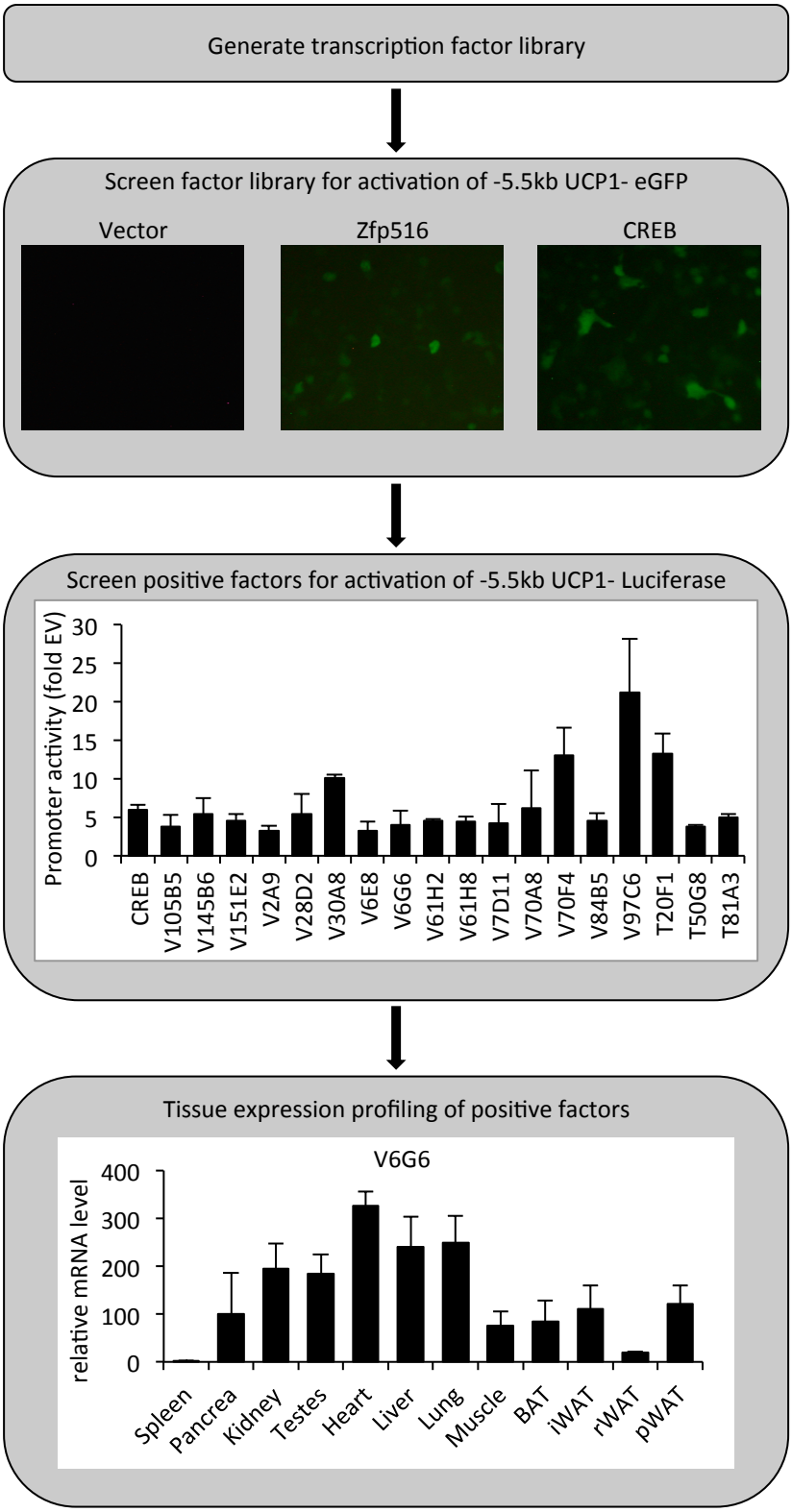
Figure S1



Final Positives:
Zfp516
Phf2
Klf10
Zc3h10
Nrf1
Klf15

Figure S1. Schematic representation of the screening of transcription factors for activation of the UCP1 promoter, related to figure 1. Top, 293FT cells were transfected with -5.5kB UCP1-eGFP and the indicated vectors. GFP fluorescence was assessed 48 hours post transfection. Middle, 293FT cells were transfected with -5.5kB UCP1-Luc and the indicated vectors and assayed at 48 hours post transfection. Positives from screening effort are shown (n=4). Bottom, RT-qPCR for V6G6 from the indicated tissues from C57BL/6 mice. For screening purposes, this is an example of a negative gene (n=3). Data shown are mean +/- SEM.

Figure S2

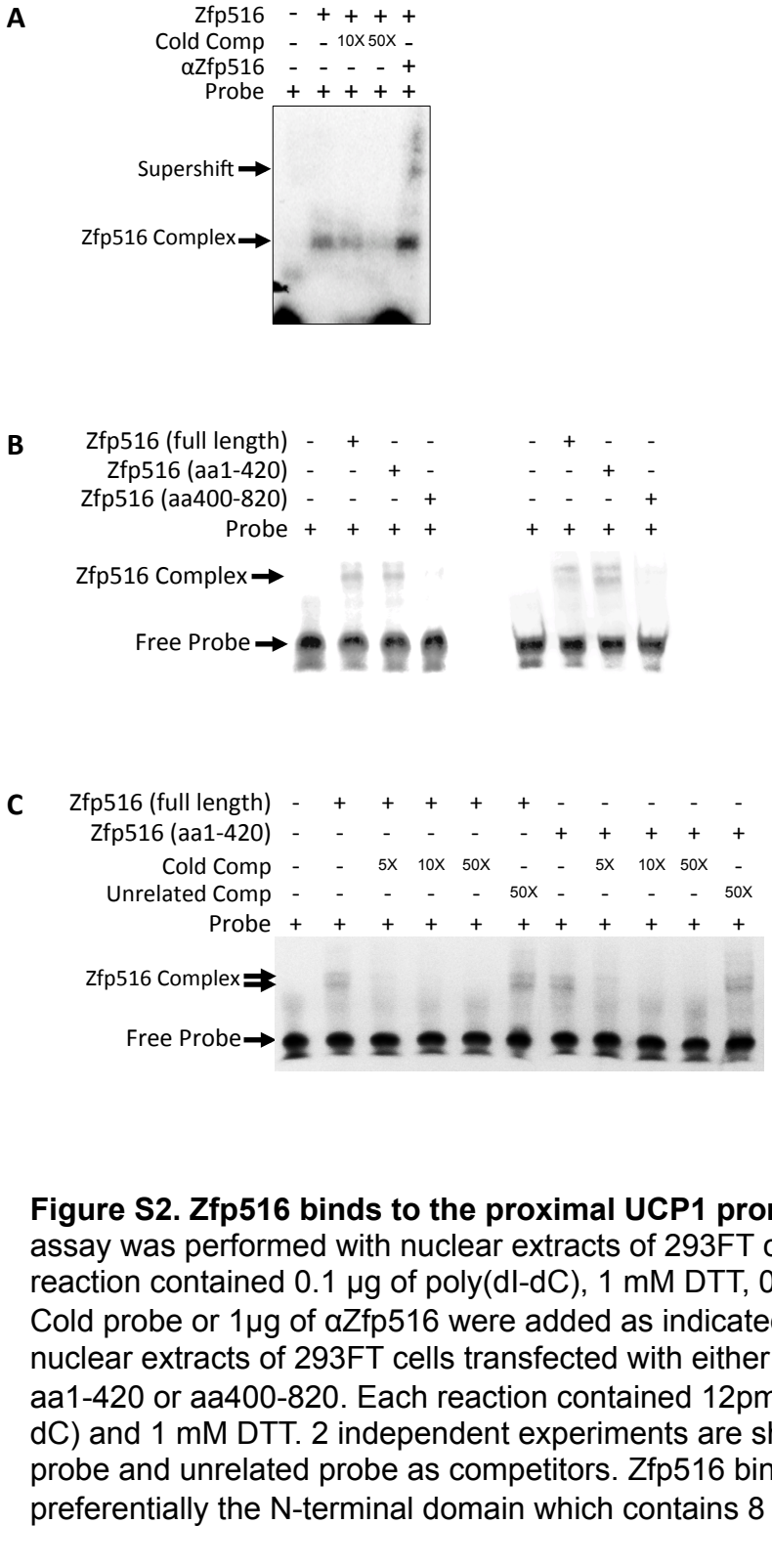


Figure S2. Zfp516 binds to the proximal UCP1 promoter, related to figure 1. A. Gel shift assay was performed with nuclear extracts of 293FT cells transfected with flag-Zfp516. Each reaction contained 0.1 µg of poly(dI-dC), 1 mM DTT, 0.1 ng of ^{32}P-labeled oligonucleotide probe. Cold probe or 1µg of αZfp516 were added as indicated. B. Gel shift assay was performed with nuclear extracts of 293FT cells transfected with either the full length Zfp516 or truncations aa1-420 or aa400-820. Each reaction contained 12pmol of biotin labelled probe, 0.1µg of poly(dI-dC) and 1 mM DTT. 2 independent experiments are shown. C. Gel shift assays as in B with cold probe and unrelated probe as competitors. Zfp516 binds to the proximal UCP1 promoter preferentially the N-terminal domain which contains 8 of the 10 total Zinc fingers.

Figure S3

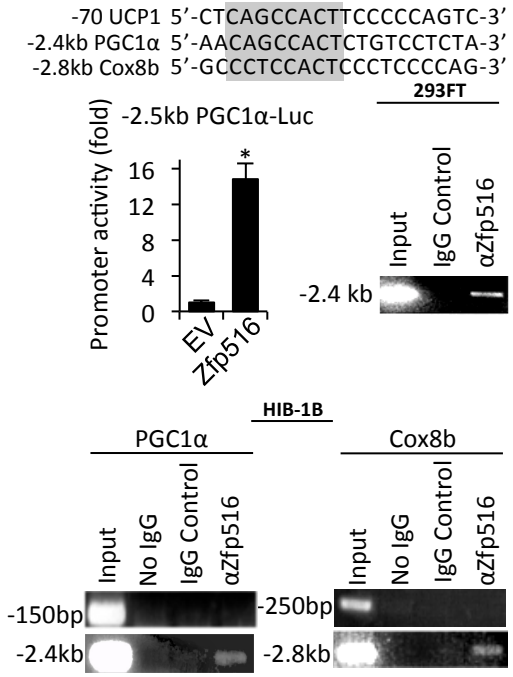


Figure S3. Zfp516 binds to PGC1α and Cox8b promoters for activation, related to figure 1. Top, sequence alignment of identified Zfp516 cis element in the UCP1 promoter to select regions of other BAT promoters with highlighted putative Zfp516 response element. Middle-left, relative luciferase activity of 293FT cells transfected with -2.5kb PGC1α- Luc with Zfp516 or vector control (n=6). Data shown are mean +/- SEM. Middle-right, ChIP for Zfp516 association to the PGC1α promoter in 293FT cells cotransfected with -2.5kb PGC1α-Luc and Zfp516 or vector control. Bottom-left, ChIP for Zfp516 association to the PGC1α promoter in HIB-1B cells. Bottom-right, ChIP for Zfp516 association to the Cox8b promoter in HIB-1B cells. Values are mean ± SEM and are normalized to the empty vector control. *$p<0.05$; **$p<0.01$. The Zfp516 binding site in UCP1 shares high sequence similarity with regions in the PGC1α and Cox8b promoters. ChIP analysis of the 293FT and HIB-1B cells transfected with the promoter-reporter and Zfp516 revealed binding of Zfp516 at the -2.5kb promoter region of the PGC1α and at the -2.8kb promoter region of Cox8b.Cotransfection of Zfp516 with the -2.5kb PGC1α promoter-luciferase construct into 293FT cells resulted in a 15-fold activation of the PGC1α promoter. This indicates that Zfp516 binds and activates the PGC1α and Cox8b promoters.

Figure S4

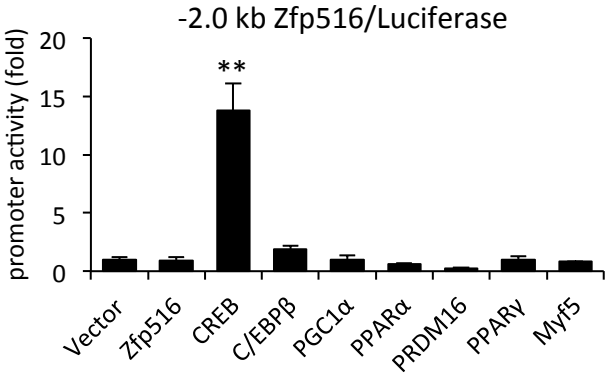
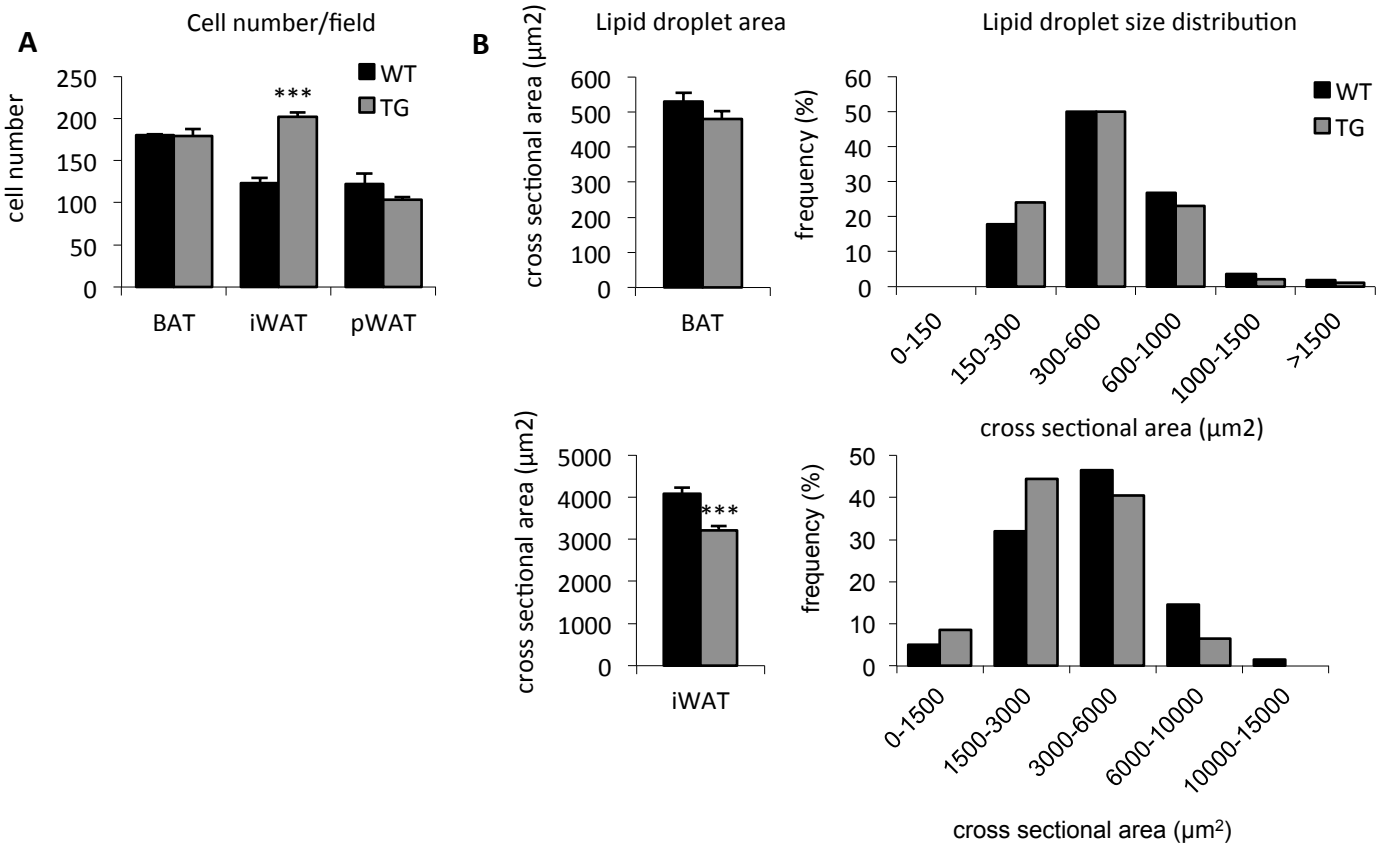
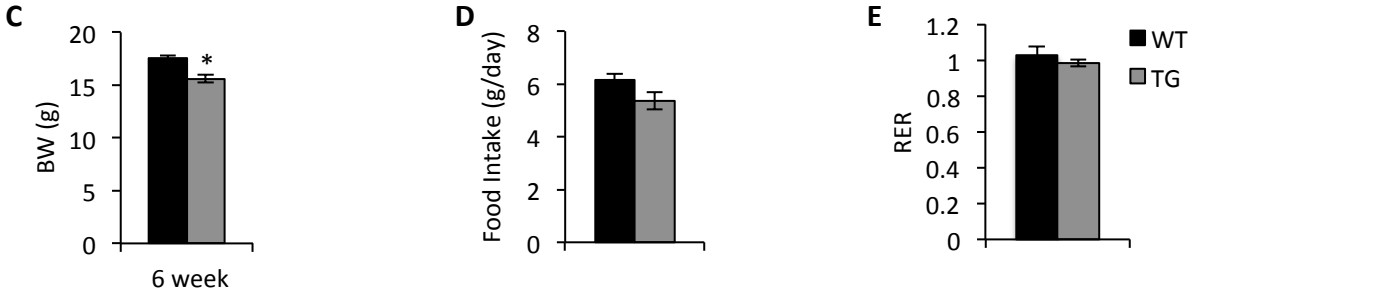
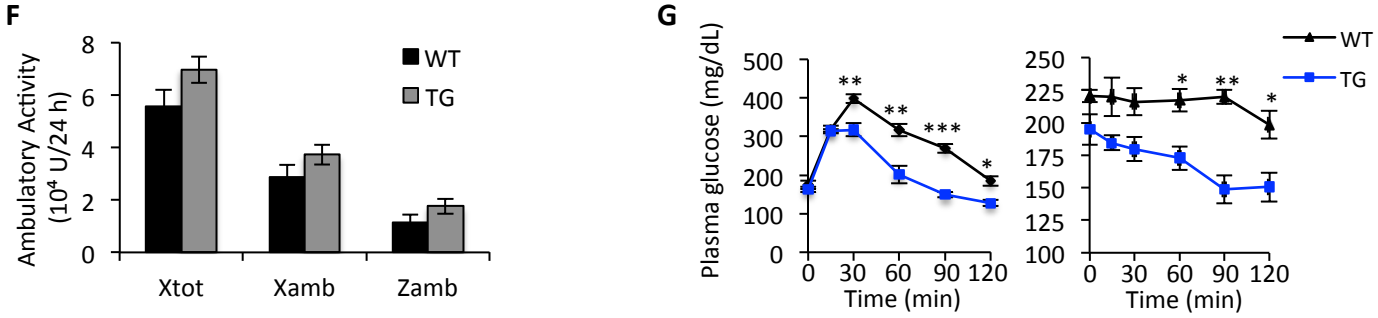


Figure S4. Zfp516 is induced via CREB/ATF pathway, related to figure 3. Relative luciferase activity of 293FT cells transfected with the -2.0kb Zfp516/Luc and indicated transcription factor expression vector. Values are normalized to empty vector control (n=4). Data shown are mean +/- SEM. **p<0.01. The -2.0kb Zfp516 promoter was activated by CREB but not the other transcription factors and co-activators tested.

Figure S5


A
Cell number/field
cell number
250
200
150
100
50
0
***
WT
TG
BAT
iWAT
pWAT
B
Lipid droplet area
cross sectional area (µm2)
600
500
400
300
200
100
0
BAT
Lipid droplet size distribution
frequency (%)
60
50
40
30
20
10
0
WT
TG
0-150
150-300
300-600
600-1000
1000-1500
>1500
cross sectional area (µm2)
5000
4000
3000
2000
1000
0
***
iWAT
frequency (%)
50
40
30
20
10
0
0-1500
1500-3000
3000-6000
6000-10000
10000-15000
cross sectional area (µm²)


C
BW (g)
20
15
10
5
0
*
6 week
D
Food Intake (g/day)
8
6
4
2
0
E
RER
1.2
1
0.8
0.6
0.4
0.2
0
WT
TG


F
Ambulatory Activity
(10⁴ U/24 h)
8
6
4
2
0
WT
TG
Xtot
Xamb
Zamb
G
Plasma glucose (mg/dL)
500
400
300
200
100
0
**
**
***
*
0 30 60 90 120
Time (min)
250
225
200
175
150
125
100
*
**
*
0 30 60 90 120
Time (min)
WT
TG

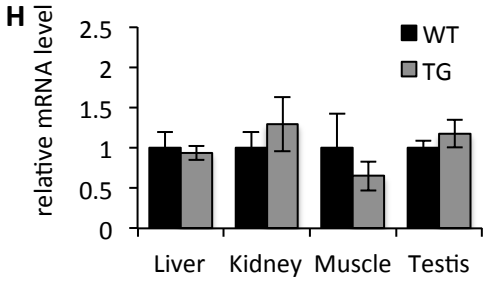

H
relative mRNA level
2.5
2
1.5
1
0.5
0
WT
TG
Liver
Kidney
Muscle
Testis

Figure S5. Zfp516 Promotes Browning of Inguinal WAT, related to figure 4. A. Left, cell number/ field in BAT from WT or TG mice (n=3). B. Top, lipid droplet area and lipid droplet size distribution in BAT from WT or TG mice. Bottom. Lipid droplet area (center) and lipid droplet size distribution (right) in iWAT from WT or TG mice (n=3). C. Body weight of WT and TG mice on CD at 6 weeks of age (n=8 mice per group). D. Average food intake of WT and TG mice on CD at 6 weeks of age (n=8 per group). E. Resting energy rate of WT and TG mice on CD at 6 week of age (n=8 per group). F. activity assayed by indirect calorimetry in WT and TG mice on CD (n=8 mice per group). G. Glucose tolerance (left) and Insulin tolerance test (right) in 10 week old mice after 4 weeks of HFD. (n=8-10). H. RT-qPCR for Zfp516 in indicated tissues from 10 week-old WT or aP2-Zfp516 mice (n=3). *$p<0.05$; **$p<0.01$; ***$p<0.001$. Data shown are mean +/- SEM. iWAT but not BAT or pWAT from TG mice had increased number of cells and decreased cell and lipid droplet size, which is indicative of the browning of iWAT. TG mice had significantly decreased body weight with no change in food intake, RER, or ambulatory activity. TG mice showed improved glucose tolerance and insulin sensitivity compared to wild-type littermates when subjected to glucose tolerance (GTT) and insulin tolerance (ITT) tests following high fat diet feeding. Zfp516 expression is the same in non-adipose depots.

Figure S6

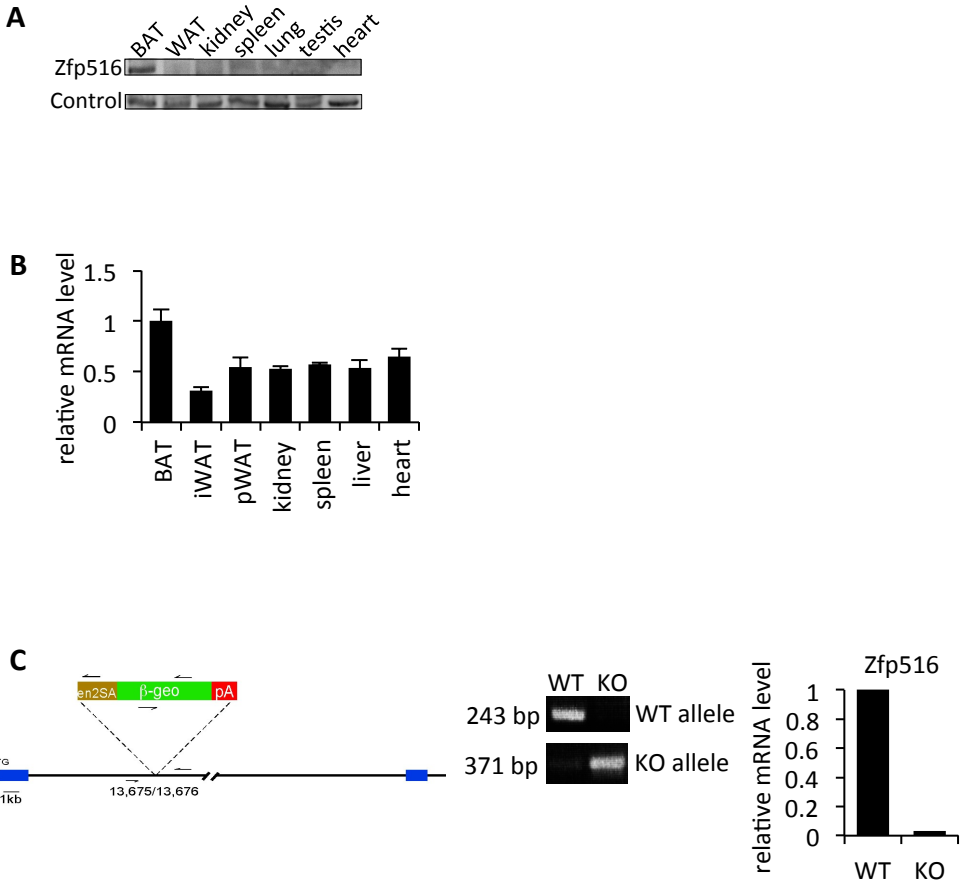


Figure S6. Generation of Zfp516 knockout mice, related to figure 5. A. Immunoblotting for Zfp516 in the indicated tissues of WT mice. B. RT-qPCR for Zfp516 in the indicated tissues. n=3. C. Left, schematic representation of gene trap insert in Intron 1 of Zfp516 with genotyping primer orientation. Center, representative genotyping gel delineating WT and KO allele. Right, RT-qPCR for Zfp516 mRNA in WT and KO BAT (n=4). Data shown are mean +/- SEM. Gene trapping construct in intron 1 of Zfp516 successfully ablates Zfp516 expression.

Table S1: Primers used for RT-qPCR

Gene	Forward Primer	Reverse Primer
Adiponectin	GCA CTG GCA AGT TCT ACT GCA A	GTA GGT GAA GAG AAC GGC CTT GT
C/EBPβ	ACG ACT TCC TCT CCG ACC TCT	CGA GGC TCA CGT AAC CGT AGT
CideA	TGC TCT TCT GTA TCG CCC AGT	GCC GTG TTA AGG AAT CTG CTG
Cox8b	GAA CCA TGA AGC CAA CGA CT	GCG AAG TTC ACA GTG GTT CC
Dio2	CAG TGT GGT GCA CGT CTC CAA TC	TGA ACC AAA GTT GAC CAC CAG
Elovl3	TCC GCG TTC TCA TGT AGG TCT	GGA CCT GAT GCA ACC CTA TGA
FABP4	ACA CCG AGA TTT CCT TCA AAC TG	CCA TCT AGG GTT ATG ATG CTC TTC A
FAS	TGC TCC CAG CTG CAG GC	GCC CGG TAG CTC CTG GGT GTA
FoxC2	TGG AGG ACA GAG CCT TTT TCT T	GCG TAG CTC GAT AGG GCA G
Igfbp3	CCA GGA AAC ATC AGT GAG TCC	GGA TGG AAC TTG GAA TCG GTC A
Mck	GCA AGC ACC CCA AGT TTG A	ACC TGT GCC GCG CTT CT
Myf5	ATC CAG GTA TTC CCA CCT GCT	ACT GGT CCC CAA ACT CAT CCT
Myf6	ATC AGC TAC ATT GAG CGT CTA CA	CCT GGA ATG ATC CGA AAC ACT TG
MyoD	CGC CAC TCC GGG ACA TAG	GAA GTC GTC TGC TGT CTC AAA GG
MyoG	AGC GCA GGT TCA AGA AAG TGA ATG	CTGTAGGCGCTCAATGTACTGGAT
Perilipin	TGT CAA TGC CTA TGA GAA GG	AGG GCG GGG ATC TTT TCC T
PGC1α	CCC TGC CAT TGT TAA GAC C	TGC TGC TGT TCC TGT TTT C
PPARα	GCG TAC GGC AAT GGC TTT AT	GAA CGG CTT CCT CAG GTT CTT
PPARγ	TCA GCT CTG TGG ACC TCT CC	ACC CTT GCA TCC TTC ACA AG
PRDM16	CAG CAC GGT GAA GCC ATT C	GCG TGC ATC CGC TTG TG
TpnI	AGA GTG TGA TGC TCC AGA TAG C	AGC AAC GTC GAT CTT CGC A
TpnT	GGA ACG CCA GAA CAG ATT GG	TGG AGG ACA GAG CCT TTT TCT T
U36B4	AGA TGC AGC AGA TCC GCA	GTT CTT GCC CAT CAG CAC C
UCP1	ACT GCC ACA CCT CCA GTC ATT	CTT TGC CTC ACT CAG GAT TGG
VEGF	TAC TGC TGT ACC TCC ACC TCC ACC ATG	TCA CTT CAT GGG ACT TCT GCT CT
VEGFR1	CGG AAG GAA GAC AGC TCA TC	CTT CAC GCG ACA GGT GTA GA
VEGFR2	GGC GGT GGT GAC AGT ATC TT	TCT CCG GCA AGC TCA AT
Zfp516	AGC GCT TGG ATA TCC TCA GTA	GAG GGG CCC TGC TGGCAC AGT

Table S2: Primers used for ChIP

Target	Forward Primer	Reverse Primer
-70 UCP1	TGT GGC CAG GGC TTT GGG AGT	AGA TTG CCC GGC ACT TCT GCG
-2.5kb UCP1	AGC GTC ACA GAG GGT CAG T	GTG AGG CTG GAT CCC CAG A
-5kb UCP1	ACA TTG CCA AGA CTG CGG CCA TC	ACC CCC AAA CAG CAG CAG CAA C
-150 PGC1α	AGC AAG CAA GCC ACA ACA CCC T	AGGGGTGGGGGCAGGTGAGT
-2.4 kb PGC1α	GCT CAC ACT GAA TTG TGG CAG GA	GGG CAG CGT GTC TGT GTT CA
-250 Cox8b	GGC TGA TCC ATC TCG CTG GCT GCT	CCC AAA CAC CGA GGC GCT GTG A
-2.8kb Cox8b	TGG CCC GAG CCC AGA AAG GCA GA	GAG GGA GGG CTC CGA TGG GGA GGT

Supplementary Methods

Plasmid Constructs

The Zfp516 sequence was subcloned into FLAG/ pcDNA3.1. The 1-420 and 400-820 Zfp516 fragments were cloned into 3Tag-3a. The -5.5kb UCP1-GFP construct was generated by PCR amplifying the -5.5kb UCP1 promoter sequence from genomic DNA from C57BL/6 mouse liver and cloning into AseI/AgeI of eGFP-N1 (Clontech). The -5.5kb UCP1-Luc construct was generated by insertion of luciferase sequence into the Mfe-1/Age-1 sites of the 5.5kb UCP1-GFP construct, which removes GFP. The -1.5kb UCP1-luc, -500bp UCP1-luc, -150bp UCP1-luc, -70bp UCP1-luc, and -25bp UCP1-luc were generated by PCR amplifying the target region from genomic DNA and inserting into SacI/NheI of pGL2 basic vector (Promega). The -45bp and -70bp + enhancer constructs were generated by PCR amplifying the enhancers region and inserting into XmaI/KpnI of the pGL2 vectors described above. The -2.0kb PGC1a-luc and PRDM16 expression vectors were from Addgene.

Antibodies, animals, and cell culture

The following antibodies were used: ZNF516 (sc-85244 and sc-85244X (ChIP) , PGC1 (sc-13067), C/EBPβ (sc-7962), PPARγ (sc-7176), CideA (sc-8732), Lamin A (sc-20680), Tubulin (sc-5546), GAPDH (sc-32233) (all Santa Cruz), V5 (ab-9137) (Abcam), PRDM16 (pab19171) (Abnova), UCP1

(SAB2501082), FLAG M2 (F1804), anti-FLAG M2-HRP (A8592)(Sigma), and anti-V5-HRP (R96125) (Invitrogen).

Embryonic stem (ES) cell lines bearing a β-galactosidase-neomycin (β-geo) trap in the Zfp516 gene (Zfp516.β-geo) were from the German Gene Trap Consortium. The entire intron 1 of Znf516 gene and inserted β-geo region of the ES cell genomic DNA was sequenced to identify the exact insertion site. ES cells were microinjected into 3.5-day blastocysts derived from C57BL/6J females, and transferred to pseudopregnant C57BL/6J recipients. Chimeric mice were then bred with C57BL/6J mice for germline transmission. The mice were bred into C57BL/6J for at least 4 generations. The presence of the targeted allele in the black-colored offspring was confirmed by PCR.

293FT cells were grown in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 250μg/mL G418. HIB-1B brown preadipocytes, C2C12 myoblasts were grown in DMEM containing 10% FBS and 1% penicillin/streptomycin.

For adenoviral infection of MEFs, C2C12 or HIB-1B cells, subconfluent cells were incubated overnight with GFP or Zfp516 adenovirus. Viral medium is replaced with induction medium for brown adipogenic or myogenic conditions. Znf516 shRNA and or GFP control lentiviral particles (Santa Cruz) were transduced into HIB-1B cells. Cells were split 48 h post transduction and were selected with Puromycin (5ug/ml) for 5 days.

Briefly, HIB-1B cells seeded in XF24 plates were induced to differentiate as described above. On the day of experiments, the cells were washed once and

maintained in XF-DMEM (Sigma-Aldrich) supplemented with 1 mM sodium pyruvate and 17.5 mM glucose. Oxygen consumption was blocked by 6 μM oligomycin. Maximal respiratory capacity was assayed by the addition of 8 μM FCCP. For tissue explants, tissues we excised and placed directly into KREB's-Heslinger buffer (121mM NaCl, 4.9mM KCl, 1.2mM $MgSO_4$, 0.33mM $CaCl_2$, 12mM Hepes, 25mM glucose, 10mM Sodium Pyruvate, 1% fatty acid free BSA, pH 7.4). 3-5μg pieces of tissue were put inXF-24 plates and secured using an islet capture screen. Tissues were incubated for 1 hr at 37°C without CO_2 prior to analysis on the XF24 Analyzer.

Functional Screen

cDNA clones in pCMV-Sport6 vector from the Mammalian Genome Collection (Invitrogen) were screened for activation of the -5.5kb UCP1 promoter-eGFP-N1 construct. 100ng of -5.5kb UCP1-eGFP-N1, 1ng of lipofectamine2000 (Invitrogen), and 44.8μl Optimem (Gibco) were added to 40ng of test vector in 48-well plates. Complexes were added to7.5×10^4 293FT cells. Cells were assayed for GFP signal at 24 and 48h post-transfection. Each plate contained positive (pCMV-Sport6-CREB) and negative (pCMV-Sport6) controls. For secondary screening, 100ng of -5.5kb UCP1-Luciferase, 0.5ng pRL-CMV, and 1.2μl Lipofectamine2000 were diluted to 45μl in Optimem before addition of 50ng of each of the positive clone from the initial screen in 48-well plates in triplicates. 7.5×10^4 293FT cells were added to transfection mixtures and incubated for 24h.

Cells were assayed for luciferase activity using Dual-Luciferase Reporter assay system (Promega).

Cryosectioning and Immunostaining

Fresh adipose tissue from indicated mice were embedded in OCT medium and snap-frozen. The tissue were sectioned on Leica CM3050S Cryostat in 10μm thick section and collected on Superfrost+ coated glass slides (Fisher). Briefly, slides were allowed to warm to room temperature for 5 minutes, fixed with 4% formalin phosphate buffered saline for 30 minutes at room temperature, blocked with 5% BSA in PBS containing 0.025% Triton X-100 (PBST), and incubated with the appropriate antibody diluted in 2.5% BSA in PBST overnight at 4°C. Slides were washed 3 times in PBST, and incubated with secondary antibody for 2 h at room temperature. Slides were then washed twice in PBST, and then stained with hematoxylin (bright field) for 30 s. Slides were washed and mounted in 15% glycerol in PBS with glass coverslips. Cell number and lipid droplet area were quantified from fresh stained BAT, iWAT and pWAT slide pictures using ImageJ software (NIH opensource) and by counting a minimum of 100 cells in 4 random fields/picture/tissue and measuring 50 lipid droplets in 4 random fields/tissue.

Electrophoretic Mobility Shift Assay

Nuclear extracts of 293FT cells transfected with flag-Zfp516, flag-Zfp516 (AA1-420), or flag-Zfp516 (AA400-820) fragment were used in electrophoretic mobility shift assay (EMSA). Sense (5’-

CCTGGGCCGGCTCAGCCACTTCCCCCAGTC-3') and antisense (5'-GACTGGGGGAAGTGGCTGAGCCGGCCCAGG-3') oligonucleotides were end-labeled by γ ^{32}P-ATP using T4 Polynucleotide kinase (NEB) or biotin (Thermo Scientific) and annealed. Samples were separated by 6% native PAGE before autoradiography or transferred to a nylon membrane and detected using streptavidin-HRP conjugate (Thermo Scientific).

ChIP

For ChIP analysis using 293FT cells, cells were transfected with 10 μg of either pcDNA3.1-FLAG-Zfp516 or Zfp516 truncations or pcDNA3.1 and 5 ug of either -5.5kb UCP1-Luciferase or -2kb PGC1α-Luciferase using calcium phosphate method. At 48 h post-transfection, cells were crosslinked for 10 min by adding 1% formaldehyde in DMEM at room temperature. Cross-linking was stopped by the addition of glycine to a final concentration of 0.125 M. After sonication, DNA sizes were 0.3–0.9kb. For HIB-1B cells, cells were cultured to confluence before crosslinking and sonication as above. For ChIP experiments in BAT, tissues were minced on ice and crosslinked using 1% formaldehyde in phosphate-buffered saline for 10 min. Crosslinking was stopped using glycine as before. Samples were dounced, washed twice, centrifuged and resuspended in RSB buffer, containing 10mM Tris pH 7.4, 10mM NaCl, 3mM $MgCl_2$, prior to sonication. Nuclei were released by douncing on ice and collected by centrifugation. Nuclei were then lysed in nuclei lysis buffer containing 50mM Tris, pH 8.0, 1% SDS 10mM EDTA supplemented with protease inhibitors, followed by

sonication. Chromatin samples were diluted 1:10 with the dilution buffer, containing 16.7mM Tris, pH 8.1, 0.01% SDS 1.1 % Triton X-100 1.2mM EDTA and 1.67mM NaCl and proteinase inhibitors. Soluble chromatin was quantified by absorbance at 260 nm, and equivalent amounts of input DNA were immunoprecipitated using 5 μg of indicated antibodies or normal mouse IgG and protein A/G beads. After the beads were washed and cross-linking was reversed, DNA fragments were extracted with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol. DNA was precipitated and resuspended in water. Samples were analyzed by endpoint and qPCR using the primer sets in Table S2.

Co-IP

293FT cells were transfected with 12.5 μg of Zfp516 expression vector and either PRDM16, C/EBPβ, PGC1α, or PPARγ using calcium phosphate method. 48 h post-transfection, nuclear extraction was carried out using the NE-PER Nuclear and Cytoplasmic Extraction kit (Thermo). Equal amounts of nuclear extracts were incubated with the specific antibodies and protein A/G agarose beads overnight at 4°C, washed and proteins separated by SDS-PAGE, transferred onto nitrocellulose membranes for immunoblotting.

For Co-IP experiments using tagged constructs, 293FT cells were transfected using Lipofectamine2000 to express FLAG-tagged Zfp516 and/or V5-tagged PRDM16. Cells were lysed in IP buffer containing 20 mM Tris, pH 7.4, 150 mM NaCl, 1mM EDTA, 10% glycerol, 1% NP-40 supplemented with

proteases inhibitors. Total cell lysates were incubated 2 h at 4°C with either anti-FLAG M2 or anti-V5 -agarose beads (Sigma). Agarose beads were washed 3 times and bound proteins were eluted by boiling in Laemmli sample buffer and analysed by immunoblotting using the indicated antibodies.

For nuclear extracts in mice, BAT from mice was excised and miced finely in sterile PBS. Following centrifugation, PBS was removed and BAT was resuspended in CERI buffer from the NE-PER kit (Thermo). BAT was dounced 10 times with a loose mortar and filtered through a 100µm screen before continuing with manufacturer protocol.

In vitro Binding Assays

GST-fused to various PRDM16 fragments (described in (Kajimura et al., 2008)) were expressed in BL21 by IPTG induction for 3 h at 37°C, purified on glutathione sepharose beads and eluted with elution buffer containing reduced glutathione. [^{35}S]-labeled Zfp516 protein was produced by using TNT coupled transcription/translation kit (Promega). 20 µg of GST fusion proteins were incubated overnight at 4°C with *in vitro* translated proteins and glutathione sepharose beads in a binding buffer containing 20mM Hepes, pH 7.7, 300mM KCl, 2.5mM $MgCl_2$, 0.05% NP40, 1mM DTT, and 10% glycerol. The sepharose beads were then washed 3 times with binding buffer. Bound proteins were eluted by boiling in Laemmli sample buffer, separated by SDS-PAGE and analysed by autoradiography.

References

Kajimura, S., Seale, P., Tomaru, T., Erdjument-Bromage, H., Cooper, M.P., Ruas, J.L., Chin, S., Tempst, P., Lazar, M.A., and Spiegelman, B.M. (2008). Regulation of the brown and white fat gene programs through a PRDM16/CtBP transcriptional complex. Genes Dev. *22*, 1397–1409.