Suppression of the C/EBP family of transcription factors in adipose tissue causes lipodystrophy

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Abstract

Adipose-specific inactivation of both AP-1 and CCAAT-enhancer-binding protein (C/EBP) families of B-ZIP transcription factors in transgenic mice causes severe lipoatrophy. To evaluate whether inactivation of only C/EBP members was critical for lipoatrophy, A-C/EBP, a dominant-negative protein that specifically inhibits the DNA binding of the C/EBP members, was expressed in adipose tissue. For the first 2 weeks after birth, aP2-A-C/EBP mice had no white adipose tissue (WAT), drastically reduced brown adipose tissue (BAT), and exhibited marked hepatic steatosis, hyperinsulinemia, and hyperlipidemia. However, WAT appeared during the third week, coinciding with significantly improved metabolic functioning. In adults, BAT remained reduced, causing cold intolerance. At 30 weeks, the aP2-A-C/EBP mice had only 35% reduced WAT, with clear morphological signs of lipodystrophy in subcutaneous fat. Circulating leptin and adiponectin levels were less than the wild-type levels, and these mice exhibited impaired triglyceride clearance. Insulin resistance, glucose intolerance, and reduced free fatty acid release in response to β 3-adrenergic agonist suggest improper functioning of the residual WAT. Gene expression analysis of inguinal WAT identified reduced mRNA levels of several enzymes involved in fatty acid synthesis and glucose metabolism that are known C/EBP α transcriptional targets. There were increased levels for genes involved in inflammation and muscle differentiation. However, when dermal fibroblasts from aP2-A-C/EBP mice were differentiated into adipocytes in tissue culture, muscle markers were elevated more than the inflammatory markers. These results demonstrate that the C/EBP family is essential for adipose tissue development during the early postnatal period, the regulation of glucose and lipid homeostasis in adults, and the suppression of the muscle lineage.

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Introduction

Studies in humans and mice demonstrate that obesity greatly increases the risk of insulin resistance, dyslipidemia, type 2 diabetes, and cardiovascular diseases (Kopelman 2000, Reitman 2004). Paradoxically, lipodystrophy, a paucity of adipose tissue, also leads to very similar metabolic conditions (Huang-Doran *et al.* 2010), indicating the importance of adipose tissue in the regulation of glucose and lipid homeostasis (Reitman *et al.* 2000, Garg & Misra 2004).

Lipodystrophy in humans comprises a heterogeneous group of rare metabolic disorders characterized by partial or complete loss of adipose tissue (Reitman *et al.* 2000, Garg & Misra 2004). Lipodystrophy is either inherited or acquired, although inherited

lipodystrophic syndromes are exceedingly rare. Etiologically, lipodystrophies are categorized as congenital or acquired, and according to the pattern of adipose tissue loss, it can be classified as generalized (affects the whole body) or partial (affects specific body regions). Mutations in several genes have been identified in congenital generalized lipodystrophy (Fiorenza *et al.* 2011) including 1-acylglycerol-3-phosphate O-acyltransferase 2 (AGPAT2; Agarwal *et al.* 2002, Garg & Misra 2004), Berardinelli-Seip congenital lipodystrophy 2 (BSCL2; Magre *et al.* 2001, Ebihara *et al.* 2004, Garg & Misra 2004), CAVI (Kim *et al.* 2008), and PTRF (Hayashi *et al.* 2009). In the case of rare familial partial lipodystrophy type 1 (FPLD1), although any genetic locus has not yet been identified, numerous genetic mutations have been implicated in other types

of inherited partial lipodystrophy (Fiorenza *et al.* 2011), including the LMNA (lamin A/C; Garg & Misra 2004), peroxisome proliferator-activated receptor- γ (PPAR γ ; Agarwal & Garg 2002, Savage *et al.* 2003), AKT2 (George *et al.* 2004, Tan *et al.* 2007), CIDEA (Rubio-Cabezas *et al.* 2009), and ZMPSTE24 (Agarwal *et al.* 2003).

Lipodystrophy is also common among human immunodeficiency virus (HIV) patients receiving highly active antiviral therapy (Gougeon *et al.* 2004, Koutkia & Grinspoon 2004). The similarity of the metabolic manifestations observed in obesity and lipodystrophy suggests that common pathways might be involved in the pathogenesis of both diseases. The identification of several fat-derived hormones, including leptin, adiponectin, and resistin, has begun to help clarify this issue, although the precise nature of such pathways remains largely unclear (Kershaw & Flier 2004, Steppan & Lazar 2004, Kadowaki & Yamauchi 2005).

Transgenic (TG) mice with fat ablation have been produced by inhibition of adipocyte differentiation and development (Moitra *et al.* 1998, Shimomura *et al.* 1998, Peterfy *et al.* 2001, Yamauchi *et al.* 2001, Laustsen *et al.* 2002) or induction of apoptosis (Ross *et al.* 1993, Burant *et al.* 1997, Pajvani *et al.* 2005). Independent of the actual cause of fat ablation, all these mutant mice demonstrate insulin resistance, hyperinsulinemia, and hyperlipidemia. In some cases, type 2 diabetes was exhibited, the phenotype characteristic of a patient with lipodystrophy. Similar to human lipodystrophy, the severity of metabolic syndrome in mice correlates with the degree of fat loss (Colombo *et al.* 2003).

The A-ZIP/F-1 mouse has severe lipodystrophic diabetes (Moitra *et al.* 1998). In this model, fat ablation was achieved by adipose-specific expression of a dominant-negative (DN) protein that inhibits the function of both the CCAAT-enhancer-binding protein (C/EBP) and the JUN families of transcription factors (Vinson *et al.* 1993) implicated in adipocyte growth and development (Herrera *et al.* 1989, Linhart *et al.* 2001). The resulting TG mice, A-ZIP/F-1, have virtually no white adipose tissue (WAT) and show many features of patients with severe congenital generalized lipodystrophy. The goal of this study was to determine whether the inactivation of only C/EBP members in adipose tissue was critical for the A-ZIP/F-1 phenotype. TG mice were generated that used the adipocyte-specific aP2 enhancer/promoter to express A-C/EBP, a DN protein that specifically inhibits the DNA binding of C/EBP family of B-ZIP transcription factors (Vinson *et al.* 2002). These aP2-A-C/EBP TG mice have a unique biphasic lipodystrophy syndrome. They have severe lipodystrophy and insulin resistance during early postnatal development, but during weaning, adipose tissue grows along with partial recovery from the metabolic syndrome.

Materials and methods

TG mice

The plasmid directing fat-specific expression using the 7621 bp aP2 gene enhancer/promoter (Bernlohr *et al.* 1985) was obtained from Dr M D Lane (Moitra *et al.* 1998). The A-C/EBP DN is a 104-amino acid protein consisting of an N-terminal 9-amino acid FLAG epitope, a 13-amino acid linker, a 21-amino acid-designed acidic amphipathic helix, and a 61-amino acid C/EBP α leucine zipper extending to the natural C-terminus (Krylov *et al.* 1995). We cloned the A-C/EBP dominant negative as a KpnI-SmaI fragment to produce the final construct 'Bluescript aP2 A-C/EBP SV40 polyA'.

For microinjection, a DNA fragment (11 410 bp) containing the aP2 promoter, A-C/EBP open reading frame, SV40 splicing site, and polyA site (Fig. 1A) was obtained by HindIII-NotI digestion and gel purification and injected into the male FVB/N mice (Taketo *et al.* 1991) pronuclei and then screened by PCR. The transgene-specific primers were 934 (5'-TTCAATAG-CACCCTCCT-3') and 933 (5'-TGTCACACCACAGAAG-TAAGGTTCC-3'), giving a 561 bp product. Zfy primer pairs (Koopman *et al.* 1991) producing a 150 bp product were included in equimolar concentration in the

Figure 1 A-C/EBP transgene is expressed in adipose tissue. (A) Schematic representation of the transgene used for adipose-specific expression of A-C/EBP. The open box represents the 7.6 kb aP2 promoter/enhancer, the solid box the 298 bp A-C/EBP DN open reading frame with a FLAG epitope, and the stippled box the ~1.0 kb SV40 small t-antigen splice site and polyadenylation sequences. The unique restriction sites used to form junctions between these three different elements are shown, as well as the 5' and 3' sites used for isolating DNA for microinjection from the plasmid. (B) mRNA expression of A-C/EBP transgene. Total RNA was isolated from wild-type and aP2-A-C/EBP BAT (B), WAT (W), and liver (L) at the indicated ages and hybridized to a A-C/EBP-specific probe (see Materials and methods) (top). All lanes contain 10 μ g RNA except for the indicated lanes. Ethidium bromide staining confirms RNA loading (bottom).

standard multiplex reaction to determine sex. All reactions were performed for 10' at 93 °C using the AmpliTaq Gold system, followed by 30 cycles of 45'' at 94 °C, 30'' at 65 °C, and 45'' at 72 °C and a final extension for 10' at 72 °C. Mice were fed a standard pellet diet (NIH-07, 11% calories from fat; Zeigler Brothers, Inc., Gardners, PA, USA). For tissue collection, mice were killed by cervical dislocation using ketamine (100 mg/kg) and xylazine (10 mg/kg) anesthetics, which were delivered intraperitoneally. All mice were kept on a 12 h light:12 h darkness cycle, with light beginning at 0600 h and dark at 1800 h. The animal study protocol was approved by the IACUC, and the study was carried out following appropriate guidelines.

Primary cultures of dermal fibroblasts

Dermal fibroblasts were cultured from newborn wild-type or TG mice that express the A-C/EBP-dominant negative under the control of the 422 adipocyte-specific promoter (Moitra *et al.* 1998) according to Lichti *et al.* (2008). Primary dermal fibroblasts were seeded at a density of one mouse dermis per 10 cm dish or equivalent in DMEM/F12: GlutaMAX medium (Invitrogen) with 10% FBS. Adipogenesis in the cultured cells was induced as described earlier (Qi *et al.* 2003, Rishi *et al.* 2010). Briefly, after cells grew to confluence, cultures were treated with 0.5 mM 3-isobutyl-1-methyl-xanthine (IBMX; Sigma), 1 µM dexamethasone (Sigma), 5 µg/ml insulin (Sigma), and 0.5 µM rosiglitazone for 48 h. Cells were then changed to medium containing only 5 µg/ml insulin (Sigma) and 0.5 µM rosiglitazone. The induction lasted for 8 days with the medium being replaced every 2 days. Cells with fat droplets, indicative of adipogenesis, were revealed by Oil Red O (Sigma) staining.

Isolation and analysis of RNA

RNA was initially extracted using RNeasy kits (Qiagen), as per the manufacturer's protocol. Nucleic acid elutes from the RNeasy spin columns were treated with DNaseI in the presence of 1 mM MgCl₂ for 30 min at 37 °C, treated twice with acid phenol (Ambion, Austin, TX, USA) in heavy Phase Lock tubes (Eppendorf, Hamburg, Germany), and the aqueous supernatant precipitated successively with isopropanol and 95% ethanol. Northern blots (Maximum Strength Nytran Plus; Schleicher & Schuell) were hybridized using UltraHyb (Ambion) at 42 °C overnight, washed for 30 min at medium stringency (2×SSC+0.2% SDS at 68 °C), and exposed to film or quantitated with a Phosphorimager. Probes used were specific for A-C/EBP (1100 bp HindIII 3'-UTR fragment including only SV40 sequences).

Microarray analysis

Total RNA was isolated from the inguinal fat of 6-month-old wild-type and aP2-A-C/EBP male mice and from primary dermal fibroblast cultures of wild type and aP2-A-C/EBP induced for adipogenic differentiation. cDNA was synthesized from 5 µg of total RNA using the SuperScript Double-Stranded cDNA Synthesis Kit (Invitrogen). This was amplified to cRNA and labeled with biotin using the BioArray RNA Transcript Labeling Kit (Enzo, New York, NY, USA). The labeled cRNA samples were hybridized to Affymetrix GeneChip Mouse Genome 430 2.0 Array allowing to analyze 39 000 transcripts. The arrays were washed and stained in the Affymetrix GeneChip Fluidics Station 450 and scanned using Affymetrix GeneChip Scanner 3000.

Biochemical assays

Blood was collected in non-fasted state mice from their tail or retro-orbital vein at 0900 h. Glucose was measured from whole blood or serum using a Glucometer Elite (Bayer). Serum insulin, leptin, adiponectin, and resistin were measured by RIA (Linco, St. Charles, MO, USA). Serum triglycerides (Thermo Electron, Louisville, CO, USA. # 2780-400H) and free fatty acids (Roche Diagnostics GmbH, #1 383 175) were measured using indicated colorimetric assay. Tissue triglycerides were measured as described (Colombo *et al.* 2003).

Body composition

Body composition was measured in non-anesthetized 30-week-old male and female mice using the Bruker minispec NMR analyzer mq10 (Bruker, Woodlands, TX, USA; males) or Echo 3-in-1 NMR analyzer (Echo Medical Systems, Houston, TX, USA; females) under the manufacture's settings (Bruker Optics, Inc., Woodlands, TX, USA). Testing was conducted in randomly fed mice between 0900 and 1100 h.

Indirect calorimetry

Oxygen consumption and carbon dioxide production were measured in 30-week-old male and female mice using Oxymax Indirect Calorimetry System (Columbus Instruments, Columbus, OH, USA), with one mouse per chamber, testing TG mice simultaneously with controls (Gavrilova *et al.* 2000b). Mice had free access to food and water. Motor activity was determined by infrared beam interruption (Opto-Varimex mini; Columbus Instruments). Daily metabolic rate was measured at 24 °C for 24 h after a 24 h acclimatization period. Resting oxygen consumption was calculated as the average of the points with less than six ambulating

beam breaks per minute. The respiratory exchange ratio (RER; the ratio of carbon dioxide produced to oxygen consumed) was calculated from the resting data points. Oxidation of carbohydrate produces RER of 1.00, whereas fatty acid oxidation results in RER of ~ 0.70 . The effect of the $\beta 3$ -adrenergic agonist, CL316, 243, was measured in 30-week-old male mice at 30 °C with each mouse serving as its own control. Mice were allowed to acclimate to calorimetry chambers for 24 h. The baseline data were collected on the following day from 1000 to 1300 h. CL316, 243 was injected i.p. (1 mg/kg in saline) at 1300 h, and after a 1 h delay, data were collected for 3 h. Oxygen consumption data were normalized to body weight and expressed in ml/kg per min.

Food intake

Food intake was measured in male and female mice at 29 weeks. Mice were caged individually, and the amount of food in the feeding container was measured at day 0 and day 5. Food intake was expressed as g/mouse per day and was also normalized to body weight (mg/g body weight per day).

Cold tolerance

Female mice aged 30 weeks were individually caged with free access to food and water, but without bedding. Mice were placed in cold room at a temperature of 4 °C at 0900 h and the body temperature was measured hourly for 8 h using a rectal probe (Thermalert TH-5, Physitemp, Clifton, NJ, USA).

In vivo assays of glucose homeostasis

Insulin tolerance test was performed at 0900 h in non-fasted 32-week-old male and female mice. Recombinant human insulin (Humulin R, Eli Lilly) was injected i.p. (0.75 IU/kg). Blood glucose levels were measured 0, 15, 30, 45, and 60 min after the injection using glucometer. Glucose tolerance was tested in 30-week-old male and female mice that had been fasted for 6 h. Glucose was injected i.p. (2 g/kg) at 1400 h and its levels in blood were measured at 0, 15, 30, 60, and 120 min after the injection. *In vivo* glucose uptake into muscle and adipose tissue was measured in 36-week-old male mice in a non-fasted state. At 0900 h, mice were injected i.p. with (1-14C) 2-deoxyglucose (2-DG) (10 μ Ci; ICN Radiochemicals, Inc., Irvine, CA, USA) and insulin (0.75 IU/kg, Humulin R, Eli Lilly). After 45 min, tissues were removed and the (14C) 6-deoxyglucose 6-phosphate in muscle and fat was quantitated (Kim *et al.* 1996).

Triglyceride clearance

Triglyceride clearance was measured in 24-week-old male and female mice fasted for 4 h (from 0800 to 1200 h) and then gavaged with 400 μ l peanut oil (Colombo *et al.* 2003). Blood was taken hourly via tail vein for 6 h, and plasma triglyceride was measured colorimetrically.

Western blotting

For protein analysis by western blotting, tissue/cell lysates were prepared from inguinal fat or induced primary dermal fibroblasts. Inguinal fat tissues from 6-month-old male mice were collected, snap frozen in liquid nitrogen, and ground with a mortar and pestle. The tissue was lysed in modified RIPA buffer containing 50 mM Tris-Cl, 150 mM NaCl, 0.5% NP-40, 1% Triton-X, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, protease inhibitor (Complete Protease Inhibitor Cocktail Tablet, Roche), 10 mM NaF, 1 mM sodium vanadate, and 1 mM phenylmethylsulphonyl fluoride (PMSF). The lysate was centrifuged two times at $15000 \times g$ at 4 °C for 30 min and the infranant was collected carefully without disturbing the upper layer of triglycerides and free fatty acids (FFA). The whole cell lysates from primary cultures were prepared in RIPA buffer containing 50 mM Tris-Cl, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1 mM EGTA, 5 mM EDTA, 10 mM NaF, 1 mM β -glycerophosphate, 1 mM sodium vanadate, and 1 mM PMSF. Protein concentrations were measured using a Bradford Protein Assay reagent (Bio-Rad) and equal amounts were loaded onto the gel. Proteins were resolved on NuPAGE 4–12% Bis-Tris gels (Invitrogen) and blotted onto polyvinylidene fluoride (PVDF) membranes (Hybond-P, Amersham Biosciences). Membranes were blocked in 5% skim milk for 1 h at room temperature and incubated for another hour with the required primary antibodies followed by three washes, at 5 min each, of PBS with 0.1% Tween 20 (Sigma Chem, Inc.). After washing, the blots were incubated for 1 h with secondary antibodies against rabbit or mouse IgG (Amersham Biosciences, 1:5000) and washed three times, at 5 min each. Blots were developed using ECL plus western blotting detection system (Amersham Biosciences). The following primary antibodies were used: polyclonal rabbit anti-myomesin-2 (sc-50435; Santa Cruz Biotechnology, Santa Cruz, CA, USA), Polyclonal goat anti-Steroyl-CoA desaturase 1 (SCD1) (sc-14719; Santa Cruz Biotechnology), and polyclonal rabbit anti-FLAG. All washes and dilutions were carried out using PBS with 0.1% Tween 20 (Sigma Chem, Inc.).

Statistical analysis

The gene expression profile consists of RMA-extracted log-transformed expression ratios measured on the full set of genes represented on the microarray. Analyses were performed with the software package BRB array tools, developed by the Biometric Research Branch of the US National Cancer Institute. Genes were filtered based on *T*-test (one tailed, two-sampled equal variance), P value ≤ 0.05 . The filtered gene list was used to classify the misregulated genes in aP2-A-C/EBP mice compared with the wild type at a cut-off value > 1.5 -fold change in expression (ratio between mean expression of two TG and three WT). The misregulated genes were used to identify the relatively enriched (P value < 0.01) Gene Ontology (GO) categories using Gene Ontology Tree Machine (GOTM) software developed and maintained by Vanderbilt University (available at: <http://bioinfo.vanderbilt.edu/gotm>). The significantly enriched GO categories were then manually grouped into functionally related classes. The statistical significance (P value) of genes appearing in these functional classes was determined using Fisher's exact test.

Values for other biochemical and physiological assays have been reported as mean $\pm$ S.E.M. Statistical significance was determined using ANOVA followed by *t*-tests, with differences considered as significant at $P < 0.05$. The Holm–Sidak test was performed for pairwise multiple comparisons.

Results

Generation of the aP2-A-C/EBP-1 TG mouse line

A-C/EBP is a DN protein that specifically heterodimerizes with C/EBP family members and is able to inhibit the DNA binding of C/EBP family members in an equimolar competition assay (Vinson *et al.* 2002). The A-C/EBP protein contains the C/EBP α leucine zipper and a designed acidic protein sequence that replaces the C/EBP basic region. The acidic protein sequence heterodimerizes with the basic region of endogenous C/EBP proteins by forming a coiled coil extension of the leucine zipper (Krylov *et al.* 1995, Olive *et al.* 1997, Ahn *et al.* 1998). We expressed the A-C/EBP protein selectively in adipose tissue by using the aP2 promoter/enhancer as described previously (Fig. 1A; Moitra *et al.* 1998). The founder with the most severe phenotype, termed aP2-A-C/EBP, is described here.

The expression of A-C/EBP DN transcript was detectable in brown adipose tissue (BAT) from 3 days after birth expressed to adulthood but was undetectable in liver (Fig. 1B). TG WAT grew into a biochemically traceable sample only after 3 weeks of age and contained less A-C/EBP transcript than BAT.

Morphological characteristics of young aP2-A-C/EBP mice

The aP2-A-C/EBP pups were similar to the wild-type littermates in either body weight or length at birth (Fig. 2A and B) but showed slower growth during the subsequent 4 weeks of life. At birth, BAT weight was 50% less in aP2-A-C/EBP mice than in wild-type mice and it progressively decreased with age (Fig. 2C). WAT was undetectable in aP2-A-C/EBP mice at birth but started to appear in its normal anatomical locations at week 3. The lower body weight of aP2-A-C/EBP mice can be partially explained by the lack of WAT. Histological examination of BAT from aP2-A-C/EBP mice identified patches of cells containing large fat droplets that were not detected in the wild-type BAT but resembled white adipocytes (Fig. 2E). The BAT from aP2-A-C/EBP mice is similar to the BAT from aP2-A-ZIP/F mice (Moitra *et al.* 1998). Subdermal white adipocytes histologically observed in 1-day-old wild-type pups were almost completely absent in the aP2-A-C/EBP pups (Fig. 2F).

At birth, the aP2-A-C/EBP liver was similar to the wild-type liver in terms of both weight and reddish color; however, by day 1, it was 75% heavier than the wild-type littermates and was pale in color (Fig. 2D and G). Histological examination of the aP2-A-C/EBP liver revealed massive accumulation of lipid in hepatocytes (Fig. 2H). The aP2-A-C/EBP liver remained large and fatty until day 21 (weaning); however, by day 28, the gross difference between the wild-type and the aP2-A-C/EBP liver in weight and color disappeared (Fig. 2D), coinciding with the appearance of WAT.

Insulin resistance and dyslipidemia in young aP2-A-C/EBP mice

During the first 4 weeks of development, serum was analyzed to determine the levels of glucose, insulin, triglyceride, and free fatty acids in wild-type and aP2-A-C/EBP mice (Fig. 2I–L). During the first week of life, the aP2-A-C/EBP mice had significantly high levels of blood glucose (Fig. 2I), whereas serum insulin levels (Fig. 2J) were dramatically elevated, 20–60 times higher than that in controls. During the second week, hyperinsulinemia gradually decreased to wild-type levels by week 4. Serum triglyceride (Fig. 2K) and free fatty acids (Fig. 2L) were also elevated in young aP2-A-C/EBP mice and gradually normalized by weaning, coinciding with appearance of WAT.

Morphological characteristics of 6-month-old aP2-A-C/EBP mice

Body weights of adult aP2-A-C/EBP mice were slightly less than wild-type mice, particularly in females (Fig. 3A). There was a reduction in fat depots

Figure 2 Morphological, metabolic, and histological characteristics of young aP2-A-C/EBP mice. (A–D) and (I–L) show data from time-course experiments (age of mice: 1, 3, 5, 10, 12, 15, 18, 21, and 28 days) on aP2-A-C/EBP (open circle) and wild-type littermate control mice (filled circle). Data are presented as mean $\pm$ S.E.M. As there was no big difference between mice of different sex before 1 month of age, data from males and females were combined together. Asterisks indicate significant (*t*-test, $P < 0.05$) difference between wild-type and aP2-A-C/EBP pups. Number of mice used for time-course experiments are shown in Table 1. (A) Body weight (in gms): the aP2-A-C/EBP pups at birth are similar in weight to wild-type littermates but they start to lag significantly (24% lighter; *t*-test, $P < 0.001$) by day 3. (B) Body length (in mm). (C) BAT (percent body weight): at birth, transgenic pups had 43% (*t*-test, $P < 0.001$) lower BAT compared with the wild type, the difference reduced while they underwent a growth spurt until day 18, then aP2-A-C/EBP BAT again regressed in weight and was on average 65% smaller (*t*-test, $P < 0.001$) than in controls by weaning. (D) Liver (percent body weight): at day 1, aP2-A-C/EBP pups had 67% larger livers than wild types (*t*-test, $P < 0.001$) and became 135% larger (*t*-test, $P < 0.001$) in a week. Then, aP2 A-C/EBP liver regressed over the next 3 weeks to reach the same relative weight as wild type. (E–H) show histology and gross appearance of selected organs in aP2-A-C/EBP pups (panels on the right) and wild-type littermates (panels on the left). (E) Hematoxylin and eosin (H and E) staining of BAT sections from 1-day-old pups ($200\times$ magnification). BAT in transgenic animals has a larger proportion of WAT-like cells but looks mostly normal. (F) H and E staining of skin section (magnification $200\times$) of 1-day-old pups. Note the almost complete absence of WAT-like cells in the subdermal region of transgenic mice. (G) Livers in the aP2-A-C/EBP mice at day 1 of life are pale in color suggesting lipid accumulation. (H) H and E staining of liver sections. Hepatocytes of transgenic mice appeared filled with lipid droplets. (I–L) Serum biochemistry in aP2-A-C/EBP pups and wild-type littermates from birth till weaning; I, serum glucose (in mg/dl); J, serum insulin (in ng/ml); K, serum triglyceride (in mg/dl); L, serum-free fatty acids (in mM).

throughout the body and a severe reduction of BAT (Fig. 3B). NMR analysis of the body composition showed a 35% decrease in the amount of fat in TG mice compared with the wild-type controls, with no change in lean body mass (Table 2). To examine whether different fat depots were affected similarly, individual fat pad weights were determined (Fig. 3C).

BAT was the most affected, being 85 and 70% smaller in males and females respectively, compared with wild-type controls. WAT depots in aP2-A-C/EBP males (inguinal, epididymal, retroperitoneal, and mesenteric) were decreased in weight by 32–46%. In TG females, all other WAT depots were significantly reduced in size except for the parametrial fat pad.

Table 1 Number of mice used for time-course experiments (Fig. 2A–D and I–L). Data on various parameters were collected on days 1, 3, 5, 7, 10, 12, 15, 18, 21 and 28 days after birth from wild-type and aP2-A-C/EBP litter mates and presented in Fig. 2. Numbers of mice used in each group are presented in this table

Parameter	Genotype	Age (days)									
		1	3	5	7	10	12	15	18	21	28
Body weight (Fig. 2A)	WT	14	27	16	14	10	11	11	6	13	17
	A-C/EBP	15	23	17	11	10	9	12	7	9	22
Body length (Fig. 2B)	WT	14	19	13	14	8	11	8	6	13	8
	A-C/EBP	20	10	14	11	8	9	7	7	9	6
BAT weight (Fig. 2C)	WT	14	19	9	8	8	9	8	6	10	6
	A-C/EBP	20	10	7	8	8	9	7	7	5	8
Liver weight (Fig. 2D)	WT	14	10	12	11	10	9	11	6	11	6
	A-C/EBP	9	10	10	11	10	9	12	7	8	8
Blood glucose (Fig. 2I)	WT	14	15	16	14	10	11	11	6	11	6
	A-C/EBP	15	20	17	11	10	9	12	7	8	8
Serum insulin (Fig. 2J)	WT	4	6	6	6	5	6	ND	6	5	ND
	A-C/EBP	6	6	6	5	5	6	ND	6	5	ND
Serum triglyceride (Fig. 2K)	WT	5	6	6	6	5	6	6	6	6	6
	A-C/EBP	4	5	5	5	4	6	4	6	5	5
Serum FFA (Fig. 2L)	WT	4	6	12	6	5	6	6	6	6	6
	A-C/EBP	6	5	12	6	3	5	4	6	5	4

Wild-type BAT consists of mitochondria-rich eosinophilic cells containing multiple lipid droplets. Wild-type WAT cells, in contrast, are larger containing a single large lipid droplet with the nucleus at the cell periphery. The aP2-A-C/EBP BAT showed sparse eosinophilic staining, a single lipid droplet per cell, and peripheral nuclei, resembling BAT observed in the aP2-A-ZIP/F-1 mouse (Moitra *et al.* 1998). Histological examination of epididymal WAT was similar in the aP2-A-C/EBP and wild-type mice. In contrast, the aP2-A-C/EBP inguinal fat revealed smaller adipocytes and presence of stretches of fibroblast-like cells unlike the wild-type fat (Fig. 3D). In addition, there was an increase in the number of lymphocytes.

In both male and female aP2-A-C/EBP mice, spleen and livers were enlarged (Table 2). In addition, TG males had bigger kidneys and hearts, similar to the A-ZIP/F model of complete lipotrophy (Moitra *et al.* 1998).

Cold sensitivity in aP2-A-C/EBP mice

BAT is a major site of adaptive thermogenesis in small mammals (Nicholls & Locke 1984, Himms-Hagen *et al.* 1994). Because aP2-A-C/EBP mice had dramatically reduced and apparently inactive BAT, we analyzed metabolic rate and cold sensitivity in 6-month-old mice. The basal metabolic rate (per mouse or normalized to fat-free mass) was comparable in the aP2-A-C/EBP and control mice (data not shown); however, when the data were normalized to total body weight, the metabolic rate of TG mice

was nearly 15% higher than the wild-type controls (Table 2). Relatively elevated metabolic rate was likely to be balanced with significantly increased food intake in the aP2-A-C/EBP mice (Table 2). To assess thermogenic capacity of the aP2-A-C/EBP male mice, we stimulated them with the β 3-adrenergic agonist, CL316243, which induces lipolysis in WAT and thermogenesis in BAT (Himms-Hagen *et al.* 1994). In response to CL316243, the wild-type mice doubled their metabolic rate, whereas the aP2-A-C/EBP mice increased their metabolic rate only by 50%, suggesting a defect in thermogenesis (Fig. 4A). Both wild-type and TG mice responded to β 3-adrenergic stimulation by lowering RER. However, the drop was significantly smaller in TG mice, indicating a relative decrease in fatty acid oxidation (Fig. 4A). To assess WAT responsiveness to the β 3-agonist, we measured *in vivo* FFA release in blood (Fig. 4B). Within 20 min of acute stimulation with CL316243, wild-type mice increased FFA threefold; however, the response was significantly less ($P=0.003$) in TG mice. Thus, the aP2-A-C/EBP mice have an impaired response to β 3-adrenergic stimulation. This disparity can lead to cold intolerance as evident in the aP2-A-C/EBP female mice that were unable to maintain normal body temperature at 4 °C (Fig. 4C).

Metabolic characteristics of the adult aP2-A-C/EBP mice

Both male and female (6-month old) aP2-A-C/EBP mice had normal glucose serum levels (Table 2). Insulin serum

Figure 3 Characteristics and gross appearance of aP2-A-C/EBP mice at 30 weeks. (A) Growth curves of male (top) and female (bottom) mice; data are mean $\pm$ s.d. for $n=7-32$. The A-C/EBP mice did not weigh any different from the wild-type littermates at birth, but by weaning, were smaller on the average by 25% for females and 30% for males respectively. This difference in weights persisted for female aP2-A-C/EBP mice throughout their lives, but for the males again becomes apparent only after 25 weeks. (B) Gross appearance of male aP2-A-C/EBP mice (mouse to the right) compared with the male wild-type littermate (mouse to the left) at 30 weeks of age. Arrows show decreased amount of s.c. fat in transgenic mouse. (C) Weight of selected fat pads in aP2-A-C/EBP mice (open bar) and wild-type littermates (filled bar); abbreviations used are: Ing, inguinal fat; Epi, epididymal fat; Para, parametrial fat; Retro, retroperitoneal fat; Mes, mesenteric fat; BAT, brown fat. Data are mean $\pm$ s.e.m., $n=7-11$ per group (males nine WT, 11 TG; females nine WT, ten TG). The amount of inguinal, retroperitoneal, and mesenteric fat and BAT was decreased by 59, 51, 40, and 70% respectively. (D) H and E staining of inguinal fat from aP2-A-C/EBP (panel on the right) and wild-type littermate (panel on the left).

levels were significantly elevated only in aP2-A-C/EBP males (76%). Both male and female aP2-A-C/EBP mice had elevated serum triglyceride levels.

Glucose and insulin tolerance tests were used to characterize glucose metabolism in more detail (Fig. 4D and E). The aP2-A-C/EBP males showed delayed clearance of glucose from the circulation after glucose load and a blunted response to insulin, the phenotype consistent with insulin resistance. Contrarily, the aP2-A-C/EBP females behaved similar to the wild-type female littermate having normal glucose serum levels. To analyze whether glucose uptake was impaired in the aP2-A-C/EBP male mice, we measured 2-deoxyglucose uptake into skeletal muscle, BAT, and epididymal and

inguinal fat (Fig. 4F). Glucose uptake into muscle and BAT was significantly reduced in TG male mice compared with controls (by 32% and 56% respectively), whereas uptake into WAT was similar in both genotypes.

To determine whether impaired clearance of triglycerides might be causing the increase in serum triglyceride, we performed a triglyceride tolerance test (Fig. 4G). After oral lipid load delivery, both male and female aP2-A-C/EBP mice demonstrated delayed triglyceride clearance, similar to mice with complete lipoatrophy (Colombo *et al.* 2003, Gavrilova *et al.* 2003). Taken together, these data demonstrate that despite only a modest reduction of total fat, the aP2-A-C/EBP mice have abnormalities in glucose and lipid metabolism.

Table 2 Characteristics of aP2-A-C/EBP mice at 30 weeks of age. Data are mean $\pm$ S.E.M. ($n=6-12$)

Characteristic	Male		Female	
	WT	A-C/EBP	WT	A-C/EBP
Body weight (g)	36.9 $\pm$ 1.4 ($n=9$)	35.5 $\pm$ 1.1 ($n=11$)	32.7 $\pm$ 2.5 ($n=7$)	30.6 $\pm$ 1.3 ($n=12$)
Fat mass (g)	4.7 $\pm$ 0.4 ($n=6$)	2.78 $\pm$ 0.3* ($n=6$)	8.4 $\pm$ 1.2 ($n=7$)	5.1 $\pm$ 0.59* ($n=9$)
Lean mass (g)	26.7 $\pm$ 1.1 ($n=6$)	25.7 $\pm$ 0.9 ($n=6$)	22.6 $\pm$ 0.6 ($n=7$)	23.5 $\pm$ 0.85 ($n=9$)
Body length (mm)	90.1 $\pm$ 1.4 ($n=9$)	89 $\pm$ 1.0 ($n=11$)	85.6 $\pm$ 1.5 ($n=7$)	84.2 $\pm$ 1.1 ($n=12$)
Liver (% body weight)	4.7 $\pm$ 0.1 ($n=9$)	5.7 $\pm$ 0.2* ($n=11$)	4.4 $\pm$ 0.2 ($n=7$)	5.6 $\pm$ 0.2* ($n=12$)
Kidney (% body weight)	1.4 $\pm$ 0.0 ($n=9$)	1.6 $\pm$ 0.1* ($n=11$)	1.1 $\pm$ 0.0 ($n=7$)	1.2 $\pm$ 0.1 ($n=12$)
Spleen (% body weight)	0.32 $\pm$ 0.1 ($n=9$)	0.47 $\pm$ 0.0* ($n=11$)	0.41 $\pm$ 0.0 ($n=7$)	0.56 $\pm$ 0.0* ($n=12$)
Heart (% body weight)	0.45 $\pm$ 0.0 ($n=9$)	0.49 $\pm$ 0.0* ($n=11$)	0.40 $\pm$ 0.0 ($n=7$)	0.45 $\pm$ 0.0 ($n=12$)
Muscle mass (% body weight)	76.6 $\pm$ 0.7 ($n=6$)	81.4 $\pm$ 0.4* ($n=6$)	71.8 $\pm$ 2.6 ($n=7$)	80.9 $\pm$ 1.4* ($n=9$)
WAT mass (% body weight)	13.4 $\pm$ 0.9 ($n=6$)	8.7 $\pm$ 0.6* ($n=6$)	26.2 $\pm$ 3.0 ($n=7$)	17.1 $\pm$ 1.4* ($n=9$)
Resting oxygen consumption (ml/kg per h)	2748 $\pm$ 93 ($n=6$)	3130 $\pm$ 74* ($n=6$)	3610 $\pm$ 120 ($n=7$)	4202 $\pm$ 122* ($n=9$)
Total oxygen consumption (ml/kg per h)	3281 $\pm$ 87 ($n=6$)	3937 $\pm$ 74* ($n=6$)	4404 $\pm$ 152 ($n=7$)	5088 $\pm$ 108* ($n=9$)
Food intake (g/mouse per day)	2.9 $\pm$ 0.2 ($n=6$)	3.5 $\pm$ 0.2* ($n=6$)	3.1 $\pm$ 0.1 ($n=7$)	3.1 $\pm$ 0.1 ($n=9$)
Food intake (mg/g per day)	84 $\pm$ 8 ($n=6$)	114 $\pm$ 12* ($n=6$)	95 $\pm$ 3 ($n=7$)	106 $\pm$ 5* ($n=9$)
Serum glucose (mg/dl)	183 $\pm$ 11 ($n=10$)	186 $\pm$ 11 ($n=12$)	172 $\pm$ 9 ($n=7$)	173 $\pm$ 9 ($n=12$)
Serum insulin (ng/ml)	1.7 $\pm$ 0.2 ($n=10$)	3.0 $\pm$ 0.6* ($n=12$)	1.0 $\pm$ 0.2 ($n=7$)	1.4 $\pm$ 0.3 ($n=12$)
Serum triglyceride (mg/dl)	216 $\pm$ 32 ($n=10$)	269 $\pm$ 22 ($n=12$)	164 $\pm$ 30 ($n=7$)	211 $\pm$ 21 ($n=12$)
Serum-free fatty acids (mM)	0.37 $\pm$ 0.4 ($n=9$)	0.32 $\pm$ 0.3 ($n=12$)	0.39 $\pm$ 0.1 ($n=6$)	0.48 $\pm$ 0.1 ($n=12$)
Serum leptin (ng/ml)	12.3 $\pm$ 2.2 ($n=9$)	4.1 $\pm$ 0.5* ($n=9$)	15.7 $\pm$ 4.1 ($n=7$)	5.6 $\pm$ 0.7* ($n=10$)
Serum adiponectin (μ g/ml)	9.6 $\pm$ 1.2 ($n=9$)	2.7 $\pm$ 0.3* ($n=9$)	20.3 $\pm$ 2.1 ($n=7$)	4.8 $\pm$ 0.7* ($n=10$)
Serum resistin (ng/ml)	9.0 $\pm$ 0.5 ($n=11$)	7.0 $\pm$ 1.1* ($n=10$)	9.1 $\pm$ 0.6 ($n=7$)	7.5 $\pm$ 0.5* ($n=9$)
Liver triglyceride (μ mol/g)	8.2 $\pm$ 0.6 ($n=6$)	8.5 $\pm$ 0.8 ($n=6$)	15.0 $\pm$ 0.6 ($n=6$)	12.7 $\pm$ 1.7 ($n=6$)
Muscle triglyceride (μ mol/g)	12.0 $\pm$ 1.2 ($n=6$)	12.5 $\pm$ 1.0 ($n=6$)	15.8 $\pm$ 1.4 ($n=6$)	12.9 $\pm$ 2.2 ($n=6$)

Asterisk (*) Indicates significant difference ($P<0.05$) between WT and A-C/EBP mice within each gender group presented in bold.

Circulating adipokine levels in aP2-A-C/EBP mice

Three serum adipokines were measured to explore the mechanisms underlying the metabolic syndrome in adult aP2-A-C/EBP mice (Fig. 5). Leptin levels were reduced in both males and females and positively correlated with percent body fat (Fig. 5A and B). Consistent with previously published data (Combs *et al.* 2003), wild-type females had higher adiponectin levels than males (Fig. 5C). In both male and female aP2-A-C/EBP mice, adiponectin levels were significantly lower when compared with wild-type controls (by 76 and 72% respectively). Interestingly, TG mice had lower adiponectin levels even after the normalization for percent of fat (Fig. 5D). Resistin levels were also significantly reduced in TG mice (22 and 18%, males and females respectively) and did not show a clear correlation with percent body fat (Fig. 5E and F).

Gene expression changes in aP2-A-C/EBP inguinal fat

We determined the global mRNA expression profiles from inguinal fat of 6-month-old wild-type and aP2-A-C/EBP male mice. Totally, 542 transcripts were significantly ($P<0.05$) misregulated by 1.5-fold, with similar numbers being downregulated (246) and upregulated (296) in the aP2-A-C/EBP mice (Fig. 6A). Potential transcriptional targets of the C/EBP family

members were among the downregulated genes. Among the enriched GO terms for downregulated genes, 37 genes were grouped in the GO category of metabolism of lipid, alcohol, or carbohydrate (Table 3). These include the well-known adipose genes (Table 4) *Scd1*, *Scd2*, *Agpat2*, adiponectin receptor 2 (*Adipor2*), and glycerol-3-phosphate dehydrogenase 1 (soluble) (*Gpd1*). The genes involved in fat cell differentiation include the adrenergic receptor, beta 1 (*Adrb1*), lipin 1 (*Lpin1*), and *Ppar γ* . The depressed mRNA expression of adipokine genes (Table 4) in the aP2-A-C/EBP inguinal depot was consistent with their lower circulating protein levels (Table 2).

Among the upregulated genes, 62 GO categories were enriched ($P<0.01$), and the most prominent enriched GO terms are muscle related (18 categories) or immune related (16 categories). Of the upregulated genes, 30 identified in the enriched GO categories were involved in the muscle-related biological processes like muscle contraction (15 genes), muscle development (nine genes), and cytoskeletal organogenesis and biogenesis (18 genes; Table 3).

Adipocytes from dermal fibroblast cultures do not express immuno-related genes

We differentiated newborn primary dermal fibroblast cultures from wild-type and aP2-A-C/EBP mice (Lichti

Figure 4 Characteristics of aP2-A-C/EBP mice at 30 weeks. (A) Oxygen consumption (panel on the left) and RER (panel on the right) were measured before (solid bars) and after CL316, 243 (hatched bars) injection at 30 °C. Note blunted response to β 3-adrenergic agonist, CL316, 243, in aP2-A-C/EBP mice. Data are mean $\pm$ S.E.M., $n=8$ per group. (B) Free fatty acid release in response to β 3 adrenergic agonist was significantly less ($P=0.003$) in aP2-A-C/EBP mice. The mice were injected with CL316, 243 (1 mg/kg i.p.), and free fatty acids in serum were measured at 0, 20, and 120 min, $n=6$ per group. (C) aP2-A-C/EBP mice are cold intolerant ($P=0.033$). The mice were placed at 4 °C, and rectal temperature was measured hourly (0–8 h). The graphs show body temperature of individual mice (four TG and five WT). Significant ($P<0.05$) differences were observed starting from hour 5. (D) Glucose tolerance test: mice (littermates) were injected with glucose (2 g/kg) i.p. Data are mean $\pm$ S.E.M., $n=7$ –12 per group (males ten WT, 12 TG; females seven WT, ten TG) at 0, 15, 30, 60, and 120 min after injection. Male aP2-A-C/EBP mice showed significantly delayed clearance of glucose ($P<0.05$ at 60 and 120 min). (E) Insulin tolerance test: mice (littermates) were injected with insulin (0.75 IU/kg). Data are mean $\pm$ S.E.M., $n=7$ –13 per group (males ten WT, 13 TG; females seven WT, ten TG) at 0, 15, 30, 45, and 60 min after injection. Male aP2-A-C/EBP mice showed significantly less response to insulin ($P<0.05$ at 45 and 60 min). (F) 2-deoxyglucose (2-DG) uptake into skeletal muscle and adipose tissue: aP2-A-C/EBP mice (open bar) and wild-type littermates (filled bar) were injected with (1–14C) 2-deoxyglucose and insulin (0.75 IU/kg). SM, skeletal muscle, gastrocnemius; BAT, brown fat; Epi, epididymal fat; Ing, inguinal fat. (G) Triglyceride clearance after oral lipid load: mice (littermates) were gavaged with 400 μ l peanut oil, and plasma triglyceride levels were measured hourly (0–6 h). Data are mean $\pm$ S.E.M., $n=7$ –11 per group (males ten WT, 11 TG; females seven WT, ten TG). aP2-A-C/EBP mice showed delayed triglyceride clearance ($P<0.05$ at 3, 4, and 5 h). The panels A, D, E, F, G, and I show data from the aP2-A-C/EBP mice (open circle) and wild-type littermates (filled circle). Asterisks (*) indicate statistical significance at $P<0.05$ from pairwise multiple comparison test (Holm–Sidak).

et al. 2008, Rishi *et al.* 2010) into adipocytes using IBMX, dexamethasone, insulin, and rosiglitazone (Qi *et al.* 2003, Rishi *et al.* 2010). Lipid droplets, a hallmark of adipogenic differentiation, appeared in differentiated wild-type cells but were almost completely absent in A-C/EBP expressing primary cultures (Fig. 6B). Comparison of mRNA expression from inguinal fat and differentiated wild-type dermal fibroblasts reveals that both the cells express similar adipocyte-specific genes, whereas immune response genes are more prevalently expressed in inguinal fat (Fig. 6A). It is well established that the adipose tissue has several important immune functions (Schaffler & Scholmerich 2010). The absence of immune genes in the primary culture suggests that adipose tissue might be infiltrated by immune cells or macrophages.

The immune cell molecular signature is more severe in inguinal fat tissue expressing A-C/EBP (Fig. 6C).

Dermal fibroblast expressing A-C/EBP express muscle markers

The genes downregulated in both inguinal fat and primary cultures expressing A-C/EBP are involved in fat metabolism (Fig. 6C and D). Over 50% of the genes (106 genes (53.5%)) downregulated in inguinal fat were also downregulated in the primary cells induced to differentiate. Genes related to fat cell differentiation were predominant among the suppressed genes, whereas genes related to muscle were overrepresented among the induced genes in primary cultures from

Figure 5 aP2-A-C/EBP mice have low circulating adipokines. (A and B) Serum leptin and (C and D) serum adiponectin in 7–10 mice per group (nine WT and nine TG males; seven WT and ten TG females). (E and F) Serum resistin in 7–11 mice per group (11 WT and ten TG males; seven WT and nine TG females). Serum was obtained from aP2-A-C/EBP mice (open bar) and wild-type littermates (filled bar) at the age of 33 weeks in non-fasted state. In panels A, C, and E, data are mean $\pm$ S.E.M., $n=7$ –11 mice per group. aP2-A-C/EBP mice had significantly (two-way ANOVA test) less levels of leptin ($P<0.001$), adiponectin ($P<0.001$), and resistin ($P=0.029$). In panels B, D, and F, the data from the aP2-A-C/EBP mice (female (open triangle); male (open circle)) and wild-type littermates (female (filled triangle); male (filled circle)) are plotted as a function of WAT mass, where WAT mass is a combined weight of inguinal, gonadal (epididymal in males or parametrial in females), retroperitoneal, and mesenteric fat pads, expressed as percent body weight.

aP2-A-C/EBP mice, as also observed in inguinal fat tissue. We have marked a few of these misregulated fat-specific (*Lpin1*, *Adrb3*, and *Scd1*) and muscle-specific (*Tnnc2* and *Myl1*) genes in Fig. 6C. Protein expression data that confirmed the expression of A-C/EBP transgene in inguinal fat and primary cultures from aP2-A-C/EBP mice also showed a decreased level of SCD1, a classical adipocyte marker and an increased level of *Myom2*, a muscle-specific gene compared with wild type (Fig. 7A and B).

Discussion

Adipocyte growth and differentiation is regulated by sequence-specific DNA binding transcription factors that include AP-1 and C/EBP B-ZIP families (Morrison

& Farmer 2000, Rosen *et al.* 2002). AP-1 promotes precursor cell proliferation (Stephens *et al.* 1992), whereas C/EBP family members mediate adipocyte differentiation via a sequential pattern of expression beginning with C/EBP β and C/EBP δ followed by C/EBP α (Mandrup & Lane 1997, Rosen *et al.* 2000). Adipose-specific expression of a promiscuous DN protein termed A-ZIP/F that inactivates both AP-1 and C/EBP families caused severe lipoatrophy in the TG mouse (Moitra *et al.* 1998). However, which of these B-ZIP families is critical for the severe lipoatrophy phenotype is not clear. In this study, A-C/EBP, a DN protein that inhibits only the DNA binding of C/EBP family members (Vinson *et al.* 1993), was expressed selectively in adipose tissue of TG mice named aP2-A-C/EBP. These mice have impaired adipocyte function.

During the first three weeks of life, the aP2-A-C/EBP TG mice have virtually no WAT and reduced BAT; hyperinsulinemia, hyperlipidemia, and hepatic steatosis are more severe in the aP2-A-C/EBP TG mice than in the A-ZIP/F-1 mice (Moitra *et al.* 1998). These data suggest that inactivation of only the C/EBP family of proteins is sufficient for induction of lipoatrophy in young TG mice. C/EBP α null mice, which died soon after birth due to hypoglycemia, had no visible WAT (Wang *et al.* 1995). C/EBP α -deficient mice rescued from early death by TG expression of C/EBP α in liver had almost no WAT but showed minimal changes in BAT (Linhart *et al.* 2001). In contrast, double knockout of C/EBP β and C/EBP δ caused dramatic reduction of BAT but only modest decrease in the amount of WAT (Tanaka *et al.* 1997). Taken together, these data suggest that *in vivo* C/EBP α is essential for differentiation of WAT, and C/EBP β and C/EBP δ play more important role in the differentiation of BAT. Lack of WAT and severe reduction of BAT in the aP2-A-C/EBP mouse are suggested to be caused by combined inhibition of C/EBP α , C/EBP β , and C/EBP δ function.

The mRNAs downregulated in adult aP2-A-C/EBP inguinal fat include GO terms for fat cell differentiation ($n=3$) including the nuclear receptor, *Ppar γ* , known to be a key regulator of adipogenesis (Rosen *et al.* 1999), lipid metabolism and transport ($n=20$), and insulin receptor signaling ($n=3$; Table 3). We observed lower mRNA levels for PPAR γ -responsive adipocyte genes, including *Scd1*, *Scd2*, *Aghat2*, and *Adipor2* (Yao-Borengasser *et al.* 2008). These data suggest that C/EBP family members are critical for the regulation of adipogenic signals.

The enriched GO terms for upregulated genes in aP2-A-C/EBP inguinal fat tissue (Table 3) were related to muscle, metabolism and energy generation, and immune response. Several skeletal muscle-specific mRNAs including regulatory factors *Myod* and *Myf4*; differentiation markers myosin light chain, myosin heavy chain, and α -actin 1 (*Acta1*); and GO terms

Figure 6 Gene expression changes in male inguinal fat from wild-type versus aP2-A-C/EBP mice. (A) cRNA was made from inguinal fat tissue or primary dermal fibroblast cultures induced for adipogenic differentiation, hybridized to 45 000 feature Affymetrix arrays microarrays, and data presented as a scatter graph. Mean fluorescence intensity ($\log_2$) values from mRNA of wild-type inguinal fat ($n=2$) versus wild-type ($n=2$) primary dermal fibroblast cultures induced for adipogenic differentiation (left panel). Genes whose expression levels changes significantly ($P \leq 0.05$) over 2.0-fold are highlighted in black dots (left panel). Mean fluorescence intensities ($\log_2$) from mRNA of wild-type ($n=3$) versus aP2-A-C/EBP ($n=2$) mice inguinal fat. Genes whose expression levels changes significantly ($P \leq 0.05$) over 1.5-fold are highlighted in black dots (middle panel). Mean fluorescence intensity ($\log_2$) values from mRNA of wild-type ($n=2$) versus aP2-A-C/EBP ($n=2$) primary dermal fibroblast cultures induced for adipogenic differentiation (right panel). Genes whose expression levels changes significantly ($P \leq 0.05$) over 3.5-fold are highlighted. (B) Oil-Red-O stained dermal dermal fibroblast cultures from newborn wild-type and aP2-A-C/EBP mice with (Diff) or without (Undiff) induction for adipogenesis. Oil-Red-O stains neutral lipids that accumulate in adipocytes. Wild-type or aP2-A-C/EBP fibroblast cultures that were not induced for adipogenesis (Undiff) did not show any lipid accumulation. Wild-type cultures showed almost complete differentiation to adipocytes 8 days after induction for adipogenesis. Induction of adipogenesis is inhibited in cultures expressing A-C/EBP. (C) cRNA was made from mRNA of wild-type ($n=3$) and aP2-A-C/EBP ($n=2$) mice inguinal fat tissue, and from wild type undifferentiated ($n=2$), wild type differentiated ($n=2$), and aP2-A-C/EBP undifferentiated ($n=2$) and aP2-A-C/EBP differentiated ($n=2$) primary dermal fibroblasts. Each of these was hybridized to 45 000 feature Affymetrix microarrays. Left panel: the fold change in mRNA abundance (mean fluorescence intensity ($\log_2$) values) in inguinal fat, wild type/aP2-A-C/EBP, were plotted against the fold change of mRNA abundance (mean fluorescence intensity ($\log_2$) values) in primary dermal fibroblasts differentiated for adipogenesis, wild type/aP2-A-C/EBP. Arrows indicate some fat-specific genes suppressed by A-C/EBP both in tissues and in primary cultures (top right quadrant), muscle-specific genes induced by A-C/EBP both in tissue and in primary cultures (bottom left quadrant), and some genes from immune system that are induced by A-C/EBP in tissue but not in the culture (top left quadrant). Right panel: the fold change in mRNA abundance (mean fluorescence intensity ($\log_2$) values) in wild-type primary dermal fibroblasts upon induction for adipogenic differentiation (ratio of Diff to Undiff) were plotted against the fold change of mRNA abundance (mean fluorescence intensity ($\log_2$) values) in aP2-A-C/EBP primary dermal fibroblasts upon induction for adipogenic differentiation. Arrows indicate the genes marked in left panel. (D) Genes (mRNA) suppressed (left panel) or induced (right panel) by A-C/EBP in inguinal fat tissue and/or primary dermal fibroblasts induced for adipogenic differentiation were plotted as Venn diagram.

muscle development ($n=9$), contraction ($n=15$), and cytoskeleton organization and biogenesis ($n=18$) were increased in the TG fat tissue. The increase in muscle genes is consistent with the increased connective tissue observed histologically. These findings possibly indicate the existence of a switch between white fat cells and muscle that is controlled directly or indirectly by C/EBP family members. A closer look to the differentiated dermal fibroblasts from A-C/EBP mice revealed a few cardiac beating cells in the culture (Fig. 7C). As reported earlier, that dermal cell preparation may contain some precursors to other cells (Lichti *et al.*

2008). Therefore, another possibility might be that when adipocyte differentiation is inhibited in A-C/EBP cells, precursors to muscle cells are prominent in the culture and may contribute to the mRNA expression.

In contrast to the aP2-A-ZIP/F-1 mice, which did not have WAT, the aP2-A-C/EBP mice developed WAT during weaning. A difference between these two dominant negatives is that the A-ZIP/F but not the A-C/EBP-dominant negative inhibits the DNA binding of the AP-1 heterodimer complex composed of an FOS and a JUN family member. Thus, one possible explanation for the delayed appearance of WAT is

Table 3 Genes significantly ($P < 0.05$) misregulated in aP2-A-C/EBP mice compared with wild-type mice. Total number of genes within each gene function group has been given in parentheses and they include genes that may be in more than one group (overlaps). The six topmost genes (with respect to fold mRNA change) have been listed for groups with more than six genes. The ratio of mean hybridization intensities from wild-type ($n=3$) over aP2-A-C/EBP ($n=2$) male inguinal fat mRNA ($n=3$) male inguinal fat mRNA is presented as mRNA fold change

Gene function	Gene symbol	Gene name	mRNA fold change (WT/A-C/EBP)
Metabolism: lipid, alcohol, and carbohydrate ($n=37$)	<i>Scd1</i>	Stearoyl-coenzyme A desaturase 2	3.5
	<i>Cidea</i>	Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A	3.4
	<i>Gpd1</i>	Glycerol-3-phosphate dehydrogenase 1 (soluble)	3.4
	<i>Acaca</i>	Acetyl-coenzyme A carboxylase alpha	2.9
	<i>Mbtps1</i>	Membrane-bound transcription factor peptidase, site 1	2.8
	<i>Pcx</i>	Pyruvate carboxylase	2.7
Transport, signaling, and cytoskeleton related ($n=15$)	<i>Sorbs1</i>	Sorbin and SH3 domain containing 1	2.3
	<i>Acss2</i>	Acyl-CoA synthetase short-chain family member 2	2.1
	<i>Akt2</i>	Thymoma viral proto-oncogene 2	2.1
	<i>Slc2a4</i>	Solute carrier family 2 (facilitated glucose transporter), member 4	2.0
	<i>Slc36a2</i>	Solute carrier family 36 (proton/amino acid symporter), member 2	2.0
	<i>Acly</i>	ATP citrate lyase	2.0
Fat cell differentiation ($n=4$)	<i>BC054059</i>	cDNA sequence BC054059	1.9
	<i>Adrb1</i>	Adrenergic receptor, beta 1	1.7
	<i>Lpin1</i>	Lipin 1	1.7
	<i>Pparγ</i>	Peroxisome proliferator-activated receptor, gamma	1.7
Muscle related ($n=30$)	<i>Myom2</i>	Myomesin 2	0.19
	<i>Trim63</i>	Tripartite motif-containing 63	0.21
	<i>Sgcg</i>	Sarcoglycan, gamma (dystrophin-associated glycoprotein)	0.22
	<i>Mybpc2</i>	Myosin binding protein C, fast type	0.24
	<i>Ryr1</i>	Ryanodine receptor 1, skeletal muscle	0.25
	<i>Tpm1</i>	Tropomyosin 1, alpha	0.26
Metabolism ($n=23$)	<i>Pgam2</i>	Phosphoglycerate mutase 2	0.23
	<i>Pygm</i>	Muscle glycogen phosphorylase	0.29
	<i>Ampd1</i>	Adenosine monophosphate deaminase 1 (isoform M)	0.31
	<i>Adssl1</i>	Adenylosuccinate synthetase like 1	0.32
	<i>Eno3</i>	Enolase 3, beta muscle	0.33
	<i>Pfkf</i>	Phosphofructokinase, muscle	0.34
Neuron differentiation ($n=4$)	<i>Bex1</i>	Brain-expressed gene 1	0.19
	<i>Cd24a</i>	CD24a antigen	0.56
	<i>Eya1</i>	Eyes absent 1 homolog (<i>Drosophila</i>)	0.63
	<i>Sema4d</i>	Sema domain, immunoglobulin domain (Ig), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D	0.67
	<i>Ptx3</i>	Pentraxin-related gene	0.29
Immune response related ($n=8$)	<i>Igh-6</i>	Immunoglobulin heavy chain 6 (heavy chain of IgM)	0.33
	<i>Fcer1a</i>	Fc receptor, IgE, high-affinity I, alpha polypeptide	0.42
	<i>Fcgr2b</i>	Fc receptor, IgG, low-affinity IIb	0.56
	<i>Cacna1s</i>	Calcium channel, voltage-dependent, L-type, alpha 1S subunit	0.30
Ion homeostasis and transport ($n=16$)	<i>Hrc</i>	Histidine-rich calcium binding protein	0.37
	<i>Atp2a1</i>	ATPase, Ca^{2+} transporting, cardiac muscle, fast twitch 1	0.37
	<i>Cacng1</i>	Calcium channel, voltage-dependent, gamma subunit 1	0.4
	<i>Sypl2</i>	Synaptophysin-like 2	0.45
	<i>Cacnb1</i>	Calcium channel, voltage-dependent, beta 1 subunit	0.48

Relatively enriched ($P < 0.01$) GO_BP (Gene Ontology Biological Process) category has been presented singly or several such categories have been grouped together in the gene function column.

that the absence of AP-1 DN activity in aP2-A-C/EBP mice allows preadipocytes to proliferate at weaning. Clonal expansion of preadipocytes is a prerequisite for WAT-type adipogenesis *in vitro* and requires AP-1 as well as c-myc (Rosen *et al.* 2000). Previously, we reported that

although A-C/EBP blocked cell proliferation associated with mitotic clonal expansion, it did not inhibit normal proliferation of 3T3-L1 preadipocytes (Zhang *et al.* 2004). Thus, once the developmental bottleneck is overcome, WAT-type adipocytes may differentiate

Table 4 mRNA levels of some known adipose tissue-expressed genes in inguinal fat of aP2-A-C/EBP (TG) mice

Gene name	TG ^a (n=2)	WT ^a (n=3)	TG/WT ^b
Leptin (<i>Lep</i>)	11.0	11.5	0.7
Resistin (<i>Retn</i>)	12.9	13.4	0.7
Insulin receptor (<i>Insr</i>)	6.5	6.7	0.8
Insulin receptor substrate 1 (<i>Irs1</i>)	11.5	11.6	1.0
Solute carrier family 2 (facilitated glucose transporter), member 4 (<i>Slc2a4</i>)	10.5	11.5	0.5
CCAAT/enhancer binding protein, beta (<i>C/EBPβ</i>)	9.2	9.6	0.8
Peroxisome proliferator-activated receptor, gamma (<i>Pparγ</i>)	10.8	11.5	0.6
Fatty acid binding protein 4, adipocytes (<i>Fabp4</i>)	12.7	12.9	0.9
Stearyl-Coenzyme A desaturase 1 (<i>Scd1</i>)	12.6	13.4	0.6
Stearyl-Coenzyme A desaturase 2 (<i>Scd2</i>)	6.9	8.7	0.3
1-acylglycerol-3-phosphate O-acyltransferase 2 (lysophosphatidic acid acyltransferase, beta) (<i>Agpat2</i>)	11.9	12.8	0.6
Adiponectin receptor 2 (<i>Adipor2</i>)	8.7	9.4	0.6

^aAverage of hybridization intensities.
^bRatio of hybridization intensities for aP2-A-C/EBP over wild-type male inguinal fat.

depending upon networks that can bypass C/EBP family members. The reappearance of WAT to near-normal amounts and in normal anatomical locations suggests that although C/EBP’s role as a transcription factor is essential for normal adipogenesis, its inhibition by A-C/EBP expression has no major effect on the number of adipocyte precursors and their ability to differentiate.

Reappearance of WAT in the aP2-A-C/EBP mice coincided with the recovery from insulin resistance, hyperlipidemia, and hepatic steatosis, suggesting that the lack of fat was primarily caused by metabolic abnormalities associated with lipodystrophy. This is in agreement with our previous data demonstrating that surgical implantation of adipose tissue reversed lipoatrophic diabetes in the AZIP/F-1 mouse (Gavrilova *et al.* 2000a). Similarly, in the FAT-ATTAC mouse, a novel model of inducible and reversible fat ablation caused by regulated apoptosis, cessation of pro-apoptotic treatment reversed many of the metabolic effects observed in the lipoatrophic state (Pajvani *et al.* 2005). Taken together, the data demonstrate the beneficial effects of a minimal amount of functional WAT on glucose and lipid metabolism.

At 6 months of age, aP2-A-C/EBP mice exhibited signs of partial lipoatrophy, including reduced fat mass and serum leptin and adiponectin, increased food intake, and mild insulin resistance observed in males. It is not clear whether this is a long-term complication of metabolic stress the mice experience early in life or a direct effect of the DN protein. As the expression of C/EBPα and C/EBPδ in mice declines with age (Karagiannides *et al.* 2001), it is possible that we may see stronger effects of the DN protein in older mice due to the relative stoichiometric increase in expression of the DN protein in WAT. Both reduced fat mass and defective WAT could be responsible for the adult

aP2-A-C/EBP phenotype. The likelihood of A-C/EBP expression in macrophages might also have some effect on the phenotype.

Dysfunction of adipose tissue can lead to the whole body insulin resistance. For example, inactivation of glucose transporter 4 (GLUT4, also known as SLC2A4) selectively in adipose tissue impairs insulin sensitivity in muscle and liver (Abel *et al.* 2001). Several studies showed that although PPARγ is sufficient to trigger the adipogenic program, C/EBPα is required for the establishment of insulin-sensitive glucose transport in mature adipocytes (El-Jack *et al.* 1999, Wu *et al.* 1999, Rosen *et al.* 2002). Insulin did not stimulate glucose uptake into adipocytes lacking C/EBPα, which were shown to have decreased levels of mRNA for insulin receptor, insulin receptor substrate 1 (IRS1), and GLUT4 (SLC2A4; Rosen *et al.* 2002). C/EBPα is also required for the intracellular retention of GLUT4 and may control the expression of the proteins that determine the basal, slow exocytosis of GLUT4 (Wertheim *et al.* 2004). Adipocytes derived from C/EBPβ- and C/EBPδ-deficient mouse embryonic fibroblasts also show reduced GLUT4 and IRS2 expression and reduced glucose uptake in response to insulin (Yamamoto *et al.* 2002). IR and GLUT4 mRNA levels in the aP2-A-C/EBP inguinal fat were reduced by 20 and 50% respectively. Yet, insulin stimulation of 2-DG uptake into epididymal and inguinal fat was similar in aP2-A-C/EBP and control mice, suggesting normal insulin sensitivity in WAT. Interestingly, aP2-A-C/EBP mice also showed significantly reduced insulin-stimulated glucose uptake into skeletal muscle, which does not express a transgene, suggesting that circulating factor(s) might be involved.

The aP2-A-C/EBP mice had low levels of circulating adipokines and less mRNA in inguinal fat suggesting that they may be transcriptional targets of C/EBP family

Figure 7 (A) Western blots using a gradient gel (4–12%) with 20 μ g of protein loaded in each lane. Immunoblotting to detect adipocyte-specific marker SCD1 (37 kDa), muscle-specific marker MYOM2 (165 kDa), and transgenic A-C/EBP (20 kDa) protein from wild-type or aP2-A-C/EBP inguinal fat tissue or primary dermal fibroblasts induced for adipogenesis were done using antibodies against SCD1, MYOM2, and FLAG. (B) Western blots using a gradient gel (4–12%) with 20 μ g of protein loaded in each lane. Blot stained with Ponceau S solution to show equal loading of protein from wild-type and A-C/EBP inguinal fat tissue (left panel). Immunoblotting to detect β -actin protein from wild-type or aP2-A-C/EBP primary dermal fibroblasts induced for adipogenesis (right panel). (C) Primary dermal fibroblast cultures from aP2-A-C/EBP transgenic mice. Beating cardiac muscle cells are marked by arrows.

members (Table 2). A large body of evidence suggests that leptin deficiency contributes to insulin resistance. The decrease in plasma leptin was proportional to the percent body fat and thus can simply result from reduction of fat mass. However, C/EBP α had been shown to activate transcription of the leptin gene (Miller *et al.* 1996, Hollenberg *et al.* 1997, Mason *et al.* 1998), and gene expression analysis showed a 34% reduction in leptin mRNA levels in the aP2-A-C/EBP mice. Thus, direct inhibition of leptin mRNA expression and reduction of fat mass may contribute to low leptin plasma levels in the aP2-A-C/EBP mice. C/EBP α activates both the adiponectin (Saito *et al.* 1999, Gustafson *et al.* 2003, Park *et al.* 2004) and the resistin genes (Hartman *et al.* 2002, Seo *et al.* 2003) that are reduced in the aP2-A-C/EBP mice (Table 2). Reduction of resistin should not contribute to the aP2-A-C/EBP phenotype (Hartman *et al.* 2002), if so, low resistin can mask insulin resistance caused by a leptin or adiponectin deficiency.

The partial lipodystrophic phenotype of the aP2-A-C/EBP mouse makes it a valuable mouse model of the human disease of partial lipodystrophy and more comparable than complete fat ablation observed in the A-ZIP/F-1 mouse (Moitra *et al.* 1998). Demethylation by 5-azacytidine treatment or by DNMT1 depletion is also reported to inhibit the adipocyte differentiation (Rishi *et al.* 2010). So, these mice will be of interest to examine any possible epigenetic consequences of inhibiting C/EBP family function in adipose tissue.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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