17.5 embryos. $n = 5$–8; all error bars are s.e.m.; $^*P < 0.05$, $^{**}P < 0.01$.

contrast, a mutant allele lacking zinc finger domain-1 (ΔZF-1; amino acids 224–447) completely lost its adipogenic function. The brown fat gene program was also induced by both wild-type and ΔPR, but not by ΔZF-1 (Supplementary Fig. 2). To avoid comparing proteomic analyses of complexes from cells of very different phenotypes, we expressed all three PRDM16 forms in bona fide brown fat cells. PRDM16 complexes were then immunopurified to apparent homogeneity (Fig. 1c), and subjected to high-resolution 'shotgun' sequencing by liquid chromatography with tandem mass spectrometry (LC–MS/MS)[18]. In total, 49 proteins were identified in differentiation-competent PRDM16 complexes, but only eight of these (*Bclaf1*, *Zfp655*, *p53* (also known as *Trp53*), *Cebpb*, *Zcchc8*, *Zkscan3*, *Zfp143* and *Vezf1*) are known or predicted transcription factors (Supplementary Table 1). Because we have assumed that the expression of a key initiating transcription factor would not be extinguished during the brown fat cell adipogenesis, and as PRDM16 is highly enriched in BAT relative to white adipose tissue (WAT)[13], we asked whether any of these factors were similarly enriched in BAT. As shown in Fig. 1d, the expression of only *Cebpb* (C/EBP-β) was co-enriched with PRDM16 in BAT versus WAT. In addition, C/EBP-β protein was enriched in BAT, and further induced by cold exposure (Supplementary Fig. 3). Notably, both primary and immortalized myoblasts express C/EBP-β at similar levels to those seen in preadipocytes (Supplementary Fig. 4), where this factor is thought to have a very important role in adipogenesis[19,20]. Our analyses have therefore been focused on C/EBP-β and its function in complex with PRDM16.

Brown fat cells express three forms of C/EBP-β, two active forms, named LAP (liver-enriched transcriptional activator protein) and a dominant-negative form, LIP (liver-enriched transcriptional inhibitory protein)[21] (Fig. 1e, left). Notably, PRDM16 preferentially bound to LAP, but not to LIP (Fig. 1e, right and Supplementary Fig. 5). Independent co-expression assays in HEK293 cells confirmed the physical binding of PRDM16 and C/EBP-β. Furthermore, PRDM16 interacts with other C/EBP family members, C/EBP-α and -δ (Supplementary Fig. 6). This interaction is likely to be direct through the two zinc finger domains, because the zinc finger domains of the purified glutathione *S*-transferase (GST)-fused PRDM16 bound to *in vitro* translated C/EBP-β (Supplementary Fig. 7). We also addressed whether PRDM16 could affect the transcriptional activity of C/EBP-β. Because C/EBP-β is known to induce *Pgc1a* (also known as *Ppargc1a*) gene expression[22], we performed a luciferase reporter assay using the −2 kilobase (kb) *Pgc1a* promoter where the C/EBP-binding sites have been characterized[22]. As shown in Fig. 1f, the expression of PRDM16 and C/EBP-β synergistically stimulated *Pgc1a* promoter activity. These

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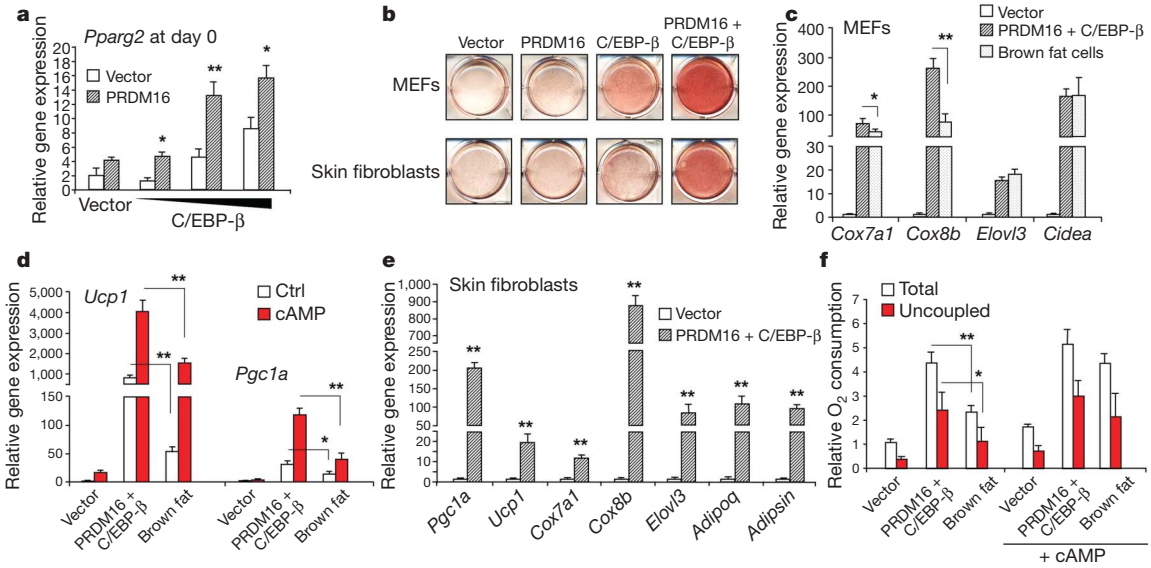

Figure 3 | Reconstitution of the brown fat gene program in fibroblasts by PRDM16 and C/EBP-β. **a**, *Pparg2* expression in undifferentiated MEFs expressing indicated viral vectors. $n = 3$. **b**, Immortalized MEFs or skin fibroblasts expressing indicated viral vectors were stained with Oil Red O 6–8 days after inducing adipocyte differentiation. **c**, BAT-selective gene expression. **d**, Thermogenic gene expression. The cells were treated with cAMP for 4 h. $n = 4$; ctrl, control. **e**, BAT-selective gene expression in primary skin fibroblasts expressing vector or PRDM16 and C/EBP-β. $n = 3$. *Adipsin* is also known as *Cfd*. **f**, Total and uncoupled cellular respiration in differentiated brown fat cells and the MEFs expressing vector or PRDM16 and C/EBP-β. The cells were treated with dibutyryl-cAMP for 12 h. $n = 3$; all error bars are s.e.m.; $*P < 0.05$, $**P < 0.01$.

data suggest that PRDM16 forms a transcriptional complex with active forms of C/EBP-β by direct interaction, and regulates their transcriptional activity.

To examine the functional role of the interaction between PRDM16 and C/EBP-β in the myoblast to brown fat conversion, retroviruses expressing a short hairpin (sh) scrambled control RNA (scr), or shRNAs targeting C/EBP-β (shβ-1 and shβ-2) were transduced together with PRDM16 or an empty vector into C2C12 myoblasts (Fig. 2a). As shown in Fig. 2b, knockdown of C/EBP-β significantly blunted the induction of *Pparg2* expression by PRDM16 in undifferentiated C2C12 myoblasts. Consistent with this result, Oil Red O staining showed that depletion of C/EBP-β blunted the adipogenesis induced by PRDM16 (Fig. 2c). Furthermore, induction of brown-fat-selective genes including *Pgc1a*, *Ucp1*, *Elovl3* and *Cox7a1* were completely or partially blocked by knockdown of C/EBP-β, correlating with the knockdown efficacy (Fig. 2d). In addition, we ectopically expressed LIP, a dominant-negative form of C/EBP-β, and this also significantly blunted PRDM16-induced adipogenesis and brown-fat-selective gene expression (Supplementary Fig. 8).

Next, we took a systematic approach to determine what fraction of the PRDM16-regulated genes requires C/EBP-β at the initiating step of the myoblast to brown fat conversion. To this end, RNAs from undifferentiated C2C12 myoblasts expressing PRDM16 or control together with scr or shβ-1, maintained under conditions non-permissive for differentiation, were subjected to Affymetrix microarray analysis. As shown in Fig. 2e, 316 genes were significantly increased or reduced by PRDM16 (>twofold, $P < 0.05$), which were clustered into four groups: (1) genes increased by PRDM16 in a C/EBP-β-dependent manner, (2) genes increased by PRDM16 in a C/EBP-β-independent manner, (3) genes repressed by PRDM16 in a C/EBP-β-dependent manner, and (4) genes repressed by PRDM16 in a C/EBP-β-independent manner. The expression of a subset of genes identified by microarray analyses was validated by PCR with reverse transcription (RT–PCR) (Supplementary Fig. 9). Notably, most genes activated by PRDM16 before differentiation (62 out of 95, 65.3%) indeed required C/EBP-β, whereas most of the repressed genes (210 out of 221, 95.0%) were not grossly altered by C/EBP-β depletion.

We explored further the genetic requirement for C/EBP-β in brown fat development by analysing C/EBP-β-deficient embryos. Defects in BAT of C/EBP-β-null newborn or adult mice have been described, although the reported phenotype was inconsistent[23,24]. Because a large number of these embryos died within the first 24 h after birth[23,25], we have re-examined this issue in late gestation (stage embryonic day (E)18.5). This should permit a clearer separation of developmental changes in the BAT, as opposed to those that might occur secondarily to abnormalities in other tissues after birth. As shown in Fig. 2f, haematoxylin and eosin staining showed that brown fat cells in knockout embryos contained significantly less lipid droplets than those in wild-type embryos, suggesting defects in brown fat development per se (Fig. 2f, top). Moreover, UCP1 expression was severely reduced in knockout embryos (Fig. 2f, bottom), consistent with the results described previously[23]. We also conducted a definitive molecular characterization of the BAT from wild-type and knockout embryos. Notably, BAT from C/EBP-β-knockout mice nearly phenocopied that from PRDM16-knockout mice at the gene expression level; that is, a broad reduction of BAT-selective gene expression, and a broad induction of the skeletal muscle gene expression (Fig. 2g). Together, these data indicate that the PRDM16–C/EBP-β transcriptional complex specifically has a critical role in the initiation of myoblast to brown fat switch. This strongly suggests that PRDM16 acts in *Myf5*-positive myoblastic precursors, at least in part, by co-activation of C/EBP-β to induce the expression of *Pparg* and *Pgc1a*. Subsequently, PRDM16 co-activates PPARγ and PGC-1α by direct binding events, which drives a complete brown fat differentiation program (Supplementary Fig. 10).

This mechanistic model suggests a critical question: are the two factors sufficient to reconstitute a brown fat program in naive cells? To this end, PRDM16 and C/EBP-β were ectopically expressed in mouse embryonic fibroblasts (MEFs) or primary skin fibroblasts with no inherent adipose or brown fat character. As shown in Fig. 3a, *Pparg2* messenger RNA expression was synergistically induced by PRDM16 and C/EBP-β in a dose-dependent manner in undifferentiated fibroblasts. After 6–8 days under adipogenic conditions, both MEFs and skin fibroblasts expressing these two factors uniformly differentiated into lipid-filled adipocytes, as shown by Oil Red O staining (Fig. 3b). The single factors alone were not sufficient

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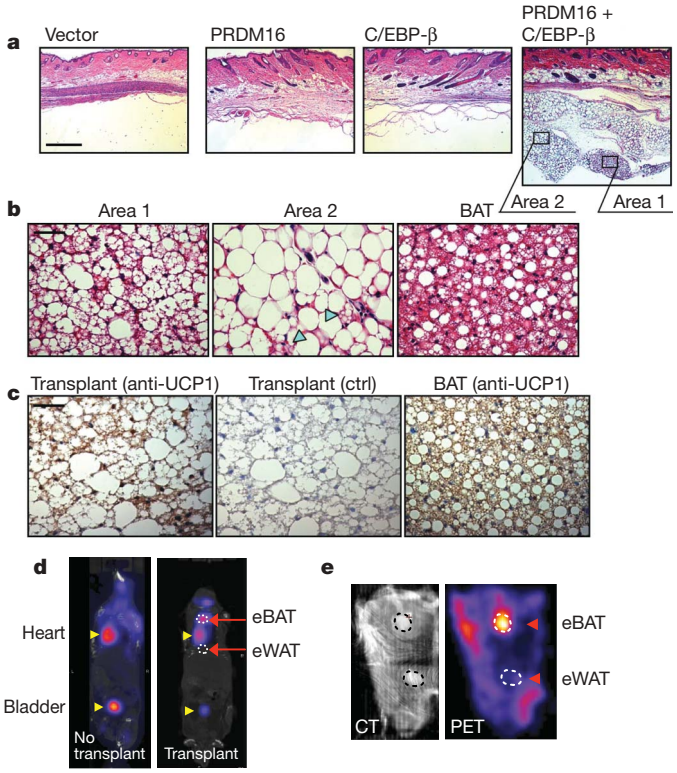

Figure 4 | Generation of functional brown adipose tissue *in vivo* by expression of PRDM16 and C/EBP-β. **a**, Fat pads from transplanted MEFs expressing indicated viral vectors were stained by H&E. Scale bar, 500 μm. **b**, High magnification images of H&E staining in the transplants expressing PRDM16 and C/EBP-β, and endogenous BAT. Arrowheads show multilocular fat cells. Scale bar, 50 μm. **c**, Immunohistochemistry to detect UCP1 expression in the transplant (left, anti-UCP1; middle, negative control) and BAT (right). Scale bar, 50 μm. **d**, PET/computed tomography image of mice with engineered BAT (eBAT) and engineered WAT (eWAT). **e**, Computed tomography (CT) image (left) and PET image (right) of mouse skin with the eBAT and eWAT.

to robustly stimulate the differentiated state. Gene expression studies showed that PRDM16 and C/EBP-β powerfully induced mRNA levels of brown fat genes including *Cox7a1* (70-fold), *Cox8b* (260-fold), *Elovl3* (16-fold) and *Cidea* (170-fold) to levels comparable with or even higher than those seen in bona fide immortalized brown fat cells (Fig. 3c). Notably, as in authentic brown fat cells, mRNA level of thermogenic genes such as *Pgc1a* and *Ucp1* were further enhanced by cyclic AMP treatment (Fig. 3d). The mechanism underlying the augmentation of cAMP effects in the engineered brown fat cells remains unknown. To our surprise, the mRNA levels of those genes at the basal state were activated to levels seen in cAMP-stimulated brown fat cells. Furthermore, the two factors were able to induce the brown fat gene program from primary mouse skin fibroblasts (Fig. 3e) and human skin fibroblasts isolated from newborn foreskin (Supplementary Fig. 11).

An important characteristic of brown fat cells is their extraordinarily high rates of respiration, particularly uncoupled respiration in response to cAMP. As shown in Fig. 3f, engineered brown fat cells induced by these two factors have significantly higher levels of total and uncoupled respiration than control cells, by 4.4- and 6.5-fold, respectively, at the basal state. It is notable that the engineered cells have greater basal respiration, both total and uncoupled, than bona fide brown fat cells. However, whereas the bona fide brown fat cells can increase both total and uncoupled respiration further (by 85% and 90%, respectively) in response to cAMP, engineered brown fat cells already seem to be at their maximal respiration. That these cells are responsive to cAMP is shown by the fact that expression of thermogenic genes, such as such as *Pgc1a* and *Ucp1*, are induced by cAMP treatment (Fig. 3d). Hence, some other aspect of the respiratory apparatus, unknown at this point, seems to be limiting in the engineered brown fat cells.

The finding that the combination of PRDM16 and C/EBP-β is sufficient to reconstitute a near complete brown fat program offers an opportunity for controlling brown fat levels and function *in vivo*. We conducted transplantation studies, as originally developed by Green and Kehinde[26], using undifferentiated MEFs expressing vector, PRDM16, C/EBP-β, or a combination of the two factors. As shown by haematoxylin and eosin staining (Fig. 4a), the cells expressing vector or PRDM16 or C/EBP-β alone did not form visible fat tissues. In contrast, the cells expressing both PRDM16 and C/EBP-β formed very distinct fat pads *in vivo*. Notably, at high magnification, the engineered fat tissue induced by the two factors contained 'multilocular' fat cells, a morphological characteristic of brown fat *in vivo* (Fig. 4b). The population of multilocular fat cells (area 1) is mixed with regions of 'unilocular' fat cells (area 2). Notably, immunohistochemical analyses showed that the engineered adipose tissue was UCP1-positive in both the multilocular and unilocular fat cells (Fig. 4c).

To characterize the activity of engineered brown fat tissue *in vivo* further, we used positron emission tomography (PET) with fluorodeoxyglucose (^{18}FDG), which has recently been used to detect active BAT in adult humans[4–7]. This technique measures glucose uptake, with brown fat functioning *in vivo* as an active 'sink' for glucose. To this end, we engineered two adipose tissues with similar sizes in the same nude mice: a 'brown' fat tissue induced by PRDM16 and C/EBP-β, and a 'white' fat tissue induced by PPARγ alone as a control (Supplementary Fig. 12a). The induction of BAT-selective genes by PRDM16 and C/EBP-β was confirmed in the cultured cells by RT–PCR (Supplementary Fig. 12b). As shown in Fig. 4d, PET scanning detected a signal in mice from the engineered BAT. To enhance the sensitivity and specificity of the PET signal from the engineered fat tissues, the skin with these fat tissues attached was removed and scanned. The combination of computed tomography image (Fig. 4e, left) and PET image (Fig. 4e, right) clearly showed that the PET signal was detected from the engineered BAT, but not from the engineered WAT. These results indicate that the engineered brown fat cells function as a sink for active glucose disposal. Given the incredible capacity of BAT to dissipate stored chemical energy and thus counteract obesity, we are optimistic that the PRDM16 pathway can be used to drive brown fat development *in vivo* in a therapeutic setting. Certainly natural or synthetic compounds that can induce PRDM16 in white fat precursors or in myoblastic cells could have great value in human metabolic disease. Alternatively, as shown here, autologous transplantation of engineered brown fat induced by PRDM16 and C/EBP-β in amounts that are both clinically acceptable and therapeutically useful may well be possible. Future experiments must define the optimal conditions to achieve maximal angiogenesis, innervation and resulting energy expenditure from autologous transplants.

METHODS SUMMARY

Cell culture. Immortalized brown fat cells have been described previously[27]. Mouse embryonic fibroblasts were isolated from E13.5 C57/Bl6 embryos, and immortalized according to the established methods[28]. R2F primary skin fibroblasts isolated from human newborn foreskin were a gift from J. G. Rheinwald and cultured following the methods described elsewhere[29]. Adipocyte differentiation in fibroblasts was induced with medium containing 5 μM dexamethasone, 850 nM insulin, 1 nM T3 and 1 μM rosiglitazone.

Identification of the PRDM16 transcriptional complex. Immortalized brown fat cells stably expressing Flag-tagged PRDM16 were homogenized to prepare nuclear extracts. The nuclear extracts were incubated with Flag M2 agarose (Sigma), washed in a binding buffer, and eluted by incubating with Flag peptide[12]. The immunoprecipitated proteins were digested by trypsin and subjected to reverse-phase LC–MS/MS, using a high-resolution hybrid mass spectrometer (LTQ-Orbitrap, Thermo Scientific) with TOP10 method[18].

Animal experiments. All animal experiments were performed according to procedures approved by Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. C/EBP-β-null mice (Cebpbtm1Vpo/J) were obtained from the Jackson Laboratory. For transplantation studies, immortalized

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MEFs, transduced with retroviral PRDM16 and/or C/EBP-β, were implanted subcutaneously into 7–9-week-old male nude mice (NCr-*Foxn1*^{nu})[13,26]. For PET scanning studies, MEFs expressing retroviral PPARγ were implanted as control. After 4–6 weeks, fat pads were dissected for histological analysis. MicroPET/computed tomography (CT) scanning was performed using the Mosaic HP microPET in conjunction with the microCT of the NanoSPECT/CT (Philips), 60 min after the injection of ^{18}FDG.

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

Received 1 June; accepted 13 July 2009.
Published online 29 July 2009.

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

Acknowledgements We are grateful to S. R. Farmer, J. Rheinwald and P. F. Johnson for providing cells and other reagents, R. Gupta for his critical comments on the manuscript, and J. Y. Choi and E. Naseri for their assistance. S.K. is supported by AHA scientist development grant (0930125N). P.S. is supported by a National Institutes of Health (NIH) grant (DK081605). This work was supported by grants from the Picower Foundation and the NIH (DK31405) to B.M.S., NIH HG3456 and GM67945 to S.P.G., and NIH/NCRR shared instrumentation grant S10-RR-023010.

Author Contributions S.K. and B.M.S. conceived and designed the experiments. S.K., K.K. and E.L. performed the experiments. All of the authors analysed the data. S.K. and B.M.S. wrote the paper.

Author Information Microarray data has been deposited in the Gene Expression Omnibus (GEO) public database under accession GSE15895. Reprints and permissions information is available at www.nature.com/reprints. Correspondence and requests for materials should be addressed to B.M.S. (bruce_spiegelman@dfci.harvard.edu.).

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METHODS

Cell culture. Immortalized brown fat cells have been described previously[27]. Mouse embryonic fibroblasts were isolated from E13.5 C57/Bl6 embryos (Jackson Laboratory), and immortalized according to the methods previously described[28]. Mouse dermal fibroblasts were obtained from Millipore. R2F primary skin fibroblasts isolated from human newborn foreskin were a gift from J. G. Rheinwald, and were cultured as per methods described elsewhere[29]. HEK293 cells and C2C12 cells were obtained from ATCC. Adipocyte differentiation in C2C12 cells was induced by treating confluent cells in DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine, 125 nM indomethacin, 5 μM dexamethasone, 850 nM insulin, 1 nM T3 and 1 μM rosiglitazone. Two days after induction, cells were switched to the maintenance medium containing 10% FBS, 850 nM insulin, 1 nM T3 and 1 μM rosiglitazone. Adipocyte differentiation in fibroblasts was induced with medium containing 5 μM dexamethosone, 850 nM insulin, 1 nM T3 and 1 μM rosiglitazone. For cAMP treatment, cells were incubated with 10 μM forskolin or 0.5 mM dibutyryl-cAMP. All chemicals for cell culture were obtained from Sigma unless otherwise indicated.

DNA constructs and viruses production. Deletion mutants of Flag-tagged PRDM16 were amplified by PCR using full-length PRDM16 as a template, and subcloned into pMSCV-puro retroviral vector (Stratagene). Various fragments of GST-fused PRDM16 fragments (1–223, 224–454, 455–680, 680–880, 881–1038 and 1039–1176) were described previously[12]. Myc-tagged C/EBP-β constructs[30] were gifts from S. R. Farmer. The sequences used for retroviral shRNA expression vectors targeting C/EBP-β were 5′-GCCCTGAGTAATCA CTTAAAG-3′ (shβ-1) and 5′-CCGGGCCCTGAGTAATCAC-3′ (shβ-2). The corresponding double-stranded DNA sequences were ligated into pSUPER-Retro (Oligoengine) for retroviral expression. For retrovirus production, Phoenix packaging cells[31] were transfected at 70% confluence by calcium phosphate method with 10 μg retroviral vectors. After 48 h, the viral supernatant was collected and filtered. Cells were incubated overnight with the viral supernatant, supplemented with 8 μg ml^{-1} polybrene. Subsequently, puromycin (PRDM16), hygromycin (C/EBP-β) or G418 (shRNAs) were used for selection. Fibroblasts expressing both PRDM16 and C/EBP-β were selected by puromycin and hygromycin to ensure expression of both constructs.

Affinity purification of PRDM16 transcriptional complex. Immortalized brown fat cells stably expressing Flag-tagged wild-type, PRΔ mutant, and ZF-1Δ mutant of PRDM16 or an empty vector were grown to confluence. The cells were homogenized to prepare nuclear extracts[12]. The nuclear extracts were incubated overnight with Flag M2 agarose (Sigma), washed in a binding buffer (180 mM KCl), and then eluted by incubating with 1× Flag peptide (0.2 mg ml^{-1}). The eluted materials were TCA precipitated, separated in a 4–20% acrylamide gradient gel, and visualized by silver staining, as described previously[12].

Mass spectrometry. The immunoprecipitated proteins were precipitated with methanol and chloroform, and precipitates were dissolved in 50 mM Tris-HCl, pH 7.5, containing 8 M urea, 50 mM EDTA and 0.005% *n*-dodecyl β-D-maltoside (DDM). Proteins were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide. After diluting urea concentration to 1 M with 50 mM Tris-HCl, pH 7.5, containing 0.005% DDM, trypsin was added and proteins were digested in solution at 37 °C for 12 h. The reaction was stopped with formic acid, and the resultant peptides were desalted with StageTips[32]. Desalted peptides were subjected to reverse-phase LC–MS/MS using a high-resolution hybrid mass spectrometer (LTQ-Orbitrap, Thermo Scientific) with TOP10 method, as described previously[18]. The obtained data were searched against the International Protein Index (IPI) mouse database[33]. Proteins were identified with at least two unique valid peptides, and the false discovery rate was estimated to be 0% using target-decoy approach[34].

Protein interaction analysis. HEK293 cells expressing PRDM16 or C/EBPs were collected 24 h after transfection. Total cell lysates were incubated overnight at 4 °C with Flag M2 agarose, washed and eluted with Flag peptide. The eluted materials were analysed by western blot using antibodies against C/EBP-α, C/EBP-β and C/EBP-δ (Santa Cruz). For *in vitro* binding assays, various fragments of the GST-fusion PRDM16 fragments were purified as described previously[12]. [^{35}S]-labelled proteins were made with a TNT reticulocyte lysate kit (Promega). Equal amounts of GST-fusion proteins (2 μg) were incubated overnight at 4 °C with *in vitro* translated proteins in a binding buffer containing 20 mM HEPES, pH 7.7, 300 mM KCl, 2.5 mM $MgCl_2$, 0.05% NP40, 1 mM DTT and 10% glycerol. The sepharose beads were then washed five times with the binding buffer. Bound proteins were separated by SDS–PAGE and analysed by autoradiography.

Gene expression analysis. Total RNA was isolated from cells or tissues using Trizol (Invitrogen). Reverse transcriptase reactions were performed using a cDNA reverse transcription kit (Applied Biosystems). The sequences of primers used in this study are found in Supplementary Table 2. Quantitative real-time PCR was performed with SYBR green fluorescent dye using an ABI9300 PCR machine. TATA-binding protein acted as an internal control.

Microarray analysis. Total RNA was isolated from undifferentiated C2C12 cells transduced with scr or shβ together with PRDM16 or vector control. 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[35]. The array data were analysed using the DNA-Chip Analyser software[36]. The statistical significance of differences in gene expression was assessed using an unpaired *t*-test ($P < 0.05$).

Reporter gene assay. The *PGC1A* (−2 kb) promoter linked to a luciferase reporter was transiently co-transfected with PRDM16 and/or C/EBP-β expression plasmids in brown preadipocytes using Lipofectamine 2000 (Invitrogen). Forty-eight hours after the transfection, cells were collected and reporter gene assays were carried out using the Dual Luciferase Kit (Promega). Transfection efficiency was normalized by measuring expression of *Renilla* luciferase.

Cellular respiration assay. Immortalized brown fat cells or MEFs transduced with retroviral PRDM16 and C/EBP-β or an empty vector were grown to confluence and induced to differentiate. At day 6 or 7 of differentiation, oxygen consumption was measured as described previously[12,13]. For cAMP-induced respiration assays, fully differentiated fat cells were incubated with 0.5 mM dibutyryl-cAMP for 12 h before measuring oxygen consumption.

Animals. All animal experiments were performed according to procedures approved by Beth Israel Deaconess Medical Center Institutional Animal Care and Use Committee. C/EBP-β-null mice (Cebpbtm1Vpo/J) were obtained from the Jackson Laboratory. For transplantation studies, male NCR-nude mice (NCr-*Foxn1^{nu}*) were obtained from Taconic.

Cell transplantations. Immortalized MEFs (3×10^7) were transduced with retroviral PRDM16, C/EBP-β, vector control, or a combination of PRDM16 and C/EBP-β, and implanted subcutaneously into 7–9-week-old male nude mice ($n = 6$ mice per group), according to the methods described previously[13,26]. For PET scanning studies, MEFs expressing retroviral PPARγ alone were implanted as a control. After 4–6 weeks, fat pads were carefully dissected and fixed in 4% paraformaldehyde for histological analysis. For immunohistochemistry, paraffin-embedded sections were incubated with anti-UCP1 antibody (Chemicon), followed by detection using the ABC Vectastain-Elite kit (Vector Labs) according to the manufacturer's instructions.

PET/CT imaging. ^{18}FDG (100 μCi) was injected intravenously to animals acclimated for at least 48 h to room temperature. Animals were imaged or euthanized at 1 h after injection in the Longwood small animal imaging facility of Harvard Medical School. PET/CT imaging was performed using a Minerve anaesthesia bed moved between a Philips Mosaic HP small animal scanner and a Bioscan CT scanner, and co-registered using custom fiducial markers. The acquired data was reconstructed by InVivoScope software (Bioscan).

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