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Cloning, expression, and differentiation-dependent regulation of *SMAF1* in adipogenesis

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Abstract

With the aim of identifying novel molecular pathways in the adipocyte, we conducted differential screening of DNA filter arrays with probes from 3T3-L1 preadipocytes and adipocytes, and discovered a novel 0.7 kb transcript we term small adipocyte factor 1 (SMAF1). SMAF1 encodes a wholly novel 10 kDa protein. Transfection and localization studies of a SMAF1–EGFP fusion construct indicate nuclear localization, suggestive of a possible regulatory role. Northern blot analysis of various murine tissues indicates adipose tissue-restricted expression, and fractionation of adipose tissue reveals that SMAF1 is expressed soley in adipocytes and not in the stromal-vascular cell population. Northern blot analysis of brown and white adipogenic conversion reveals that expression of SMAF1 closely parallels emergence of an adipocyte phenotype and that TNFα-mediated dedifferentiation of 3T3-L1 adipocytes results in a rapid decline of SMAF1 transcript. These data indicate that SMAF1 is closely tied to the adipocyte phenotype and predict a novel and possibly regulatory role for this gene in adipocyte function.

Keywords: Adipocyte; Differentiation; 3T3-L1; Obesity; Brown adipogenesis; White adipogenesis; SMAF1; Nuclear localization; Novel gene

In higher eukaryotes a primary role of white adipose tissue is the storage of excess energy in the form of triglyceride. In response to nutritional and hormonal cues these stores are mobilized to meet the energy demands of the organism [1,2]. Adipose tissue is now also recognized as an endocrine organ that synthesizes and secretes a number of soluble factors that function in systemic energy balance including leptin, adiponectin, and TNFα [3–5]. Adipogenesis is the process of new adipocytes arising from the differentiation of preadipocyte precursors. For several decades, the 3T3-L1 preadipocyte cell line, primary preadipocytes in culture, and in vivo analyses have been utilized to define genes central to adipocyte function and differentiation. These include genes that encode molecules central to lipogenesis, lipolysis, lipid transport, and hormone signaling [3]. Such studies have defined PPARγ to be the master transcriptional regulator of the adipogenic program [4–10], and have illustrated the important contribution of the C/EBP family transcriptional regulators to adipogenesis [9,11–14]. Molecular signals that inhibit adipogenesis have also been identified, including the secreted protein Wnt10b, GATA transcription factors, and Pref-1 [15–17].

In addition to a number of well-defined adipogenic modulators, the advent and application of DNA array gene expression profiling has revealed that multiple additional signaling networks and protein families are regulated during adipogenesis [18–22]. However, which of this multitude of gene expression alterations are most integral to the molecular definition of the adipogenic program and/or the mature adipocyte remains largely undetermined. Thus, while studies to date have led to

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detailed functional characterization of a number of the central components of adipogenesis, the picture of adipocyte differentiation and function in normal and pathological states is far from complete. We report here the identification and characterization of small adipocyte factor 1 (SMAF1), a new adipocyte-specific gene encoding an 80 amino acid protein with a leucine-rich N-terminal half and a C-terminal portion particularly enriched in acidic amino acids. The expression and regulation of SMAF1 suggests that this novel protein occupies a unique niche in the adipocyte transcriptome and in the physiology and pathophysiology of adipose tissue.

Materials and methods

Cell culture and adipocyte differentiation. All chemicals used in studies of white and brown adipogenesis were from Sigma Chemical (Sigma–Aldrich, St. Louis MO), unless otherwise noted. 3T3-L1 cells [23] were obtained from American Type Culture Collection (ATCC) and propagated in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% calf serum. For in vitro differentiation to adipocytes, 3T3-L1 cells were treated at two days post-confluence with DMEM supplemented with 10% fetal calf serum (FCS) in the presence of the adipogenic inducers 0.5 mM methylisobutylxanthine and 1 μM dexamethasone for 48 h. Adipogenic agents were then removed and growth of cultures was continued in DMEM containing 10% FCS. At five days post-induction of differentiation, adipose conversion had occurred in approximately 90% of the cells, as judged by lipid accumulation and cell rounding. For time course studies of regulation by TNFα, 3T3-L1 adipocytes were incubated with 10 ng/ml TNFα for the indicated periods and RNA extracted. For studies on brown adipocyte differentiation, a brown preadipocyte cell line [24] was obtained from C.R. Kahn, Joslin Diabetes Foundation, Harvard Medical School, Boston. For differentiation of brown preadipocytes, cells were cultured to confluence in DMEM with 10% FCS, 20 nM insulin, and 1 nM triiodotyronine (differentiation medium per Kahn and co-workers [24]). Confluent cells were incubated in differentiation medium that contained 0.5 mM methylisobutylxanthine, 0.5 μM dexamethasone, and 0.125 mM indomethacin for 24–48 h. Following this period nearly 100% of cells showed adipogenic conversion at which time medium was replaced with differentiation medium and replenished every two days.

For collection, culture, and differentiation of primary cells, white adipose tissue collected from male rats was removed, rinsed in sterile Hank's buffered saline solution (HBSS), and minced with sterile scissors. Tissue was transferred to a 50 ml sterile tube and digested with 1 mg/ml of type I collagenase for 40 min with shaking at 37 °C. Following digestion, material was filtered through a 300-μm pore size nylon mesh (Sefar America, Lumberton NJ) and the filtrate was centrifuged at 2000 rpm for 5 min. The floating adipocyte fraction was removed and the pellet of stromal-vascular cells was resuspended in DMEM containing 10% FCS and plated. Upon confluence these cells were either collected or subjected to differentiation media consisting of DMEM containing 10% FCS, 0.1 μM dexamethasone, 0.25 mM methylisobutylxanthine, and 17 nM insulin for three days. At this time, the differentiation media were removed and culture was continued in DMEM in 10% FCS with insulin. RNA was prepared at confluence (day 0), and at 2, 5, and 7 days post-induction of differentiation and Northern blot analysis was conducted using a rat partial SMAF1 cDNA clone as probe.

Cellular fractionation of adipose tissue. To assess expression of SMAF1 in adipose tissue components in vivo, tissue was fractionated into adipocyte and stromal vascular fractions. For this, epididymal adipose tissue was removed from male C57BL/6 mice, rinsed three times in sterile HBSS, and minced with scissors. Tissue was transferred to a 50 ml sterile tube with 15 ml HBSS containing 0.1 mg/ml of type I collagenase (Sigma–Aldrich). After digestion for 40 min at 37 °C with constant agitation, material was filtered through 300-μm pore size nylon mesh. Filtrate was collected into sterile 50 ml centrifuge tubes and centrifuged at 2000 rpm for 5 min, and the floating adipocyte cell fraction was recovered into 10 volumes of Trizol reagent (Invitrogen, Carlsbad CA) and the stromal vascular pellet fraction was lysed in 2 ml Trizol reagent. RNA was extracted and analyzed by Northern blot analysis as described above.

RNA preparation, Northern blot analysis, and DNA array hybridization. For RNA preparation from cultured cells, media were removed and cell monolayers were lysed in Trizol reagent. RNA was purified from this lysate according to manufacturer's instructions via chloroform extraction and isopropanol precipitation. For RNA preparation from mouse tissues, tissues were dissected and frozen in liquid nitrogen, followed by maceration and homogenization in Trizol reagent with a polytron. For studies of SMAF1 expression in murine tissues, 8-wk-old C57BL/6 male mice were utilized. For Northern blot analysis, RNA was fractionated in 1% formaldehyde gels in Mops buffer. Following staining and photography of the gel, RNA was transferred by capillary blotting to Hybond N membrane (Amersham Biosciences, Piscataway NJ), and after overnight transfer nucleic acid was cross-linked to the membrane. Blots were prehybridized at 65 °C for 1 h in ExpressHyb (BD Biosciences Clontech, Palo Alto CA) and hybridized at 65 °C for 1 h with [α-^{32}P]dATP-labeled cDNA probes generated by random-primed synthesis of gel-purified cDNA inserts. For SMAF1 transcript analysis of murine and 3T3-L1 cells, a full-length murine SMAF1 cDNA was used. For studies of primary adipocyte differentiation a rat partial SMAF1 cDNA was used as probe. Post-hybridization, Northern blot filters were washed for 30 min in 1× SSC/1% SDS at 65 °C followed for 30 min in 0.1× SSC/0.1% SDS at 65 °C, and exposed at −80 °C to Kodak Biomax film with a Kodak Biomax intensifying screen.

For array analysis, duplicate mouse ResGen GeneFilter Arrays (Invitrogen) containing 5184 cDNAs were processed exactly per manufacturer instructions. Probes were synthesized by reverse transcription of 8 μg of total RNA with the incorporation of [α-^{33}P]dCTP. Following high stringency washes, filters were exposed to Kodak Biomax film with a Kodak Biomax intensifying screen at −80 °C and differentially expressed signals were identified.

SMAF1 cDNA sequence and 5′ RACE analysis .The filter position of the differentially expressed signal was cross-referenced with the GeneFilter database (Invitrogen) and the corresponding IMAGE clone(s) were obtained from the IMAGE clone collection at Incyte Genomics or ATCC and fully sequenced. For 5′ RACE analysis poly(A)$^+$ 3T3-L1 adipocyte mRNA was used as template utilizing the SMART-RACE analysis kit according to manufacturer's instructions (BD Biosciences Clontech). For these analyses a primer positioned at (+)631 of SMAF1 (5′-GCTCTGTTAGGGGCACAAAAGCACC-3′) was combined with the SMART IIA oligo primer to amplify the 5′ end. The gene-specific primer for the PCR was positioned at (+) 419 of SMAF1 (5′-CTCCCTCCATCCACTTAGGCCCAG-3′). Amplified RACE products were cloned into pCR2.1 using TA cloning (Invitrogen) and multiple clones were assessed for insert size by restriction digest and sequenced.

Expression and analysis of SMAF1 protein by transient transfection. A SMAF1 expression construct was prepared by PCR amplification of the SMAF1 coding sequence using the primer (5′-GAAGG TACCGCCGCCATGAAGTACCCTCTGGTGCCTCTG-3′) that included a *Kpn*I site (underlined), incorporation of a Kozak consensus sequence, and the initiator ATG. To prepare HA-tagged versions of SMAF1 in the mammalian expression vector pcDNA3.1/TOPO (Invitrogen), this primer was employed with the 3′ primer (5′-

ACAGGGATCCTCAAAGAGCGTAATCTGGAACATCGTATG GGTACCAGTGGAGTCCGTCCTCCTCA-3′) that resulted in fusion of an in-frame C-terminal HA tag, followed by a stop codon and a *Bam*HI site (underlined). Purified PCR products were inserted into pcDNA3.1/TOPO according to manufacturer's instructions. Five micrograms of HA-tagged SMAF1 construct was transfected into COS cells using Superfect reagent (Qiagen, Valencia CA). Four days after transfection, cells were lysed in lysis buffer (10 mM Tris, pH 8.0, 120 mM NaCl, 0.5% Nonidet P-40, and 1 mM EDTA), and protease inhibitors (0.1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 μg/ml leupeptin, 1 μg/ml pepstatin, and 250 μg/ml 4-(2-aminoethyl)benzenesulfonyl fluoride) on ice for 30 min. Following centrifugation at 14,000 rpm for 20 min at 4 °C, cell lysate supernatants were assessed for protein content using the Bio-Rad protein assay kit (Bio-Rad Incorporated, Hercules CA). Fifty micrograms of protein was loaded per lane of 10% Tris–Tricine gels. For Western blot analysis, protein was transferred to 0.2 μm pore size Immobilon polyvinylidene difluoride membranes (Millipore, Bedford MA) and blocked in 5% non-fat milk in PBS supplemented with 0.5% Tween 20 (PBS-T). Primary anti-HA antibody (Santa Cruz Biotechnology, Santa Cruz CA) was used at a dilution of 1:500 in blocking buffer, and signal was visualized by incubation in goat anti-rabbit conjugated horseradish peroxidase with enhanced chemiluminescence detection (Supersignal, Pierce Biochemical, Rockford IL), and exposure to X-ray film. For analysis of SMAF1 by green fluorescent protein fusion expression, the complete SMAF1 open reading frame, excluding the initiator methionine, was ligated in-frame to the pEGFP-C2 fusion vector (Clontech) by PCR-based cloning. Cloning junction and the SMAF1 region were sequenced. The resultant EGFP–SMAF1 fusion construct, wherein SMAF1 coding sequence is fused C-terminal to EGFP, was transfected into COS cells using Lipofectamine 2000 (Invitrogen), or transfection was conducted with EGFP-C2 empty vector control. At one day post-transfection cells were observed by fluorescence microscopy and digital images were captured with an Olympus IX70 inverted microscope with Spot software (Diagnostic Instruments, Sterling Heights, MI).

Isolation and characterization of SMAF1 genomic clones and gene structure. Genomic clones for SMAF1 were isolated from an SVJ129 mouse genomic library in the vector λ FIXII (Stratagene, La Jolla, CA). Phage plaques were screened by standard plate-lift filter hybridization method using random-primed ^{32}P-labeled full-length SMAF1 cDNA as probe. Two genomic clones that were determined by Southern blot hybridization of phage DNA to SMAF1 probe to likely contain the complete SMAF1 structural gene and portions of the 5′ and 3′ flanking regions were further analyzed by DNA sequencing, restriction mapping, and PCR analysis. This was combined with available database information on the murine SMAF1 gene using www.ensemble.org to determine intron–exon structure.

Results and discussion

DNA array identifies induction of a novel gene, SMAF1, during adipocyte conversion

To identify and characterize new genes important in adipocyte function, we undertook DNA array analysis of 5184 cDNAs utilizing ResGen GeneFilter arrays (Invitrogen). Duplicate filters were hybridized with reverse-transcribed probes prepared from total RNA obtained from either murine 3T3-L1 preadipocytes or from 3T3-L1 adipocytes resulting from dexamethasone and methylisobutylxanthine induced in vitro differentiation. In the latter case, seven days following initiation with adipogenic inducers, approximately 90% of the cells had undergone adipose conversion upon visual inspection. Following hybridization, washing, and exposure to film, signals were analyzed visually. After conducting a subsequent round of hybridization with reverse transcribed probes prepared from kidney and brain, we identified differentially expressed clones showing specific expression in adipocytes; one of these was chosen for additional studies. Array hybridization patterns indicated that this particular clone was induced during adipose conversion of 3T3-L1 cells and was not detectable with the probes derived from kidney or brain. Cross-referencing with the array database indicated that this cDNA corresponded to an unknown EST clone derived from 19.5-day mouse embryo library. We have termed this new gene SMAF1. A portion of the array that contains the SMAF1 signal is shown in Fig. 1A. A comparison of the barely visible SMAF1 signal intensity with those of other signals on the array indicates that SMAF1 is likely present at low abundance in adipocytes. An EST corresponding to this signal was obtained as an IMAGE clone (GenBank Accession No. AI390834) and the insert used to verify differential expression in 3T3-L1 preadipocytes and adipocytes by Northern blot analysis shown in Fig. 1B. This figure also indicates that SMAF1 transcript is detected in adipose tissue at a level similar to that present in 3T3-L1

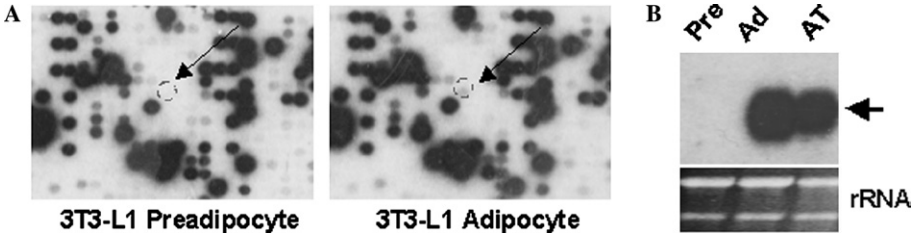

Fig. 1. Differential hybridization identification of SMAF1 transcript. (A) DNA array analysis. Nylon arrays were hybridized to reverse-transcribed probes from 3T3-L1 preadipocytes (left panel) or 3T3-L1 adipocytes (right panel) at 7 days post-induction of adipogenic differentiation. The arrow and circled area indicate position of the SMAF1 signal on the array. (B) Validation of DNA array signals by Northern blot analysis. Northern blot indicates SMAF1 level (arrow) in 3T3-L1 preadipocytes (Pre) and mature day 7 adipocytes (Ad), and white adipose tissue (AT). EtBr stained rRNA is shown below.

adipocytes. Since SMAF1 represented a new gene regulated during adipogenic conversion we performed additional studies to further characterize its expression and regulation in white and brown adipogenesis.

The SMAF1 gene encodes a unique bipartite 80 amino acid protein with nuclear localization

To determine the full-length SMAF1 cDNA sequence six additional SMAF1 cDNA IMAGE clones (BE629860, BE629856, AA218259, AA189269, AI413624, BE624674, and AI118813) were obtained and we identified those with the largest insert sizes, approximately 600–700 nucleotides. Since this insert size corresponded to that of the mRNA transcript detected in Northern blot analysis, it was deduced to likely correspond to full-length SMAF1. To ascertain if additional length of 5′ transcript was present, 5′ RACE analysis was performed using the SMART-RACE analysis kit (BD Biosciences Clontech). This 5′ RACE data combined with our sequencing of multiple SMAF1 EST clones on both strands resulted in full-length sequence for SMAF1. Fig. 2 shows the SMAF1 cDNA sequence and the corresponding predicted protein sequence. The murine SMAF1 cDNA is 633 nucleotides with an additional length of poly(A) tail. This sequence has been deposited in GenBank, Accession No. AY092026. The SMAF1 ORF was identified with GeneScan software and via manual inspection for the presence of initiator methionine and stop codons. In silico analysis of the relative conservation of predicted coding sequences between regions of human, rat, and cow SMAF1 was also utilized in the prediction of the SMAF1 open reading frame. The SMAF1 gene product is 80 amino acids with a p*I* of 4.25. Isolation and analysis of genomic clones for SMAF1 combined with searches of the Ensembl murine databases indicates that the murine SMAF1 gene consists of three exons and two introns spanning 5.6 kb, Fig. 3A. These analyses also reveal that the SMAF1 gene maps to the distal portion of murine chromosome 2 and to the syntenic region of human 20q11 (Fig. 3B). We have also identified SMAF1 homologs in rat and cow, in silico, with overall regions of homology of SMAF1 proteins in these various species shown in Fig. 3C. However, database searches have failed to identify SMAF1 homologs in *Danio rerio*, *Xenopus laevis*, *Gallus gallus* nor in the invertebrates *Caenorhabditis elegans* or *Drosophila melanogaster*, suggesting that the SMAF1 gene may be restricted to mammals.

The SMAF1 protein sequence shows a striking bipartite primary structure with distinct compositions in the amino- and carboxyl halves of the protein. The N-terminal half of the molecule is rich in leucine, comprising 14 of the initial 40 residues. The spacing of the leucines, however, is not that found in either leucine-rich repeats, leucine zipper, or other leucine-based protein motifs, but do exactly match that of a consensus nuclear export signal, $LX_{2-3}(F/I/L/V/M)X_{1-3}LX(I/V/L)$ [25]. SMAF1 also has a single short stretch of several basic amino acids flanked by helix-breakers, PWSKRP, that is suggestive of a nuclear localization signal [26,27]. In contrast to the N-terminal region, the C-terminal half of SMAF1 is enriched in acidic amino acids, with aspartic acid and glutamic acid comprising 12 of 40 residues. Acidic-rich elements, a so-called "acidic blob," are a common activation region found in many transcriptional activators [28–31]. Western blot analysis of an HA epitope-tagged SMAF1 in transfected COS cells reveals SMAF1 to be present as a single protein species of

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  1 cttccttgttacatcacactctccgtaactaagtcacaattggactcaaagtagatcctg
 61 ccaagctgacaggtcttcaggagcagccctcccgctgaatccaggtggcccgcagggcc
                M  K  Y  P  L  V  P  L  V  S  D  L  T  L  S  F   16
121 ccaggtatgcgATGAAGTACCCTCTGGTGCCTCTGGTGAGCGACCTCACACTCGTCTTTC
    L  V  F  W  L  C  L  P  V  A  L  L  L  F  L  T  I  V  W  L   36
181 CTGGTTTTCTGCTCTGCCTGCCCGTGGCTTTGCTGCTGTTCTTGACGATCGTCTGGTTGC
    H  F  L  L  S  Q  E  S  K  E  D  D  S  D  L  C  F  N  W  E   56
241 ATTTCTTACTTAGTCAAGAGTCAAAGGAAGATGATTCAGATTTGTGCTTCAACTGGGAGC
    P  W  S  K  R  P  S  E  C  G  C  E  E  T  F  P  G  E  E  D   76
301 CCTGGAGCAAAAGACCATCTGAGTGTGGCTGTGAGGAGACATTTCCTGGTGAGGAGGACG
    G  L  H  W  *                                                 80
361 GACTCCACTGGTGAtcctgctctaccaggtaccatctgggcctaagtggatggagggaga
421 acactggtgaccgatggccttccctgggatctcccgttgtcagacctgccaagcctcttg
481 ccaaaccatcccttctccatcaccacagatgacccggaacctggaagagccaggcccaag
541 cggcttatgggggccagttggccagtcaactcacagggtgtgttggatacctattaaaga
601 aatgggggtgcttttgtgcccctaacagagctt
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Fig. 2. Nucleotide and predicted protein sequence for SMAF1. The nucleotide sequence and predicted encoded 80 amino acid protein is shown. The numbers on the left of the sequence indicate nucleic acid positions and to the right the amino acid positions. Untranslated regions of the nucleic acid sequence are shown in lowercase and translated portion in uppercase, with the stop codon indicated by an asterisk. The nucleotide sequence has been deposited in GenBank as Accession No. AY092026.

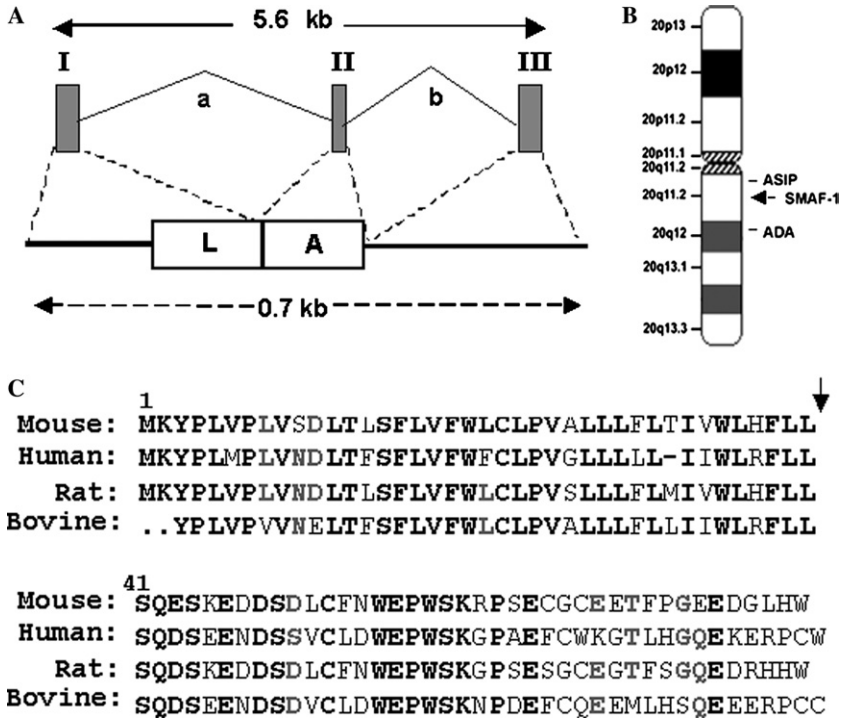

Fig. 3. Gene structure, chromosomal location, and species conservation of SMAF1. (A) SMAF1 gene contains 3 exons (I, II, and III) and 2 introns (a and b) spanning 5.6 kb, indicated with solid arrow above the diagram. The dashed arrows below the diagram indicate the 0.7 kb size of SMAF1 cDNA. The dashed lines from the exon boxes indicate corresponding regions of the SMAF1 transcript, shown as a solid line representing the SMAF1 untranslated regions, and the boxes designating the coding region, with L indicating the leucine-rich exon I and A indicating the acidic exon II. (B) Chromosomal location of human SMAF1. The SMAF1 gene (arrow) is located on human chromosome 20 at 20q11, between agouti signaling protein (ASIP) and adenosine deaminase (ADA). (C) Sequence conservation of SMAF1 protein across several mammalian species. The downward arrow indicates the placement of the intron that separates the N-terminal leucine-rich portion from the C-terminal acidic region. Invariant amino acids are shown in dark bold and similar amino acids are shown in grey bold.

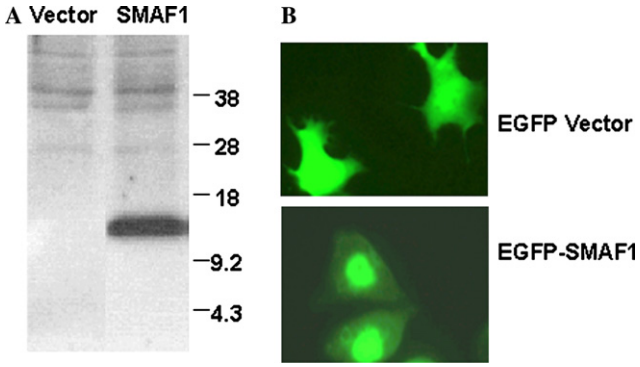

Fig. 4. Expression and intracellular localization of SMAF1 protein. (A) Expression of SMAF1 protein in COS cells. COS cells were transfected with empty pcDNA3.1 vector or a HA-tagged SMAF1 and analyzed by Western blot using anti-HA primary antibody. (B) COS cells were transfected with empty EGFP vector or EGFP–SMAF1. The EGFP fluorescence was visualized using the FITC channel.

10 kDa, Fig. 4A. Expression of EGFP–SMAF1 in COS cells shows a light cytoplasmic signal and intense nuclear signal, while control cells transfected with EGFP-C2 empty vector show uniform fluorescent signal, Fig. 4B. Thus, while the SMAF1 protein sequence and localization is suggestive of a role in nuclear regulatory processes, the PFAM, Prosite, and GenBank searches indicate that SMAF1 is not a member of any known protein family but is a wholly novel gene.

SMAF1 is selectively expressed in adipose tissue

To determine the tissue distribution of SMAF1 in detail, we conducted Northern blot analysis on a panel of murine tissue RNAs. Fig. 5A indicates that of the variety of tissues examined, SMAF1 signal is present only in adipose. Adipose tissue is heterogeneous in composition in regard to cell type. To assess which cell type(s) within white adipose tissue expressed the SMAF1 transcript, murine adipose tissue was subjected to fractionation into the stromal vascular and adipocyte populations, Fig. 5B. As expected, SCD1 and resistin are found in the adipocyte fraction. For a marker for the stromal vascular fraction we utilized TSC-36, a secreted follistatin-like molecule, also known as Fstl1 [32,33], which we have recently determined to be a preadipocyte-expressed gene in in vivo and in vitro adipogenesis (manuscript in preparation). SMAF1 is detected only in the adipocyte fraction of adipose tissue and is completely absent from the stromal vascular fraction.

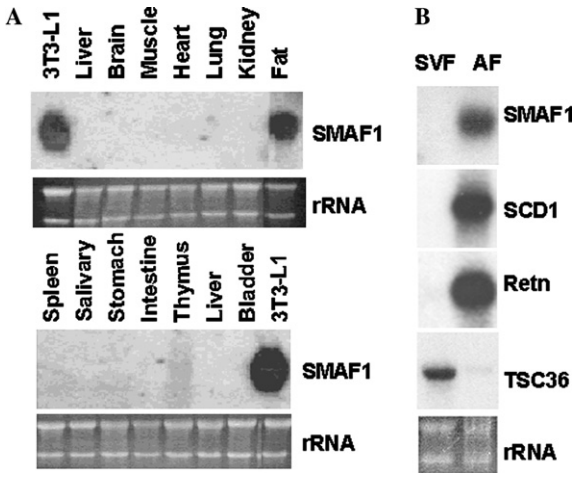

Fig. 5. Adipose tissue expression of SMAF1 transcript. (A) SMAF1 transcript level in a panel of murine tissues. Five micrograms of total RNA from indicated mouse tissues was analyzed by Northern blot using SMAF1 cDNA probe; fat indicates white adipose tissue. 3T3-L1 adipocyte RNA was loaded for positive control. (B) SMAF1 transcript in fractionated murine adipose tissue. Northern blot shows transcript levels for SMAF1, stearoyl-CoA desaturase 1 (SCD1), resistin (Retn), and TSC36 in stromal vascular fraction (SVF) and adipose fraction (AF). For (A) and (B) above, EtBr staining of rRNA was used as a gel loading control.

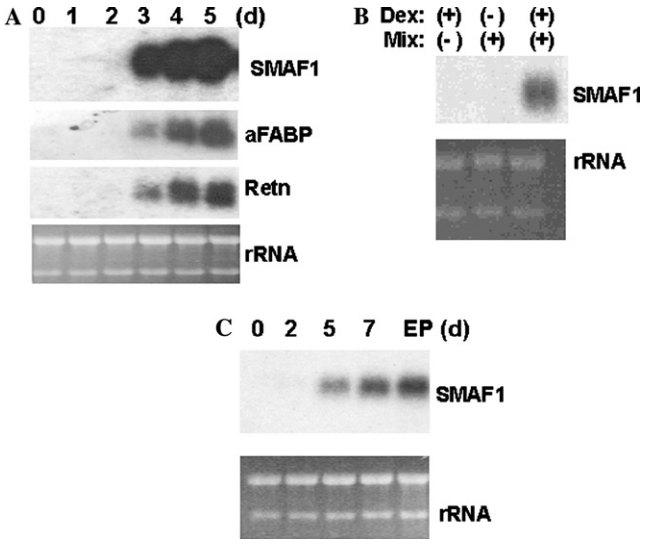

Fig. 6. Upregulation of SMAF1 transcript during white adipogenesis. (A) SMAF1 upregulation in 3T3-L1 in vitro adipocyte differentiation. 3T3-L1 cells were harvested prior to induction of adipogenesis as confluent preadipocytes (day 0), or at indicated daily interval post-induction of adipogenic differentiation with the standard dexamethasone and methylisobutylxanthine protocol. RNA was isolated at indicated time points and analyzed by Northern blot using SMAF1, aFABP, and resistin (Retn) cDNA probes. (B) SMAF1 transcript expression in the presence of Dex/Mix. 3T3-L1 preadipocytes were treated with 1 μM dexamethasone (Dex), 0.5 mM methylisobutylxanthine (Mix), or by the standard combination of Dex and Mix for two days post-confluence. RNA was harvested at day 5 of differentiation. (C) SMAF1 upregulation in adipocyte differentiation of rat primary preadipocytes. Stromal vascular fraction cells from rat white adipose tissue were harvested and cultured in DMEM containing 10% FCS. Confluent cells were subjected to induction of adipogenic differentiation with dexamethasone, methylisobutylxanthine, and insulin. RNA was prepared at confluence (day 0), and at 2, 5, and 7 days post-induction and hybridized with SMAF1 cDNA probe. Murine epididymal adipose tissue (EP) was included as a positive control. For (A)–(C) above, EtBr staining of rRNA was used as a gel loading control.

Differentiation-dependent expression of SMAF1 in models of white adipogenesis

If SMAF1 is integral to the adipocyte phenotype, it would be expected to be upregulated in various models of adipogenic conversion. Northern blot analysis in Fig. 6A shows that SMAF1 is completely absent from murine 3T3-L1 preadipocytes and is induced dramatically during their adipocyte differentiation. SMAF1 transcript is first noted the day after removal of differentiation inducers (day 3) and parallels emergence of transcripts for aFABP and resistin. Fig. 6B indicates that treatment of 3T3-L1 preadipocytes with either the dexamethasone or the methylisobutylxanthine components of the differentiation cocktail individually does not result in upregulation of SMAF1 transcript. This indicates that SMAF1 gene is not merely responding to one or the other of these two differentiation agents, but rather that the temporal upregulation of SMAF1 is very closely tied to the full program of adipogenesis. Fig. 6C reveals that SMAF1 transcript is not detected in primary cultures of rat stromal vascular cells, but is dramatically upregulated during differentiation of these primary cultures to white adipocytes. Here the SMAF1 transcript level in mature fat cells (day 7) was similar to that found in epididymal adipose tissue. Together these studies indicate that conversion of preadipocytes to mature fat cells is accompanied by striking upregulation of SMAF1 transcript and argue that expression of SMAF1 may be an important aspect of adipogenesis in vitro and in vivo.

Regulation of SMAF1 transcript in TNFα-induced 3T3-L1 adipocyte dedifferentiation

To begin to examine regulation of SMAF1 in the context of adipose tissue physiology and pathophysiology, we tested SMAF1 regulation by TNFα, a mediator of insulin resistance [34]. Treatment of mature 3T3-L1 adipocytes with TNFα is known to result in adipocyte dedifferentiation and one mechanism of its action is via downregulation of PPARγ [35]. Recent microarray studies reveal that TNFα treatment of adipocytes results in a coordinated downregulation of adipocyte-expressed genes and a concomitant re-expression of select preadipocyte genes [22,36]. As shown in Fig. 7, exposure of 3T3-L1 adipocytes to 10 ng/ml TNFα treatment resulted in a marked decrease of SMAF1 transcript; this is first noted at 4 h and is markedly decreased by 12 h post-treatment. The downregulation of SMAF1 was preceded by a decrease in PPARγ transcript level, suggesting that

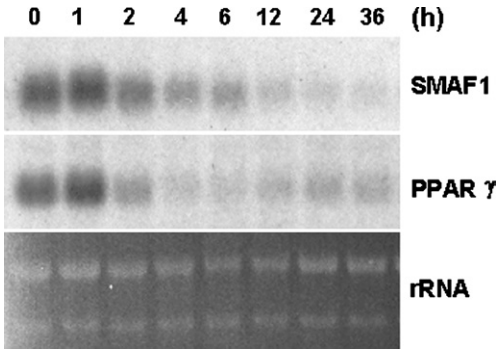

Fig. 7. Downregulation of SMAF1 transcript by TNFα. Mature 3T3-L1 adipocytes at day 5 post-induction of differentiation were treated with 10 ng/ml TNF for the indicated time points. RNA was analyzed by Northern blot using murine PPARγ or SMAF1 radiolabeled cDNA probes. EtBr staining of rRNA was used as a RNA gel loading control.

the TNFα-mediated reduction in SMAF1 transcript may occur via its effects on PPARγ.

Brown adipose tissue is the predominant adipose depot site for SMAF1 transcript

To determine if SMAF1 is also present in brown adipose tissues and to compare its relative transcript level in white versus brown adipose tissue, RNA was prepared from interscapular brown adipose tissue, and various white adipose tissue depots including retroperitoneal, epididymal, and subcutaneous white adipose tissue, and the transcript level was determined by Northern blot analysis. As shown in Fig. 8A, SMAF1 is expressed in brown adipose tissue, and in fact among all the adipose depots examined, brown adipose tissue shows the highest level of SMAF1 transcript. To determine the expression of SMAF1 transcript during brown adipogenesis we utilized a brown preadipocyte cell line [24]. As has been previously described for this cell line, and which we show in Fig. 8B, in vitro brown adipogenesis is indicated by the expression of the brown adipocyte gene, UCP1, as well as morphological conversion. Brown adipogenesis is accompanied by a robust upregulation of SMAF1, which is non-detectable in brown preadipocytes. Interestingly, SMAF1 is expressed at highest levels midway through differentiation, prior to UCP1 expression, although it is also detected in fully mature brown adipocytes.

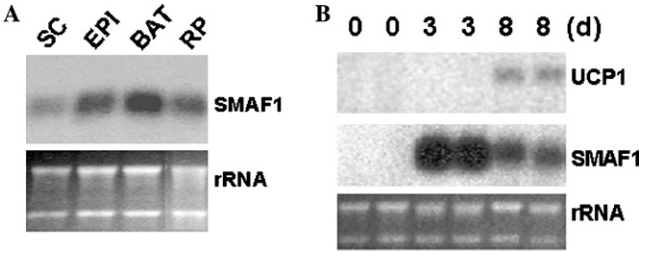

Fig. 8. SMAF1 transcript expression is particularly enriched in brown adipogenesis. (A) SMAF1 transcript distribution in murine adipose tissue depots. Subcutaneous fat (SC), epididymal fat (EPI), brown adipose tissue (BAT), and retroperitoneal fat (RP) RNA were isolated from C57BL/6 mice. Five micrograms of total RNA was analyzed by Northern blot using SMAF1 cDNA probe. (B) SMAF1 transcript expression in brown adipogenesis. Brown preadipocytes were differentiated as described under Materials and methods at confluence (day 0). Brown adipocytes were harvested in duplicate at 0, 3, and 8 days post-onset of differentiation. Five micrograms of total RNA was hybridized on a Northern blot with SMAF1 and UCP1 probes. For (A) and (B), above, EtBr staining of rRNA was used as a gel loading control.

Overall our findings indicate SMAF1 upregulation is an integral part of the adipogenic program for white and brown adipocytes, and dysfunction of this program, as evidenced by TNFα-induced dedifferentiation of white adipocytes, involves dysregulation of SMAF1 transcript levels in adipocytes. Interestingly, in addition to our studies herein, the relevance of SMAF1 in an in vivo setting is illuminated by data recently reported as part of a DNA microarray study by Reddy and co-workers [37] which assessed the effects of ectopic adenoviral expression of PPARγ1 in the liver of PPARα null mice. These mice manifested an adipogenic transformation of hepatocytes, termed adipogenic hepatic steatosis, which was accompanied by increased expression of a number of key adipocyte genes in liver including adiponectin and aFABP. As we had recently deposited the SMAF1 sequence into GenBank (Accession No. AY092026), these data were cross-referenced in the Affymetrix database, and enabled the assessment that SMAF1 showed an 8-fold increase in the liver of mice with PPARγ1-induced adipogenic hepatic steatosis.

In conclusion, although the exact function of SMAF1 remains to be uncovered, we argue that the characteristics we have described herein place SMAF1 in a very select group of adipocyte genes whose expression defines the molecular nature of fat cells. The timely application of DNA array technologies to adipocyte differentiation/function has confirmed long-standing observations [38] that adipogenesis is accompanied by a complex pattern of gene expression [39]. DNA microarray profiling of approximately 12,000 genes by the Friedman laboratory found changes in over one thousand genes, with 27 distinct temporal expression patterns identified and distinctions noted for in vitro versus in vivo adipogenesis [21]. Such data indicate that, in addition to those proteins whose function in adipocytes has been under study for some time, many additional signaling networks and protein families are likely involved in the regulation and/or maintenance of adipocyte differentiation and function. However, high-throughput studies have also, paradoxically, revealed that those genes that truly define the adipocyte phenotype are likely to be limited in number. Microarray studies by MacDougald and co-workers [18] examined roughly 12,000 murine genes and

identified a subset of 120 genes they suggest may define the adipocyte phenotype, using the criteria of a similar degree of upregulation during both in vivo and in vitro adipogenesis. If we impose the additional criteria of adipocyte-specific expression on this subset, relatively few genes would be in this highly select group. Thus, while a large number of genes are altered during adipogenesis, relatively few of these would be predicted to show the expression pattern illustrated by SMAF1. Together our data indicate that SMAF1 may occupy a unique functional niche in the repertoire of the adipocyte-specific transcriptome and work in the laboratory is currently underway to assess the function of SMAF1 in adipose tissue in vivo.

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