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Initiation of myoblast/brown fat switch through a PRDM16-C/EBP- β transcriptional complex

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Abstract

Brown adipose cells are specialized to dissipate chemical energy in the form of heat, as a physiological defense against cold and obesity¹. PRDM16 (PRD1-BF1-RIZ1 homologous domain containing 16) is a 140 kDa zinc finger protein that robustly induces brown fat determination and differentiation². Recent data suggests that brown fat cells arise *in vivo* from a *myf5*-positive, myoblastic lineage through the action of PRDM16³; 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 (LAP), serving 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 naïve 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. As with endogenous brown fat, this synthetic brown fat tissue serves as a sink for glucose uptake, as determined by ¹⁸F-DG-PET scanning. These data indicate that the PRDM16-C/EBP- β complex initiates brown fat development from myoblastic precursors, and may provide opportunities for the development of novel therapeutics for obesity and type-2 diabetes.

Because of the importance of brown adipose tissue (BAT) as a natural defense against hypothermia and obesity¹, and its demonstrated presence in adult humans^{4–7}, understanding the formation of this cell type in mechanistic detail may open new avenues to the development of novel classes of 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 Rb8, p1079, RIP14010 and FoxC211. Most

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recently, we have shown that PRDM16, a 140 kDa zinc finger protein, functions as a bi-directional switch in brown fat cell fate by stimulating the development of brown fat cells from white preadipocytes^{12,13} and from *myf5*-positive myoblastic precursors³ *in vitro* and *in vivo*. At a molecular level, PRDM16 works as a transcriptional coregulatory protein by coactivating PPAR- γ (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 myoblast to brown adipocyte conversion³. However, both isoforms of PPAR- γ are expressed at very low levels in both primary and immortalized myoblasts, while 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 coactivation of PPAR- γ .

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 analyzed and compared to that of PRDM16. Subsequently, we examined their function in the process of myoblast-brown fat conversion through PRDM16.

As shown in Fig. 1b, wild type (WT) and a mutant protein lacking the PR (*PRD1-BF-1-RIZ1* homologous)-domain (PR; a.a.91–223), sharing high homology with a SET domain^{16,17}, induced brown fat cell differentiation from myoblasts. In contrast, a mutant allele lacking zinc finger domain-1 (ZF-1; a.a. 224–447) completely lost its adipogenic function. The brown fat gene program was also induced by both WT and PR, but not by ZF-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 8 of these (*Bclaf1*, *Zfp655*, *p53*, *C/ebp- β* , *Zcchc8*, *Zkscan3*, *Zfp143* and *Vezf1*) are known or predicted transcription factors (Supplementary Table 1). Since we have assumed that the expression of a key *initiating* transcription factor would not be extinguished during the brown fat cell adipogenesis, and 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 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). Importantly, 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 play 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.

Supplementary Information contains Supplementary Figures 1–12 and Tables 1–2. Microarray data has been deposited in Gene Expression Omnibus (GEO): GSE15895.

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). Interestingly, 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 . In addition, 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). Lastly, we asked if PRDM16 could affect the transcriptional activity of C/EBP- β . Since C/EBP- β is known to induce PGC-1 α gene expression²², we performed a luciferase reporter assay using the -2kb *PGC-1 α* promoter where the C/EBP binding sites have been characterized²². As shown in Fig. 1f, the expression of PRDM16 and C/EBP- β synergistically stimulated *PGC-1 α* promoter activity. These data suggest that PRDM16 forms a transcriptional complex with active forms of C/EBP- β through direct interaction, and regulates its transcriptional activity.

To examine the functional role of the interaction between PRDM16 and C/EBP- β in the myoblast-brown fat conversion, retroviruses expressing a short hairpin (sh) scrambled control RNA (scr) or shRNAs targeting C/EBP- β (sh β -1 and -2) were transduced together with PRDM16 or an empty vector to C2C12 myoblasts (Fig. 2a). As shown in Fig. 2b, knock-down of C/EBP- β significantly blunted the induction of *Ppar γ 2* 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 *Pgc-1 α* , *Ucp1*, *Elovl3* and *Cox7a1* were completely or partially blocked by knock-down of C/EBP- β , correlating with the knock-down 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 ask what fraction of the PRDM16-regulated genes require C/EBP- β at the *initiating* step of the myoblast-brown fat conversion. To this end, RNAs from undifferentiated C2C12 myoblasts expressing PRDM16 or control together with scr or sh-C/EBP- β , maintained under conditions non-permissive for differentiation, were subjected to Affymetrix microarray analysis. As shown in Fig. 2e, 316 genes were significantly elevated or reduced by PRDM16 (>2-fold, $P < 0.05$), which were clustered into 4 groups: 1) genes elevated by PRDM16 in a C/EBP- β dependent manner; 2) genes elevated 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 RT-PCR (Supplementary Fig. 9). Strikingly, the majority of genes activated by PRDM16 before differentiation (62/95, 65.3%) indeed required C/EBP- β , whereas most of the repressed genes (210/221, 95.0%) were not grossly altered by C/EBP- β depletion.

We further explored the genetic requirement for C/EBP- β in brown fat development by analyzing C/EBP- β deficient (KO) embryos. Defects in BAT of C/EBP- β null newborn or

adult mice have been described, although the reported phenotype was inconsistent^{23,24}. Since a large number of these embryos died within first 24 h after birth^{23,25}, we have re-examined this issue in late gestation (stage E18.5). This should permit a clearer separation of developmental changes in the BAT, as opposed to those that might occur secondary to abnormalities in other tissues after birth. As shown in Fig. 2f, haematoxylin and eosin (H&E) staining showed that brown fat cells in KO embryos contained significantly less lipid droplets compared with those in WT, suggesting a defects in brown fat development *per se* (Fig. 2f, top). Moreover, UCPI expression was severely reduced in KO embryos (Fig. 2f, bottom), consistent with the results of Tanaka *et al*²³. We also conducted a definitive molecular characterization of the BAT from WT and KO embryos. Remarkably, BAT from C/EBP- β KO mice nearly phenocopied that from PRDM16 KO mice at the gene expression level; *i.e.* 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 plays a critical role in the initiation of myoblast-brown fat switch. This strongly suggests that PRDM16 acts in *myf5*-positive myoblastic precursors, at least in part, by coactivation of C/EBP- β to induce the expression of PPAR γ and PGC-1 α . Subsequently, PRDM16 coactivates PPAR γ and PGC-1 α through 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 naïve 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, *Ppar γ 2* mRNA 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 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). Importantly, as in authentic brown fat cells, mRNA level of thermogenic genes such as *Pgc-1 α* and *Ucp1* were further enhanced by cAMP treatment (Fig. 3d). The mechanism underlying the augmentation of cAMP effects in the engineered brown fat cells remains unknown. To our surprise, mRNA level of those genes at the basal state were activated to levels seen in cAMP-stimulated brown fat cells. Lastly, 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, while the *bona-fide* brown fat cells can further increase both total and uncoupled respiration (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 *Pgc-1 α* 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 Kehinde²⁶, using undifferentiated MEFs expressing either: vector, PRDM16, C/EBP- β or combination of the two factors. As shown in hematoxylin and eosin staining (Fig. 4a), the cells expressing vector nor either 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). Importantly, immunohistochemical analyses showed that the engineered adipose tissue was UCP1 positive both in the multilocular and unilocular fat cells (Fig. 4c).

To further characterize the activity of engineered brown fat tissue *in vivo*, we utilized positron emission tomography (PET) with fluorodeoxyglucose (¹⁸FDG), 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 PPAR γ alone as a control (Supplementary Fig. 12a). Induction of BAT-selective genes by PRDM16 and C/EBP- β were 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 were removed and scanned. The combination of computed tomography (CT) 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 previously²⁷. Mouse embryonic fibroblasts were isolated from E13.5 C57/B16 embryos, and immortalized according to the established methods²⁸. R2F primary skin fibroblasts isolated from human newborn foreskin were a kind gift from Dr. J.G. Rheinwald (Harvard Medical School) and cultured following the methods described elsewhere²⁹. Adipocyte differentiation in fibroblasts was induced with medium containing 5 μ M dexamethosone, 850 nM insulin, 1 nM T3 and 1 μ M 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 peptide¹². The immunoprecipitated proteins were digested by trypsin and subjected to reverse-phase liquid chromatography with tandem mass spectrometry (LC-MS/MS), using a high resolution hybrid mass spectrometer (LTQ-Orbitrap, Thermo Scientific) with TOP10 method¹⁸.

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 MEFs, transduced with retroviral PRDM16 and/or *C/EBP- β* , were implanted subcutaneously into 7–9 week old male nude mice (*NCr-Foxn1^{tmu}*)^{13,26}. For PET scanning studies, MEFs expressing retroviral PPAR γ were implanted as control. After 4–6 weeks, fat pads were dissected for histological analysis. MicroPET/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 ¹⁸FDG.

Full Materials and Methods

Cell Culture

Immortalized brown fat cells have been described previously²⁷. Mouse embryonic fibroblasts were isolated from E13.5 C57/B16 embryos (Jackson Laboratory), and immortalized according to the methods established by Todaro and Green²⁸. Mouse dermal fibroblasts were obtained from Millipore. R2F primary skin fibroblasts isolated from human newborn foreskin were kind gift from Dr. J.G. Rheinwald (Harvard Medical School) and cultured as per methods described elsewhere²⁹. 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 μ M dexamethasone, 850 nM insulin, 1 nM T3, and 1 μ M rosiglitazone. Two days after induction, cells were switched to the maintenance medium containing 10% FBS, 850 nM insulin, 1 nM T3, 1 μ M rosiglitazone. Adipocyte differentiation in fibroblasts was induced with medium containing 5 μ M dexamethosone, 850 nM insulin, 1 nM T3 and 1 μ M

rosiglitazone. For cAMP treatment, cells were incubated with 10 μ M forskolin or 0.5 mM dibutyryl cyclic AMP. 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 previously¹². Myc-tagged C/EBP- β constructs³¹ were kind gifts from Dr. S.R. Farmer (Boston University). The sequences used for retroviral shRNA expression vectors targeting C/EBP- β were 5'-GCC CTG AGT AAT CAC TTA AAG-3' (sh β -1) and 5'-CCG GGC CCT GAG TAA TCA C-3' (sh β -2). The corresponding double-stranded DNA sequences were ligated into **pSUPER-Retro** (Oligoengine) for retroviral expression. For retrovirus production, Phoenix packaging cells³² were transfected at 70% confluence by calcium phosphate method with 10 μ g retroviral vectors. After 48 hours, the viral supernatant was harvested and filtered. Cells were incubated overnight with the viral supernatant, supplemented with 8 μ g/ml 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, PR-domain deletion mutant, and ZF-1 deletion mutant of PRDM16 or an empty vector were grown to confluence. The cells were homogenized to prepare nuclear extracts¹². 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 \times flag peptide (0.2 mg/ml). The eluted materials were TCA precipitated, separated in a 4%–20% acrylamide gradient gel and visualized by silver stain, as described previously¹².

Mass spectrometry

The immunoprecipitated proteins were precipitated with methanol/chloroform, and precipitates were dissolved in 50 mM Tris-Cl, pH 7.5, containing 8 M urea, 50 mM EDTA and 0.005% n-dodecyl β -D-maltoside (DM). Proteins were reduced with dithiothreitol (DTT) and alkylated with iodoacetamide. After diluting urea concentration to 1 M with 50 mM Tris-Cl, pH 7.5, containing 0.005% DM, trypsin was added and proteins were digested in solution at 37°C for 12 hours. Reaction was stopped with formic acid (FA), and the resultant peptides were desalted with StageTips³³. Desalted peptides were subjected to reverse-phase liquid chromatography with tandem mass spectrometry (LC-MS/MS) using a high resolution hybrid mass spectrometer (LTQ-Orbitrap, Thermo Scientific) with TOP10 method, as described previously¹⁸. The obtained data were searched against IPI mouse database³⁰. Proteins were identified with at least two unique valid peptides, and the false discovery rate was estimated to be 0% using target-decoy approach³⁴.

Protein interaction analysis—HEK293 cells expressing PRDM16 or C/EBPs were harvested 24 hours 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 analyzed by Western blot using antibodies for 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 previously¹². [³⁵S]-labeled proteins were made with a TNT reticulocyte lysate kit (Promega). Equal amounts of GST fusion proteins (2 μ g) were incubated overnight at 4°C with *in vitro* translated proteins in a binding buffer containing 20mM HEPES pH 7.7, 300 mM KCl, 2.5 mM MgCl₂, 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 analyzed by autoradiography.

Gene expression analysis

Total RNA was isolated from cells or tissues using Trizol (Invitrogen). Reverse transcriptase reactions were performed using 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 (TBP) served as an internal control.

Microarray analysis

Total RNA was isolated from undifferentiated C2C12 cells transduced with scr or sh-CEBP- β , 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 methods³⁵. The array data were analyzed using the DNA-Chip Analyzer (dChip) software³⁶. The statistical significance of differences in gene expression was assessed by unpaired t-test ($p < 0.05$).

Reporter gene assay

PGC-1 α (-2kb) promoter linked to a luciferase reporter was transiently cotransfected with PRDM16 and/or C/EBP- β expression plasmids in brown preadipocytes using Lipofectamine 2000 (Invitrogen). Forty-eight hours after the transfection, cells were harvested 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 cyclic AMP for 12 hours prior to measuring oxygen consumption.

Animals

All animal experiments were performed according to procedures approved by 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-*Foxn1tm*) were obtained from Taconic.

Cell transplantations

Immortalized MEFs (3×10^7) were transduced with retroviral PRDM16, C/EBP- β , vector control, or combination of PRDM16 and C/EBP- β , and implanted subcutaneously into 7–9 weeks old male nude mice (n=6 mice/group), according to the methods described previously^{13,26}. For PET scanning studies, MEFs expressing retroviral PPAR- γ 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 manufacturer's instructions.

PET/CT imaging

¹⁸FDG (100 μ Ci) was injected intravenously to animals acclimated for at least 48 hours to a room temperature. Animals were imaged or sacrificed at 1 h post-injection in the Longwood small animal imaging facility of Harvard medical school. PET/CT imaging was performed using a Minerve anesthesia 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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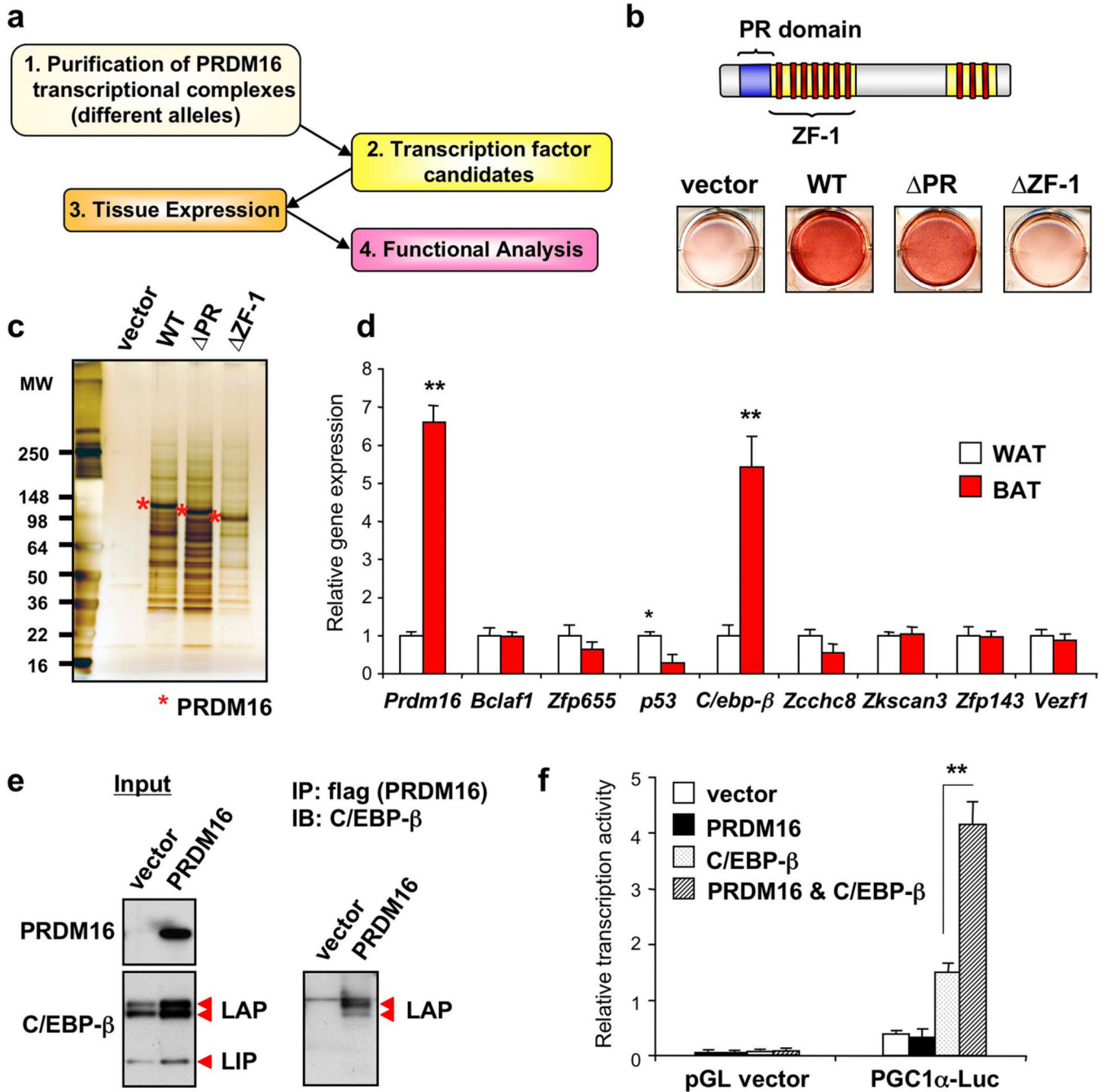


Figure 1. Identification of C/EBP- β as a critical binding partner in the PRDM16 transcriptional complex

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 was shown in left. **f**, Transcriptional activity of *PGC-1 α* promoter in response to PRDM16 and/or C/EBP- β . n=3; error bars are s.e.m.; * P <0.05, ** P <0.01.

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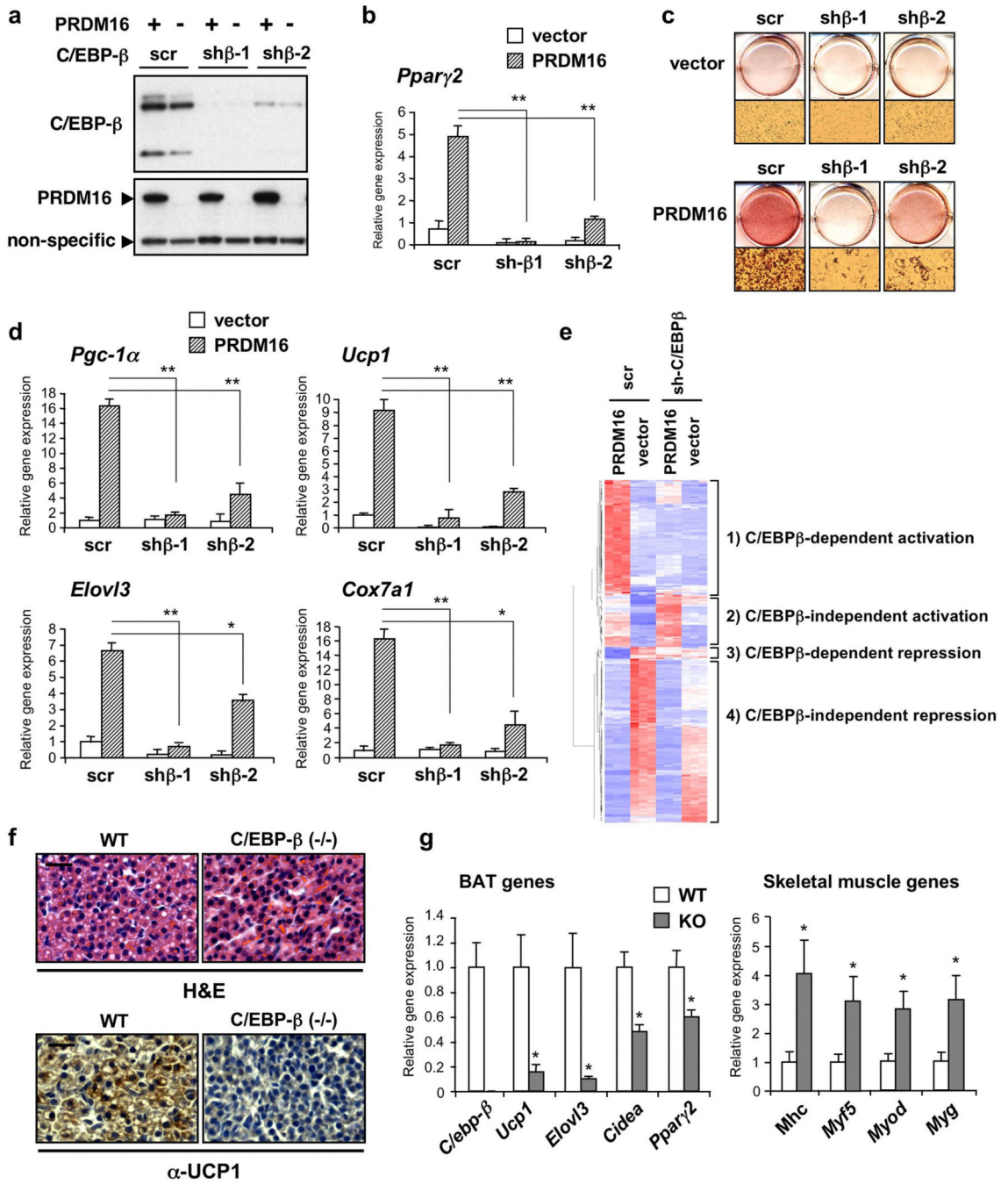


Figure 2. C/EBP-β is required for the initiation of the myoblast to brown fat conversion through PRDM16

a, Western blot analysis for C/EBP-β and PRDM16 in C2C12 myoblasts expressing scr or sh-C/EBP-β with PRDM16 or vector. **b**, *Pparg2* gene expression. n=3. **c**, These 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 sh-C/EBP-β with PRDM16 or vector. n=3. **f**, Top: H&E staining of BAT from WT and C/EBP-β KO mice. Bottom: Immunohistochemistry to detect UCP1

expression. Scale bar, 20 μm . **g**, mRNA expression of BAT and skeletal-muscle-selective genes in BAT from E17.5 embryos. $n=5-8$; error bars are s.e.m.; * $P<0.05$, ** $P<0.01$.

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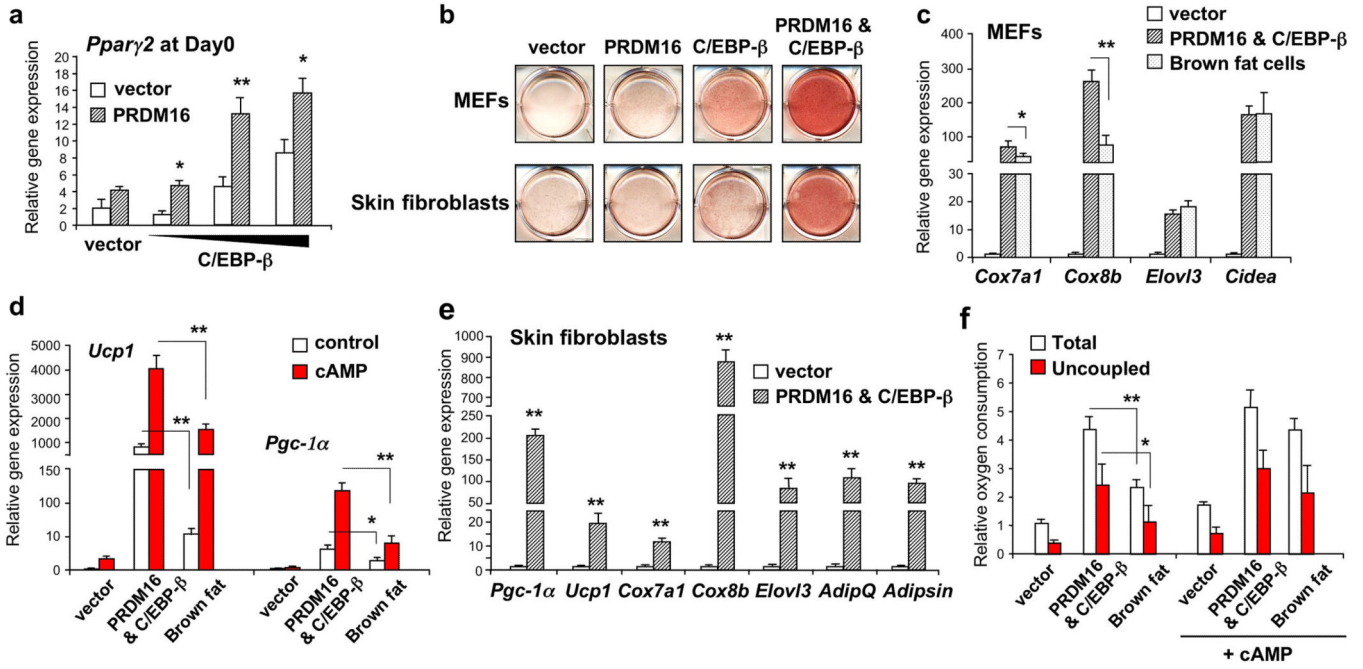


Figure 3. Reconstitution of the brown fat gene program in fibroblasts through PRDM16 and C/EBP-β

a, *Pparγ2* 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 hours. n=4. **e**, BAT-selective gene expression in primary skin fibroblasts expressing vector or PRDM16 and C/EBP-β. n=3. **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 dbcAMP for 12 hours. n=3; error bars are s.e.m.; **P*<0.05. ***P*<0.01.

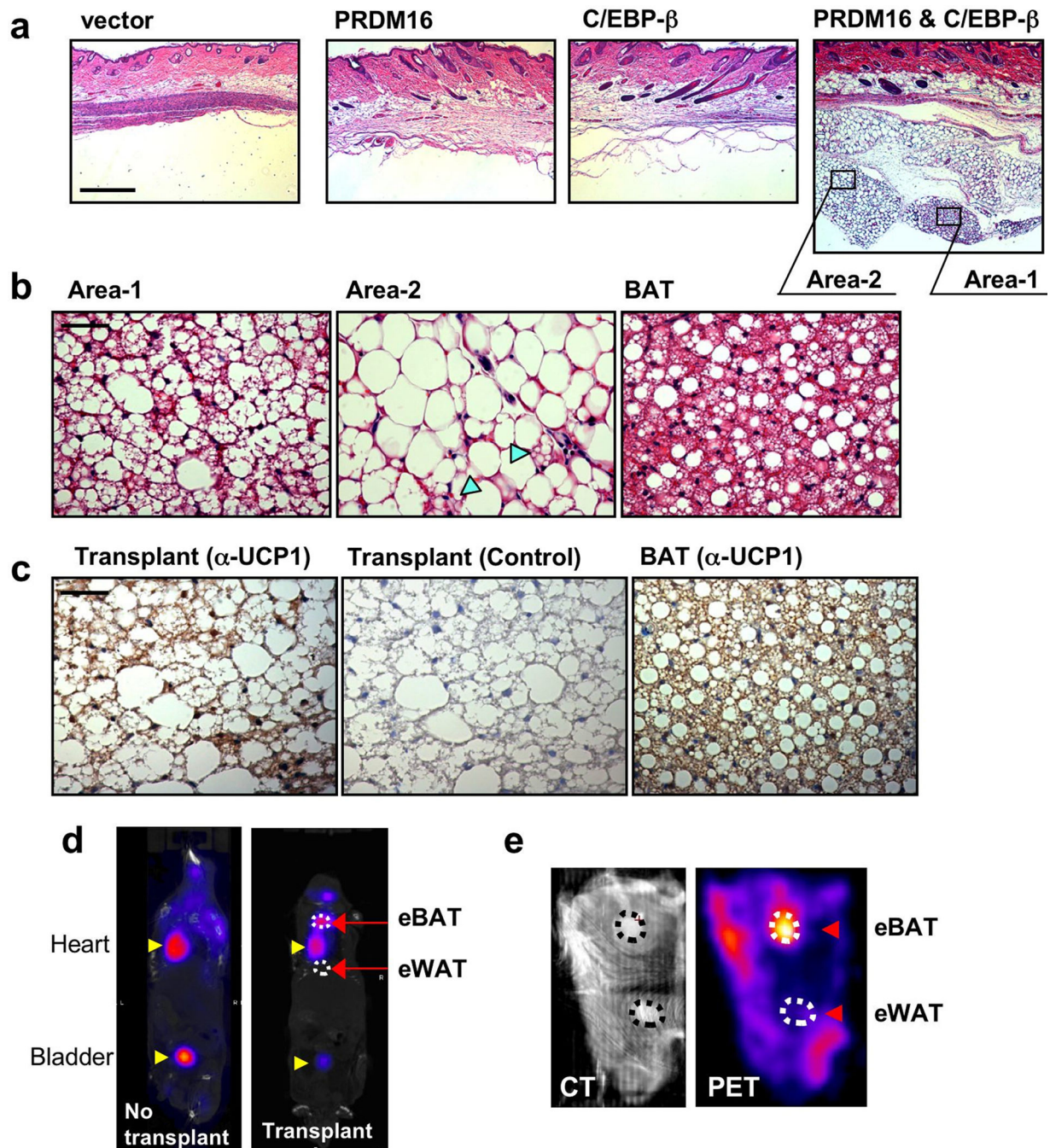


Figure 4. Generation of functional brown adipose tissue *in vivo* through expression of PRDM16 and C/EBP- β

a, Fat pads from transplanted MEFs expressing indicated viral vectors were stained by H&E. Scale bar, 500 μ m. **b**, High magnification images of H&E staining in the transplants expressing PRDM16 and C/EBP- β , and endogenous BAT. Arrow-heads show multilocular fat cells. Scale bar, 50 μ m. **c**, Immunohistochemistry to detect UCP1 expression in the transplant (left, anti-UCP1; middle, negative control) and BAT (right). Scale bar, 50 μ m. **d**,

PET/CT image of mice with engineered BAT (eBAT) and engineered WAT (eWAT). **e**, CT image (left) and PET image (right) of mouse skin with the eBAT and eWAT.

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