SUPPLEMENTARY DATA

Id1 promotes obesity by suppressing brown adipose thermogenesis and white adipose browning

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Supplementary Information.

Id1 partially suppresses Ebf2-mediated re-programing of C2C12 myoblasts into brown adipocytes

Forced expression of *Ebf2* alone is sufficient to reprogram C2C12 myoblasts into brown adipocytes (1). Since Id1 directly interacted with Ebf2 and suppressed its transcriptional activity (Fig 8D-F), we asked if Id1 inhibits Ebf2-mediated reprograming of C2C12 myoblasts into brown adipocytes. We generated Ebf2-expressing C2C12 cells (Suppl. Fig 4A), and as demonstrated previously (1), Ebf2-expressing cells differentiated into adipocytes as determined by aP2 expression and Oil-Red-O staining (Suppl. Fig 4B-C). We then expressed *Id1* in Ebf2-C2C12 cells (Suppl. Fig 4D) and observed reduced differentiation in Ebf2/Id1-C2C12 cells compared to Ebf2-C2C12 cells on day 4 (Suppl. Fig 4E). Consequently, we detected reduced induction of *Prdm16, PGC1α* and *Ucp1* mRNA in *Id1* overexpressing day 4 differentiated Ebf2-C2C12 cells, whereas in non-differentiated cells, except for *PGC1α,* expression of the other genes was not affected (Suppl. Fig 4F-G). However, by day 8 the overall differentiation was not different between Ebf2-C2C12 and Ebf2/Id1-C2C12 cells as determined by Oil-Red-O staining (Suppl. Fig 4H). Similarly, the expression of *Prdm16, PGC1α* and *Ucp1* were also unchanged in day 8 differentiated Ebf2-C2C12 and Ebf2/Id1-C2C12 cells (Suppl. Fig 4I). This is mainly due to the fact that Ebf2 expression is very strongly induced during C2C12 differentiation and Id1 expression is down-regulated (Suppl. Fig 4J); therefore, Ebf2 is able to escape from Id1-mediated inhibition. Consistent with this explanation, we detected a direct interaction between Id1 and Ebf2 in day 0 cells (Suppl. Fig 4K). However, we were unable to immunoprecipitate Id1from latter time points (day 2-6) due to the fact that Id1 levels are very low. These results together suggest that Id1 was unable to sustain the inhibition on Ebf2-mediated programing of C2C12 cells into brown adipocytes due to the fact that Id1 is strongly downregulated during C2C12 differentiation.

Additional Information

Immunoblotting and co-immunoprecipitation

Whole-cell protein lysates were prepared from cells and tissues using RIPA lysis buffer (ThermoScientific # 89901) supplemented with complete mini-protease inhibitor tablet (Roche # 11836153001). For Western blot analysis, 50μg of protein was separated on NuPAGE precast gels (Invitrogen), transferred using a XCell II Blot module (Invitrogen # 090707-098) onto Immobilon-FL membranes (Millipore # IPFL00010), and probed with specific primary antibodies: mouse Id1 (Biocheck # BCH-1, 37-2), mouse/human Id1 (Biocheck # BCH-1/195-14), Rb (Santa Cruz # (sc-50), pRb (Cell Signaling, # 8516), RIP140 (Santa Cruz # sc-8997), Foxc2 (abcam # ab5060), SRC1 (Santa Cruz # sc-136077), PPARγ (Thermo Scientific # MA5-14889), PPARγ (Cell Signaling # 2443), Ebf2 (R&D Systems # AF7006), CREB (Cell Signaling # 9197), PGC1α (Santa Cruz # sc-13067), aP2 (Santa Cruz # sc-18661), Ucp1 (abcam # ab10983), PRDM16 (abcam # ab106410), Sirt3 (Cell Signaling # 5490), IRF4 (Santa Cruz # sc-6059), HA-Tag (Cell Signaling # 3724), Cyt-c (Cell Signaling # 4280), Dio2 (Proteintech # 26513-1-AP) and β-actin (Sigma # A5441). All the antibodies were used at a dilution of 1:1000 except Id1 (1:500), Ebf2 (1:500) and β-actin (1:10,000). The following IRDye-conjugated secondary antibodies were used: donkey anti-mouse IRDye800CW (Licor # 926-32212) and donkey anti-rabbit IRDye800CW (Licor # 926-32213), donkey anti-mouse

IRDye680RD (Licor # 925-68072) and donkey anti-rabbit IRDye680RD (Licor # 925-68073). Licor Odyssey Classic Imager was used to detect Western blots. For co-immunoprecipitation assays, 1mg of protein from whole-cell lysates and 20 µl of Protein A/G PLUS-Agarose immunoprecipitation reagent (sc-2003), Rabbit IgG (Santa Cruz # sc-2027) and Mouse IgG (Santa Cruz # sc-2025) were used and co-IPs were performed as described previously (2).

Oxymax/CLAMS metabolic analysis

Mice (*aP2-Id1*$^{Tg+}$ and *aP2-Id1*$^{Tg-}$) were acclimated for 12 h in the metabolic cages, and their metabolic rates were measured for 96 h in an indirect open-circuit calorimeter (Oxymax Comprehensive Lab Animal Monitoring System; Columbus Instruments). O_2 consumption, CO_2 release, heat production, food consumption and physical activity were measured at room temperature (RT) and normalized to body weight to account for the disparity in body weight between the two groups.

Multi-slice CT scans

The fat volume in *aP2-Id1*$^{Tg+}$ and control *aP2-Id1*$^{Tg-}$ mice was determined by multi-slice microCT imaging (Mediso Imaging System # Nanoscan SpectCT) as described previously (3). Lean mass and fluid content was measured by LF90II BCA-Analyzer (Bruker). Bone mass was measured by Lunar PIXImus2.

H&E and Oil-Red-O staining

Oil-Red-O staining and the staining intensity measurements on differentiated adipocytes, and H&E staining on different tissues were performed as described previously (4).

Adipocyte size measurement

H&E stained WAT sections were photographed under 40X magnification using AmScope Inverted microscope equipped with 9MP Camera and ToupView software. The adipocyte area was measured using imageJ software and presented as arbitrary units. A total of 200 adipocytes were measured from each genotype.

Immunocytochemical staining

Day 4 differentiated HIB1B cells cultured on coverslips in 6-well plates were washed twice with cold PBS, fixed with ice-cold acetone: methanol (1:1) for 5 min, and washed 3 times with ice-cold PBS and incubated with Id1 (#BCH-1, 37-2; BioCheck) and aP2 (Santa Cruz # sc-18661) antibodies at 4°C overnight. The cells were then washed 3 times with PBS/0.2% Tween 20 and incubated with AlexaFluor 555 goat anti-rabbit (Life Technologies # A21428) and AlexaFluor 488 goat anti-mouse (abcam # ab150105) secondary antibodies for 1 h at room temperature. The coverslips were washed 3 times with PBS/0.2% Tween 20, air dried for 10 min, and mounted with fluorescence mounting medium (Vector Laboratories Vectashield # H-1200) containing DAPI. The optical single-slice images were captured with the Zeiss Axio fluorescence laser-scanning microscope.

Immunohistochemistry (IHC)

For IHC on iWAT, slides were deparaffinized in xylol, and antigen retrieval was performed by boiling the slides for 30 min in H_2O. After cooling for 20 min and washing in PBS, endogenous peroxidase was blocked with 0.3% hydrogen peroxide for 30 min. The slides were then incubated

with UCP1 antibody (1:300 dilution, abcam # ab10983) overnight at 4°C. UCP1 staining was detected using UltraVision Quanto Detection System HRP DAB (Thermofisher # TL-060-QHL). The staining pattern was analyzed and photographed using AmScope inverted microscope equipped with 9MP camera and ToupView software. For Id1, CD45 and Ebf2 stainings, slides were incubated after antigen retrieval with primary antibodies Id1, 1:100 dilution (Biocheck # BCH-1, 37-2), Ebf2, 1:50 dilution (R&D Systems # AF7006) and CD45, 1:200 dilution (abcam # ab10558) overnight at 4°C. The slides were then washed 3 times with PBS/0.2% Tween 20 and incubated with AlexaFluor 555 goat anti-rabbit (Life Technologies # A21428) and AlexaFluor 488 anti-mouse (abcam # ab150105) secondary antibodies for 1 h at RT. The slides were washed 3 times with PBS/0.2% Tween 20, air dried for 10 min, and mounted with fluorescence mounting medium (Vector Laboratories Vectashield # H-1200) containing DAPI. For CD45/Id1, CD45/Ebf2 and Id1/Ebf2 double stainings, primary antibody incubations were performed sequentially on consecutive days. The optical images were captured with the Zeiss Axio fluorescence laser-scanning microscope.

Brown adipocyte differentiation in HIB1B cells, MEFs and C2C12 myoblasts

HIB1B brown pre-adipocyte cells were a kind gift from Dr. Bruce Spiegelman (Harvard Medical School). HIB1B cells were cultured in 10cm dishes in DMEM (HyClone Laboratories # SH30022.01) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Gemini Bio-Products #100106) and 1% penicillin/streptomycin (Life Technologies # 15140) in a humidified cell culture incubator (Thermo Fisher Scientific # Heracell 150i) maintained at 37°C, 5% CO2, and 20% O2. After the cells reached 100% confluence, the media was replaced with adipogenesis differentiation media [DMEM, 10% FBS, 20nM insulin (I2643; Sigma) and 1nM triiodothyronine (T3) (Sigma # T6397) for 48 h. Adipocyte differentiation was induced by treating cells for an additional 48h in differentiation medium further supplemented with 0.5μM dexamethasone (Sigma # D1756), 0.5mM 3-isobutyl-1-methylxanthine (Sigma # I7018), and 125 μM indomethacin (Sigma # I7378) (induction media). After induction, cells were returned to differentiation medium, and the differentiated cells were collected at different time points for further analysis. Mouse embryonic fibroblasts (MEFs) were cultured in 100mm dishes and induced to differentiate into brown adipocytes as described previously (5). Briefly, E13.5 $Id1^{+/+}$ and $Id1^{-/-}$ MEFs (passage 2) were cultured in DMEM supplemented with 10% FBS and 1% penicillin/streptomycin. After the cells became fully confluent, the cells were treated for 48h with media containing 10% FBS, 0.5 mM 3-isobutyl-1-methylxanthine, 125 μM indomethacin, 1 μM dexamethasone, 850 nM insulin, 1 nM T3, and 1μM rosiglitazone (Sigma # R2408). Two days after induction, cells were switched to maintenance medium containing 10% FBS, 850 nM insulin, 1 nM T3, and 1 μM rosiglitazone, and the differentiated cells were collected at different time points for further analysis. C2C12 cells were purchased from ATCC (CRL-1772, lot # 62042837). The full-length mouse Ebf2 gene was PCR amplified and sub-cloned into the retroviral vector PLNCX2. PLNCX2-Ebf2 was used for viral packaging in ampho-phoenix cells. C2C12 cells were transduced with retrovirus expressing Ebf2 using polybrene (Millipore # TR-1003-G) at a concentration of 5μg/ml at 35°C. Viral transduction was conducted twice with an interval of 24h and the cells were then selected with G418 (750μg/ml, Sigma # G8168) to generate stable Ebf2-expressing C2C12 cells. The Ebf2-C2C12 cells were transiently transfected with pcDNA3 mId1 (gift from Robert Benezra, Addgene plasmid # 16060) to overexpress Id1, and brown adipogenesis was induced in C2C12, Ebf2-C2C12 and Ebf2/Id1-C2C12 cells as described previously (1).

Retrovirus-mediated Id1 overexpression in HIB1B cells

The Id1 gene was excised from pCDNA3 mId1 (Addgene plasmid # 16060) by restriction digestion and sub-cloned into the retroviral vector PLNCX2. PLNCX2-Id1 was then used for viral packaging in ampho-phoenix cells. HIB1B cells were transduced with retrovirus expressing Id1 using polybrene at a concentration of 5μg/ml at 35°C. Viral transduction was conducted twice with an interval of 24h,

and the cells were then selected with G418 (200μg/ml) to generate stable Id1-expressing HIB1B cells.

Mitochondrial staining and FACS analysis

For measuring mitochondrial content in MEFs and HIB1B cells, cells growing in complete medium were incubated with 10 nM NAO (Nonyl Acridine Orange, Molecular Probes # A1372) dissolved in PBS/5% FBS for 15 min at 37°C. Medium containing NAO was removed from the cells, then the cells were washed twice with PBS, trypsinized, resuspended in PBS, and analyzed by the FACSCalibur flow cytometry system equipped with CellQuest Pro Software (BD Biosciences).

O_2 consumption measurement

Cellular respiration analysis was done as described previously (6). Cells were trypsinized, a single-cell suspension was placed in respiration buffer, and the O_2 consumption rate was measured using OxygraphPlus System (Hansatech Instruments). For tissues, BAT was weighed and 25mg of tissue was minced using a razor blade and equal amount of wet tissue was used to measure O2 consumption rate. After measuring basal respiration, 1μM (final conc.) oligomycin was added to the respiration buffer to measure uncoupled respiration. After the measurements, cells and tissues were lysed using RIPA lysis buffer, and whole cell protein concentration was measured. O2 consumption was normalized to protein concentration.

Glucose, insulin, and adipokine measurements

Blood glucose levels were measured from the tail blood by One Touch Ultra II Glucometer (LifeScan) and One Touch Ultra glucose strips (LifeScan). For insulin and leptin measurements, mice were starved 4 h, and blood serum was prepared, aliquoted and stored at -80°C until use. Insulin concentration was determined by insulin (mouse) ultrasensitive EIA kit (Alpco # 80-INSMSU-E01). Leptin and adiponectin levels were measured by leptin (murine/rat) EIA kit (Alpco # 22-LEPMS-E01) and adiponectin EIA Kit (Alpco # 17-ADPMS-E01) according to the manufacturer's protocols. The ELISA plates were read using Synergy HTX Multi-Mode Plate Reader (BioTek).

Transient transfection, shRNA and overexpression vectors

Continuum™ Transfection Reagent (Gemini Bio-products # 400-700) was used to transfect the cells with control, shRNA, or expression vectors according to the manufacturer's protocol. pCF-CREB was a gift from Marc Montminy (addgene plasmid # 22968) (7), RcCMV/Rb was a gift from Bob Weinberg (Addgene plasmid # 1763), pBabe human FOXC2 was a gift from Bob Weinberg (Addgene plasmid # 15535) (8), pAd-Track HA PGC1α was a gift from Pere Puigserver (Addgene plasmid # 14427) (9), pcDNA3 hId1 was a gift from Robert Benezra (Addgene plasmid # 16061), pBABE puro PPARγ2 was a gift from Bruce Spiegelman (Addgene plasmid # 8859) (10), pMIG-IRF4 was a gift from David Baltimore (Addgene plasmid # 58987) (11), SIRT3 Flag was a gift from Eric Verdin (Addgene Plasmid # 13814) (12), and pBabe hygro human RXRα was a gift from Ronald Kahn (Addgene plasmid # 11440). Control shRNA (shGL) and Id1-shRNA vectors were a kind gift from Jonathan Keller (NCIFrederick) and were described previously (13).

Luciferase reporter assays

Gal4-UAS and full-length PGC1α fused with DNA-binding domain (DBD) of yeast Gal4 vectors were kind gift from Dr. Bruce Spiegelman (Harvard Medical School). For the luciferase reporter

assays, Cos7 cells ($3x10^4$) were seeded onto 24-well plates. The next day, cells were co-transfected with Gal4-UAS (40ng/well), DBD-Gal4-PGC1α (40ng/well), Id1 plasmid (different concentrations, 0-120ng) and galactosidase expression vector (control reporter) (100ng/well). The mass of transfected plasmids was equalized with an empty vector (pCDNA3.1). After 48 hr, cells were harvested and the luciferase activity was measured using the Luciferase assay system (Promega # E1500) and Synergy HTX Multi- Mode Plate Reader (BioTek). Luciferase activity was divided by β-gal (control reporter) to normalize for transfection efficiency. For the *Prdm16* promoter driven luciferase assay, the -25Kb *Prdm16* promoter-driven luciferase vector was obtained from Dr. Patrick Seale (UPenn). HEK293 cells were seeded at ~65% confluency in 12-well plates. The next day, cells were co-transfected with -25Kb *Prdm16* promoter-driven luciferase vector (100ng/well), PPARγ2 (100ng/well), RXRα (100ng/well), Id1 plasmid (different concentrations, 0-120ng) and galactosidase expression vector (control reporter) (100ng/well). The mass of transfected plasmids was equalized with an empty vector (pCDNA3.1). After 48 hr, cells were harvested and the luciferase activity was measured as described above. *Ucp1* promoter-driven luciferase assays were carried out in a similar fashion in Cos7 cells.

ChIP-qPCR

ChIP-qPCR was performed as described previously (1) using the ChIP assay kit (Millipore cat# 17-295). Briefly, HIB1B preadipocytes were differentiated for 4 days and cross-linked with 1% formaldehyde (final conc.) in the growth media at 37^0C for 10 min. Cells were then harvested and re-suspended in SDS lysis buffer at a final concentration of 10^7 cells per ml. Then the cells were sonicated for 18 cycles with 10 second intervals and centrifuged at 13000 RPM for 10 min. The supernatant was diluted 10 fold in ChIP dilution buffer/cocktail inhibitor and pre-cleared using 75µl of salmon sperm DNA/ProteinG agarose (Cell Signaling # 9007). Immunoprecipitations were carried out using IgG, Ebf2, PGC1α and Id1 antibodies overnight and washed twice sequentially with low salt, high salt, lithium chloride and TE buffers. DNA/protein complexes were eluted using SDS/NaHCO3 buffer and reverse cross linked at 65^0C for 4 h in the presence of 0.2M NaCl. The elutes were then treated with proteinase K for 1 h and DNA was extracted using phenol chloroform and used for qPCR. Primer sequences were provided in Suppl. Table 1.

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Supplementary Table 1. Mouse primers used for real-time qPCR

Gene	Forward primer (5'-3')	Reverse primer (5'-3')
Id1	GGTCCGAGGCAGAGTATTACA	CCTGAAAAGTAAGGAAGGGGGA
aP2	ACACCG AGATTTCCTTCAAACTG	CCATCTAGGGTTATGATGCTCTTC A
Cidea	TGCTCTTCTGTATCGCCCAGT	GCCGTGTTAAGGAATCTGCTG
Dio2	CAGTGTGGTGCACGTCTCCAATC	TGAACCAAAGTTGACCACCAG
Pgc1α	CCCTGCCATTGTTAAGACC	TGCTGCTGTTCCTGTTTTC
Pparγ	GTGCCAGTTTCGATCCGTAGA	GGCCAGCATCGTGTAGATGA
Prdm16	CAGCACGGTGAAGCCATTC	GCGTGCATCCGCTTGTG
Ebf2	GCTGCGGGAACCGGAACGAGA	ACACGACCTGGAACCGCCTCA
Ucp1	ACTGCCACACCTCCAGTCATT	CTTTGCCTCACTCAGGATTGG
Elovl3	TCCGCGTTCTCATGTAGGTCT	GGACCTGATGCAACCCTATGA
Elovl6	TGCTGCATCCAGTTGAAGAC	TGCCATGTTCATCACCTTGT
Nrf1	GAACTGCCAACCACAGTCAC	TTTGTTCCACCTCTCCATCA
Nrf2	GCTTTTGGCAGAGACATTCC	ATCAGCCAGCTGCTTGTTTT

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Cytc	GCAAGCATAAGACTGGACCAAA	TTGTTGGCATCTGTGTAAGAGAATC
Cox4	ACCAAGCGAATGCTGGACAT	GGCGGAGAAGCCCTGAA
Cox5b	GCTGCATCTGTGAAGAGGACAAC	CAGCTTGTAATGGGTTCCACAGT
Cox7a	CAGCGTCATGGTCAGTCTGT	AGAAAACCGTGTGGCAGAGA
Cpt1b	CGAGGATTCTCTGGAACTGC	GGTCGCTTCTTCAAGGTCTG
Errα	TTCTGCACAGCTTCCACATC	GGAAGAATTCGTCACCCTCA
Cd40	TTGTTGACAGCGGTCCATCTA	CCATCGTGGAGGTACTGTTTG
Cd137	CGTGCAGAACTCCTGTGATAAC	GTCCACCTATGCTGGAGAAGG
Tbx1	GGCAGGCAGACGAATGTTC	TTGTCATCTACGGGCACAAAG
Tmem26	ACCCTGTCATCCCACAGAG	TGTTTGGTGGAGTCCTAAGGTC
18S	GTAACCCGTTGAACCCCATT	CCATCCAATCGGTAGTAGCG
Prdm16 ChIP	GACCTCCTGCCTTCCCTGAGG	GCTGCCTGAGCTGGGCCAGCC
UCP1-ChIP	AGTGAAGCTTGCTGTCACTC	GTCTGAGGAAAGGGTTGACC
18s ChIP	AGTCCCTGCCCTTTGTACACA	CGATCCGAGGGCCTCACT

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Supplementary Table 2. Metabolic parameters in *aP2-Id1*$^{Tg-}$ and *aP2-Id1*$^{Tg+}$ mice (n=7). *p<0.05

Blood Glucose(mg/dL)	***aP2-Id1*$^{Tg-}$**	***aP2-Id1*$^{Tg+}$**
2-month-old	138.7±8.8	158.7±19.9
6-month-old	166.6±5	169.3±22
High-fat diet*	148.6±46.1	191±51.3 *
Insulin (ng/ml)		
2-month-old	0.6±0.13	1.12±0.42

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6-month-old	0.91±0.55	1.41±0.77
High-fat diet*	4.2±2.32	15.32±4.02 *
Leptin (ng)		
2-month-old	1.2±0.2	1.3±0.3
6-month-old	6.7±2.1	8.3±3.5
High-fat diet*	22.4±4.7	34.6±2.2 *
Adiponectin (µg/ml)		
2-month-old	8.5±2	8.4±1.4
6-month-old	6.5±1.61	9±1.09
High-fat diet*	5.6±2.19	8.84±0.8

*12 Weeks of high-fat diet.

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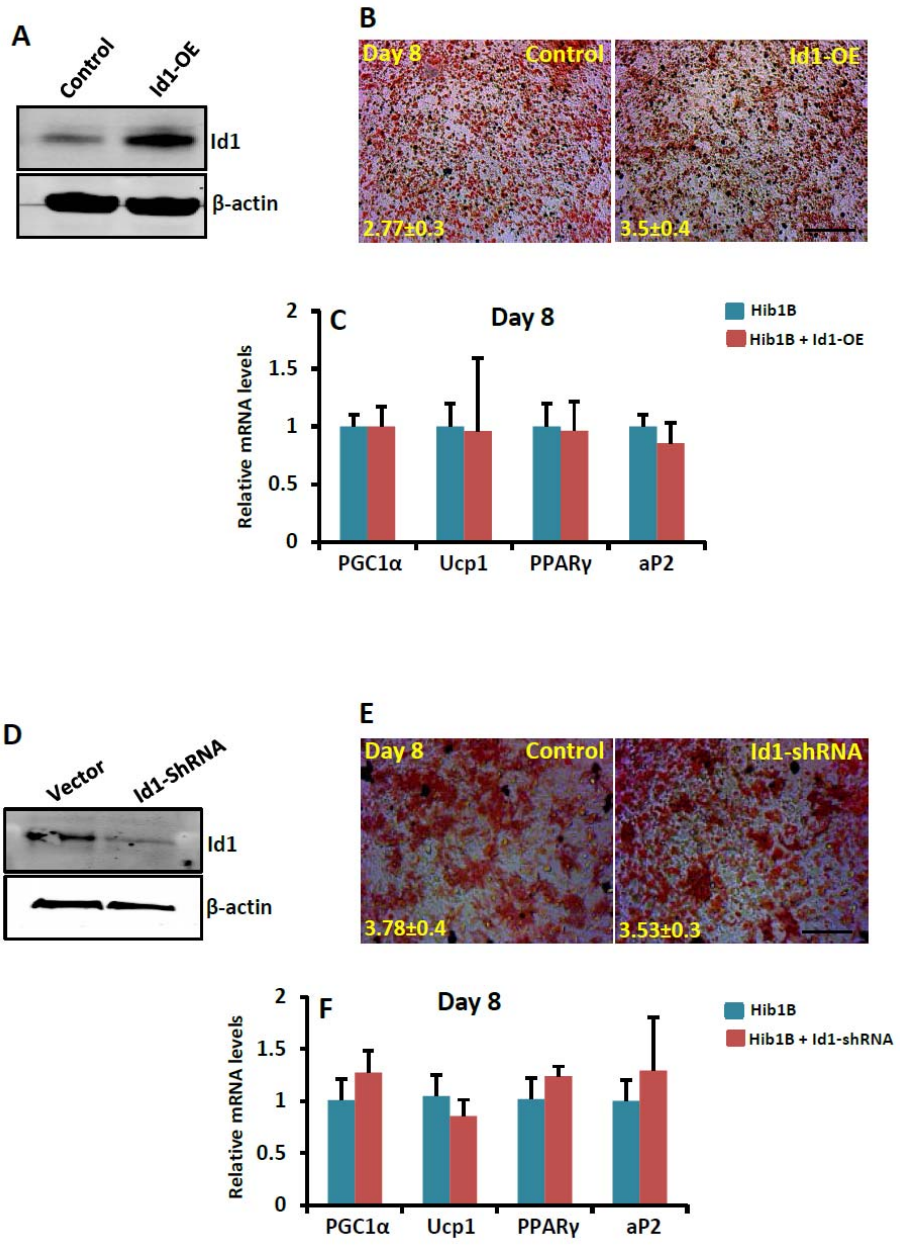
Supplementary Figure 1. Id1 is dispensable for brown adipocyte differentiation. (A) Id1 and β-actin levels in vector control and mouse-Id1 overexpressed (Id1-OE) HIB1B cells. **(B)** Oil-Red-O staining of day 8 differentiated control and Id1-OE HIB1B brown adipocytes. Scale bar 20μm. **(C)** Relative mRNA transcript levels of indicated genes in control and Id1-OE day 8 differentiated HIB1B cells. Expression data are mean ± SD; n=3. **(D)** Id1 and β-actin levels in scramble-shRNA or Id1-shRNA expressing HIB1B cells. **(E)** Oil-Red-O staining of day 8 differentiated control or Id1-shRNA HIB1B adipocytes. Scale bar 10μm. **(F)** Relative mRNA transcript levels of indicated genes in control and Id1-shRNA day 8 differentiated HIB1B cells. Expression data are mean ± SD; n=3. Oil Red O-stained cells were de-stained with isopropanol and the amount of staining was quantified by reading absorbance at 510nm. The staining intensity was not significantly different between control and Id1-shRNA cells, and between control and Id1-OE cells.



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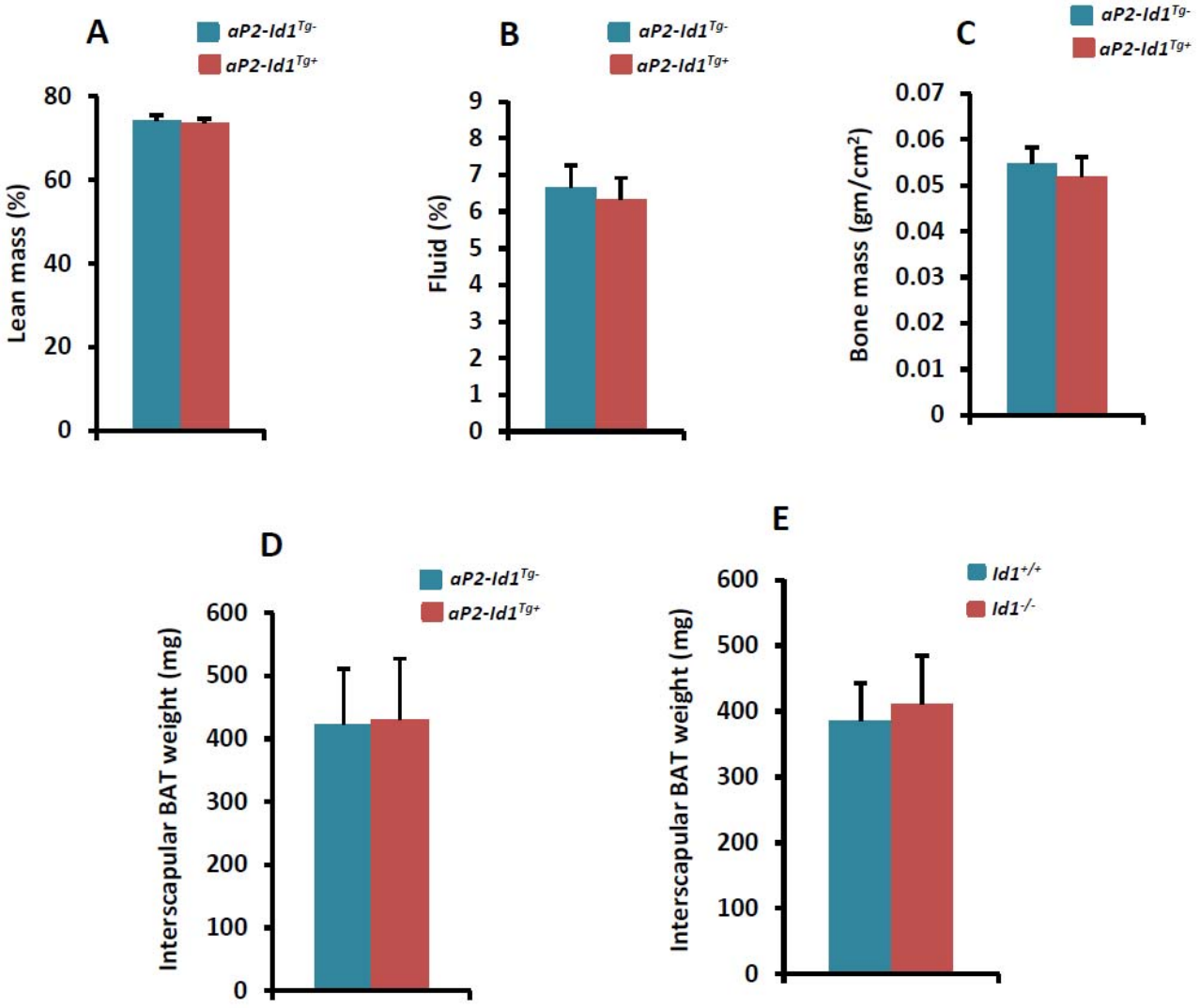
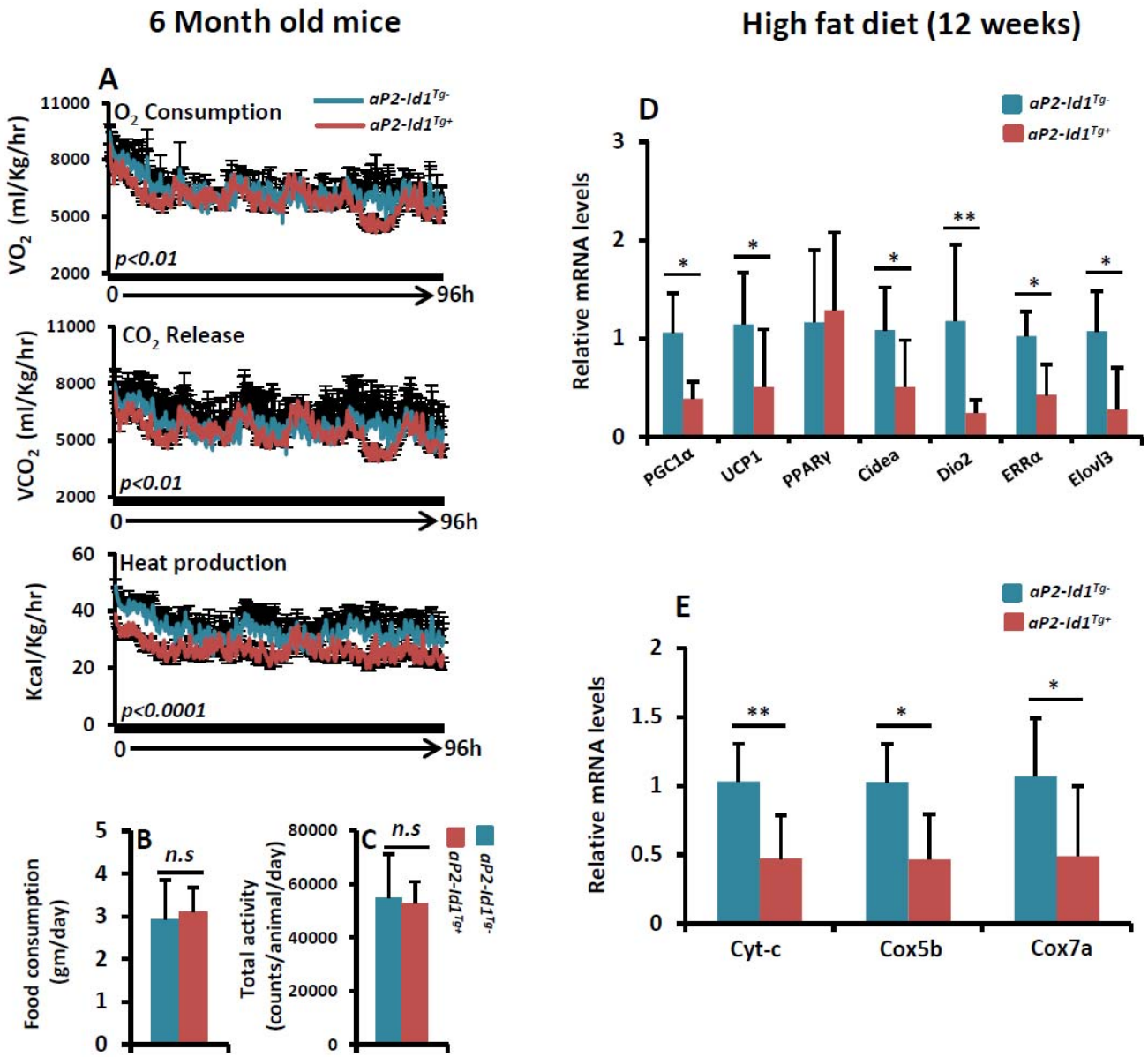
Supplementary Figure 2. Lean mass, bone mass and BAT content are unchanged in *aP2-Id1*$^{Tg+}$ mice. (A-B) Percentage of lean mass (A) and fluid content (B) in 6-month old *aP2-Id1*$^{Tg-}$ and *aP2-Id1*$^{Tg+}$ male mice measured by LF90II BCA-Analyzer (n=5). **(C)** Bone mass in 6-month old *aP2-Id1*$^{Tg-}$ and *aP2-Id1*$^{Tg+}$ male mice measured by Lunar PIXImus2 (n=5). (D-E) Interscapular BAT weight in 6 month old *aP2-Id1*$^{Tg-}$ and *aP2-Id1*$^{Tg+}$, and *Id1*$^{+/+}$ and *Id1*$^{-/-}$ male mice (n=5). All the data are mean ± SD.



Supplementary Figure 3. Reduced energy expenditure in adult *aP2-Id1*$^{Tg+}$ mice, and reduced expression of thermogenic genes in HFD-fed *aP2-Id1*$^{Tg+}$ mice. (A-C) O_2 consumption rate, CO_2 release**,** heat production, food consumption and physical activity in 6 month old *aP2-Id1*$^{Tg-}$ and *aP2-Id1*$^{Tg+}$ male mice measured for 4 days using Oxymax/CLAMS animal monitoring system (n=3). Data are mean ± SD, **p*<0.01, ****p*<0.0001. **(D-E)** Relative mRNA transcript levels of thermogenic and mitochondrial genes in the BAT of *aP2-Id1*$^{Tg-}$ and *aP2-Id1*$^{Tg+}$ male mice after 12 weeks of HFD. Data are mean ± SD, n=5; **p*<0.05, ***p*<0.005.



SUPPLEMENTARY DATA

Supplementary Figure 4. Id1 partially suppresses Ebf2-mediated differentiation of C2C12 cells into brown adipocytes. (A) Ebf2 protein levels in C2C12 and C2C12-Ebf2-expressing cells. **(B)** aP2 expression levels in C2C12 and Ebf2-C2C12 cells at the indicated time points after inducing brown adipogenesis. **(C)** Oil-Red-O staining pictures of day 4 differentiated control and Ebf2-C2C12 cells. Scale bar 20µm. **(D)** Expression levels of Ebf2 and Id1 after co-expressing Id1 in Ebf2-expressing C2C12 cells. **(E)** Oil-Red-O staining pictures of day 4 differentiated Ebf2-C2C12 and Ebf2/Id1-C2C12 cells. Scale bar 20µm. **(F-G)** Relative mRNA transcript levels of indicated genes in day 0 and 4 differentiated Ebf2-C2C12 and Ebf2/Id1-C2C12 cells. Data are mean ± SD, n=3; **p*<0.05, ***p*<0.005. **(H)** Oil-Red-O staining pictures of day 8 differentiated Ebf2-C2C12 and Ebf2/Id1-C2C12 cells. Scale bar 20µm. **(I)** Relative mRNA transcript levels of indicated genes in day 8 differentiated Ebf2-C2C12 and Ebf2/Id1-C2C12 cells. Data are mean ± SD, n=3. **(J)** Western blot showing the expression patterns of Ebf2 and Id1 at the indicated time points after inducing brown adipocyte differentiation in Ebf2-C2C12 and Ebf2/Id1-C2C12 cells. **(K)** Co-IP followed by Western blot showing a direct interaction between Id1 and Ebf2 in C2C12 cells. Input: 2% of IP reaction.