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

Raghunath Chatterjee, Paramita Bhattacharya, Oksana Gavrilova1, Kimberly Glass2, Jaideep Moitra, Max Myakishev, Stephanie Pack1, William Jou1, Lionel Feigenbaum3, Michael Eckhaus4 and Charles Vinson

Laboratory of Metabolism, National Cancer Institute, National Institutes of Health, Building 37, Room 3128, Bethesda, Maryland 20892, USA
1Mouse Metabolism Laboratory, NIDDK, NIH, Bethesda, Maryland 20892, USA
2Physics Department, University of Maryland, College Park, Maryland 20783, USA
3SAIC Frederick, NCI-Frederick Cancer Research and Development Center, Frederick, Maryland 21702, USA
4Veterinary Resources Program, Office of Research Services, NIH, Bethesda, Maryland 20892, USA

(Correspondence should be addressed to C Vinson; Email: vinsonc@mail.nih.gov)

(J Moitra is now at Section of Pulmonary and Critical Care Medicine, Department of Medicine, University of Chicago, Chicago, Illinois, USA)

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 \( \beta \)-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\( \alpha \) 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.

Journal of Molecular Endocrinology (2011) 46, 175–192

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). 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), CAV1 (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

Lipodystrophy is also common among human immunodeficiency virus (HIV) patients receiving highly active antiretroviral 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 2002). These A-P2-A-C/EBP TG mice have a unique family of B-ZIP transcription factors (Vinson 1998) that were generated that used the adipocyte-specific aP2 enhancer/promoter to express A-C/EBP, a DN 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/EBPα 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′-TTCAATAGGCTGGGCTGTT-3′) and 935 (5′-GTTACCCGAGACACCACGTAAGGTTCC-3′) extending to the natural C-terminus, giving a 561 bp product. ZfY primer pairs (Koopman et al. 1991) producing a 150 bp product were included in equimolar concentration in the

---

**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/EBPα 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′-TTCAATAGGCTGGGCTGTT-3′) and 935 (5′-GTTACCCGAGACACCACGTAAGGTTCC-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 μg 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 ºC using the
AmpliTaq Gold system, followed by 30 cycles of 45/ºC
at 94 ºC, 30/ºC at 55 ºC, and 45/ºC at 72 ºC and a final extension
for 10’ at 72 ºC. 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 (Invitro-
gen) 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 rosigli-
tazone 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 ºC, 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 ºC overnight, washed for
30 min at medium stringency (2×SSC+0.2% SDS at
68 ºC), 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 differ-
etiation. 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 ºC 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 β3-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 μCi; 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 μl 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× g at 4 °C for 30 min and the infranatant 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 ± 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 leucine zipper and a designed acidic protein sequence that replaces the C/EBP basic region. The acidic protein sequence heterodimerizes with C/EBP family members and is able to inhibit the DNA binding of C/EBP family members. 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 (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.

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. 1B-L). During the first week of life, the aP2-A-C/EBP mice had significantly high levels of blood glucose (Fig. 1I), whereas serum insulin levels (Fig. 1J) 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. 1K) and free fatty acids (Fig. 1L) were also elevated in young aP2-A-C/EBP mice and gradually normalized by weaning, coinciding with appearance of WAT.

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.

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
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.
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 lipoatrophy (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 β3-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 β3-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 β3-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 β3-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

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

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.
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.
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 also showed lower adiponectin levels even after the normalization for percent of fat (Fig. 5D). Resistin levels were also significantly reduced in TG mice (Fig. 5C). In both sexes, adiponectin levels were significantly (*P<0.05) between WT and A-C/EBP mice within each gender group presented in bold.

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

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>WT</td>
<td>A-C/EBP</td>
</tr>
<tr>
<td>Body weight (g)</td>
<td>36.9 ± 1.4 (n=9)</td>
<td>35.4 ± 1.1 (n=11)</td>
</tr>
<tr>
<td>Fat mass (g)</td>
<td>4.7 ± 0.4 (n=6)</td>
<td>2.78 ± 0.3* (n=6)</td>
</tr>
<tr>
<td>Lean mass (g)</td>
<td>26.7 ± 1.1 (n=6)</td>
<td>25.7 ± 0.9 (n=6)</td>
</tr>
<tr>
<td>Body length (mm)</td>
<td>90.1 ± 1.4 (n=9)</td>
<td>89 ± 1.0 (n=11)</td>
</tr>
<tr>
<td>Liver (% body weight)</td>
<td>1.4 ± 0.0 (n=9)</td>
<td>1.6 ± 0.1* (n=11)</td>
</tr>
<tr>
<td>Spleen (% body weight)</td>
<td>0.43 ± 0.1 (n=9)</td>
<td>0.47 ± 0.0* (n=11)</td>
</tr>
<tr>
<td>Heart (% body weight)</td>
<td>0.45 ± 0.0 (n=9)</td>
<td>0.49 ± 0.0* (n=11)</td>
</tr>
<tr>
<td>Muscle mass (% body weight)</td>
<td>76.6 ± 0.7 (n=6)</td>
<td>81.4 ± 0.4* (n=6)</td>
</tr>
<tr>
<td>WAT mass (% body weight)</td>
<td>13.4 ± 0.9 (n=6)</td>
<td>8.7 ± 0.6* (n=6)</td>
</tr>
<tr>
<td>Total oxygen consumption (ml/kg per h)</td>
<td>278 ± 93 (n=6)</td>
<td>313 ± 74* (n=6)</td>
</tr>
<tr>
<td>Food intake (g/mouse per day)</td>
<td>2.9 ± 0.2 (n=6)</td>
<td>3.5 ± 0.2* (n=6)</td>
</tr>
<tr>
<td>Food intake (mg/g per day)</td>
<td>84 ± 8 (n=6)</td>
<td>114 ± 12* (n=6)</td>
</tr>
<tr>
<td>Serum glucose (mg/dl)</td>
<td>183 ± 11 (n=10)</td>
<td>185 ± 11 (n=12)</td>
</tr>
<tr>
<td>Serum insulin (ng/ml)</td>
<td>1.7 ± 0.2 (n=10)</td>
<td>3.0 ± 0.6* (n=6)</td>
</tr>
<tr>
<td>Serum triglyceride (mg/dl)</td>
<td>216 ± 32 (n=10)</td>
<td>269 ± 22 (n=12)</td>
</tr>
<tr>
<td>Serum-free fatty acids (mM)</td>
<td>0.37 ± 0.4 (n=9)</td>
<td>0.32 ± 0.3 (n=12)</td>
</tr>
<tr>
<td>Serum leptin (ng/ml)</td>
<td>12.3 ± 2.2 (n=9)</td>
<td>4.1 ± 0.5* (n=9)</td>
</tr>
<tr>
<td>Serum adiponectin (μg/ml)</td>
<td>9.6 ± 1.2 (n=9)</td>
<td>2.7 ± 0.3* (n=9)</td>
</tr>
<tr>
<td>Serum resistin (ng/ml)</td>
<td>9.0 ± 0.5 (n=11)</td>
<td>7.0 ± 1.1* (n=10)</td>
</tr>
<tr>
<td>Liver triglyceride (μmol/g)</td>
<td>8.2 ± 0.6 (n=6)</td>
<td>8.0 ± 0.8 (n=6)</td>
</tr>
<tr>
<td>Muscle triglyceride (μmol/g)</td>
<td>12.0 ± 1.2 (n=6)</td>
<td>12.1 ± 1.0 (n=6)</td>
</tr>
</tbody>
</table>

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

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 Pparγ. 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...
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 (53-55%)) 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
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/EBPβ and C/EBPδ followed by C/EBPα (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/EBPα null mice, which died soon after birth due to hypoglycemia, had no visible WAT (Wang et al. 1995). C/EBPα-deficient mice rescued from early death by TG expression of C/EBPα in liver had almost no WAT but showed minimal changes in BAT (Linhardt et al. 2001). In contrast, double knockout of C/EBPβ and C/EBPδ 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/EBPα is essential for differentiation of WAT, and C/EBPβ and C/EBPδ 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/EBPα, C/EBPβ, and C/EBPδ function.

The mRNAs downregulated in adult aP2-A-C/EBP inguinal fat tissue include GO terms for fat cell differentiation (n = 3) including the nuclear receptor, Pparγ, 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 PPARγ-responsive adipocyte genes, including Scd1, Scd2, Agpat2, 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 Myf5; 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 (log2) 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≤0.05) over 2.0-fold are highlighted in black dots (left panel). Mean fluorescence intensities (log2) from mRNA of wild-type (n=3) versus aP2-A-C/EBP (n=2) mice inguinal fat. Genes whose expression levels changes significantly (P≤0.05) over 1.5-fold are highlighted in black dots (middle panel). Mean fluorescence intensity (log2) 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≤0.05) over 3.0-fold are highlighted. (B) Oil-Red-O stained 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 (log2) values) in inguinal fat, wild type/aP2-A-C/EBP, were plotted against the fold change of mRNA abundance (mean fluorescence intensity (log2) 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 (log2) 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 (log2) 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
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 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

<table>
<thead>
<tr>
<th>Gene function</th>
<th>Gene symbol</th>
<th>Gene name</th>
<th>mRNA fold change (WT/A-CEBP)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Metabolism: lipid, alcohol, and carbohydrate (n=37)</td>
<td>Scd1</td>
<td>Stearoyl-coenzyme A desaturase 2</td>
<td>3.5</td>
</tr>
<tr>
<td></td>
<td>Cidea</td>
<td>Cell death-inducing DNA fragmentation factor, alpha subunit-like effector A</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Gpd1</td>
<td>Glycerol-3-phosphate dehydrogenase 1 (soluble)</td>
<td>3.4</td>
</tr>
<tr>
<td></td>
<td>Acaca</td>
<td>Acetyl-coenzyme A carboxylase alpha</td>
<td>2.9</td>
</tr>
<tr>
<td></td>
<td>Mtbp1</td>
<td>Membrane-bound transcription factor peptidase, site 1</td>
<td>2.8</td>
</tr>
<tr>
<td></td>
<td>Pcx</td>
<td>Pyruvate carboxylase</td>
<td>2.7</td>
</tr>
<tr>
<td>Transport, signaling, and cytoskeleton related (n=15)</td>
<td>Sorbs1</td>
<td>Sorbin and SH3 domain containing 1</td>
<td>2.3</td>
</tr>
<tr>
<td></td>
<td>Acss2</td>
<td>Acyl-CoA synthetase short-chain family member 2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Akt2</td>
<td>Thymoma viral proto-oncogene 2</td>
<td>2.1</td>
</tr>
<tr>
<td></td>
<td>Slc2a4</td>
<td>Solute carrier family 2 (facilitated glucose transporter), member 4</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>Slc36a2</td>
<td>Solute carrier family 36 (proton/amino acid symporter), member 2</td>
<td>2.0</td>
</tr>
<tr>
<td>Fat cell differentiation (n=4)</td>
<td>Acly</td>
<td>ATP citrate lyase</td>
<td>2.0</td>
</tr>
<tr>
<td></td>
<td>BC054059</td>
<td>cDNA sequence BC054059</td>
<td>1.9</td>
</tr>
<tr>
<td></td>
<td>Adrb1</td>
<td>Adrenergic receptor, beta 1</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Lpin1</td>
<td>Lipin 1</td>
<td>1.7</td>
</tr>
<tr>
<td></td>
<td>Pparγ</td>
<td>Peroxisome proliferator-activated receptor, gamma</td>
<td>1.7</td>
</tr>
<tr>
<td>Muscle related (n=30)</td>
<td>Myom2</td>
<td>Myomesin 2</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Trim63</td>
<td>Tripartite motif-containing 63</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>Sipgc</td>
<td>Sarcoglycan, gamma (dystrophin-associated glycoprotein)</td>
<td>0.22</td>
</tr>
<tr>
<td></td>
<td>Mybp2c</td>
<td>Myosin binding protein C, fast type</td>
<td>0.24</td>
</tr>
<tr>
<td></td>
<td>Ryr1</td>
<td>Ryanodine receptor 1, skeletal muscle</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Tpm1</td>
<td>Tropomyosin 1, alpha</td>
<td>0.26</td>
</tr>
<tr>
<td>Metabolism (n=23)</td>
<td>Pgam2</td>
<td>Phosphoglycerate mutase 2</td>
<td>0.23</td>
</tr>
<tr>
<td></td>
<td>Pygm</td>
<td>Muscle glycogen phosphorylase</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Ampd1</td>
<td>Adenosine monophosphate deaminase 1 (isoform M)</td>
<td>0.31</td>
</tr>
<tr>
<td></td>
<td>Adss1</td>
<td>Adenylosuccinate synthetase like 1</td>
<td>0.32</td>
</tr>
<tr>
<td></td>
<td>Eno3</td>
<td>Enolase 3, beta muscle</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Pkm</td>
<td>Phosphofructokinase, muscle</td>
<td>0.34</td>
</tr>
<tr>
<td>Neuron differentiation (n=4)</td>
<td>Bex1</td>
<td>Brain-expressed gene 1</td>
<td>0.19</td>
</tr>
<tr>
<td></td>
<td>Cd24a</td>
<td>CD24a antigen</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Eya1</td>
<td>Eyes absent 1 homolog (Drosophila)</td>
<td>0.63</td>
</tr>
<tr>
<td></td>
<td>Sema4d</td>
<td>Sema domain, immunoglobulin domain (lg), transmembrane domain (TM) and short cytoplasmic domain, (semaphorin) 4D</td>
<td>0.67</td>
</tr>
<tr>
<td>Immune response related (n=8)</td>
<td>Ptx3</td>
<td>Pentraxin-related gene</td>
<td>0.29</td>
</tr>
<tr>
<td></td>
<td>Igh-6</td>
<td>Immunoglobulin heavy chain 6 (heavy chain of IgM)</td>
<td>0.33</td>
</tr>
<tr>
<td></td>
<td>Fcera1a</td>
<td>Fc receptor, IgE, high-affinity I, alpha polypeptide</td>
<td>0.42</td>
</tr>
<tr>
<td></td>
<td>Fcgr2b</td>
<td>Fc receptor, IgG, low-affinity Ilib</td>
<td>0.56</td>
</tr>
<tr>
<td>Ion homeostasis and transport (n=16)</td>
<td>Cacna1s</td>
<td>Calcium channel, voltage-dependent, L-type, alpha 1S subunit</td>
<td>0.30</td>
</tr>
<tr>
<td></td>
<td>Hrc</td>
<td>Histidine-rich calcium binding protein</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Atp2a1</td>
<td>ATPase, Ca²⁺ transporting, cardiac muscle, fast twitch 1</td>
<td>0.37</td>
</tr>
<tr>
<td></td>
<td>Cacng1</td>
<td>Calcium channel, voltage-dependent, gamma subunit 1</td>
<td>0.4</td>
</tr>
<tr>
<td></td>
<td>Syp12</td>
<td>Synaptophysin-like 2</td>
<td>0.45</td>
</tr>
<tr>
<td></td>
<td>Cacnb1</td>
<td>Calcium channel, voltage-dependent, beta 1 subunit</td>
<td>0.48</td>
</tr>
</tbody>
</table>

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.
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 lipodystrophic 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

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

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

\(^a\)Average of hybridization intensities.

\(^b\)Ratio of hybridization intensities for aP2-A-C/EBP over wild-type male inguinal fat
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/EBPα 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/EBPα 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.

Figure 7 (A) Western blots using a gradient gel (4–12%) with 20 μg 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 μg 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.

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.

Funding

This research was supported by the Intramural Research Program of the Center for Cancer Research, National Cancer Institute, National
Institutes of Health (NIH), and NIDDK, NIH. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade names, commercial products, or organizations imply endorsement by the US government.

Acknowledgements

Microarray analyses were performed using BRB-Array Tools Version 3.7.0 Patch_1 developed by Dr Richard Simon and Amy Peng Lam (available at: http://linus.nci.nih.gov/BRB-ArrayTools.html). Gene Ontology analyses were carried out using Gene Ontology Tree Machine (GOTM) developed and maintained by Bing Zhang. Machine (GOTM) developed and maintained by Bing Zhang, Ontology analyses were carried out using Gene Ontology Tree (available at: http://bioinfo.vanderbilt.edu/gotm ). We thank Eric Rios-Doria for his editing and valuable comments on the manuscript.

References


Agarwal AK, Arioglu E, De Almeida S, Akkoc N, Taylor SI, Bowcock AM, Barnes RJ & Garg A 2002 A PPARa2 is mutated in congenital generalized lipodystrophy linked to chromosome 9q34. Nature Genetics 31 21–25. (doi:10.1038/ng880)


Downloaded from Bioscientifica.com at 01/27/2019 10:16:32PM


Received in final form 21 January 2011

Accepted 14 February 2011

Made available online as an Accepted Preprint 14 February 2011