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A Novel pro-adipogenesis factor abundant in adipose tissues and over-expressed in obesity acts upstream of PPARγ and C/EBPα

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Abstract An important question about adipogenesis is how master adipogenesis factors (defined as being able to initiate adipogenesis when expressed alone) peroxisome proliferator-activated receptor (PPAR) initiate adipogenesis only in differentiating preadipocytes. The objective of our research was to find previously unidentified factors that are unique or highly enriched in cells of the adipocyte lineage during adipogenesis that may provide functional tissue specificity to preadipocytes. We reasoned that such factors may alter expression profile specifically in obese individuals. Omental adipose tissues were obtained from obese and non-obese male patients undergoing emergency abdominal surgery. mRNAs extracted from either group were used for suppression subtraction hybridization (SSH). Genes corresponding to mRNAs enriched in obese versus non-obese patients were identified through sequencing and further analyzed for tissue distribution. Out of ~20 genes, we found several that showed clear fat cell specific expression patterns. In this study, we functionally studied one of these genes, previously designated as open reading frame C10orf116. Our data demonstrated that C10orf116 is highly expressed in adipose tissue and is localized primarily within the nucleus. Over-expression studies in 3T3-L1 cells indicated that it up-regulates the levels of CCAAT/enhancer binding protein α (C/EBPα) and PPARγ and promotes adipogenic differentiation starting from the early stage of adipogenesis. Over-expressed in omental tissues from obese patients, C10orf16 manifested the characteristics of an adipocyte lineage-specific nuclear factor that can modulate the master adipogenesis transcription factors early during differentiation. Further studies of this factor should help reveal tissue-specific events leading to fat cell development at the transcriptional level.

Keywords PPARγ · C/EBPα · Master adipogenesis factors · Pre-adipocytes differentiation · Adipocyte-specific factors

Introduction

Excessive and unbalanced fat tissue mass builds up in obese individuals. Adipose tissue mass can accumulate from an increasing number of adipocytes through adipogenesis or increasing amount of triglycerides per cell (adipocyte hypertrophy) (Haslam et al. 2005). Various hormones, cytokines, transcription factors, and receptors produced in human omental adipose tissue, such as leptin, leptin receptor, adiponectin, peroxisome proliferator-activated receptor γ (PPARγ) and CCAAT/enhancer binding protein α (C/EBPα), have been suggested to participate in the regulation of adipose tissue mass in humans (Farmer et al. 2004; Galic et al. 2010).

It has been a major focus of obesity research to elucidate the molecular mechanism of adipogenesis. Most of the current understanding of this process came from gene function studies both in vitro and in vivo, where conversion of preadipocytes to mature adipocytes is a strictly regulated

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procedure by sequential activation of key pro-adipogenesis factors. For instance, in the commonly used mouse preadipocyte model 3T3-L1 cells, upon induction to differentiate, adipogenesis repressor preadipocyte factor 1 (Pref-1) is down-regulated and the immediate early proadipogenesis genes C/EBPβ and C/EBPδ are transcriptionally turned on (Cristancho et al. 2011). These two factors then synergize to activate PPARγ, which is capable of controlling full differentiation of adipocytes in coordination with C/EBPα. Each step of adipogenesis is influenced by a number of other factors with relevance to cell cycle controls and extracellular differentiation cues, such as hormones and metabolites.

When ectopically expressed, PPARγ can initiate adipogenesis in cells that have not committed to the adipocyte lineage, demonstrating its capability as the "master adipogenesis factor" (Gregoire et al. 1998). Still, PPARγ is subject to regulations by different ligands and transcriptional co-activators. Among a large number of factors that have been known to influence adipogenesis, PPARγ2, a splicing isoform of PPARγ, is perhaps the only factor that has clear adipose tissue specificity. Relative to post-translational activation of PPARγ, less is known about the transcriptional activation of PPARγ in preadipocytes at the beginning of adipogenic differentiation. C/EBP family members are known to activate PPARγ in a positive feedback loop once adipogenesis starts; however, PPARγ, C/EBP proteins are not adipose-specific themselves.

There are more than 200 genes or chromosomal regions that have been associated with obesity phenotypes and this number keeps growing (Rankinen et al. 2006). It is possible that some of the previously unknown, adipose-specific factors provide tissue-specificity to the PPARγ and C/EBPs transcriptional expression when adipogenesis is initiated. Apparently, more obese-associated candidate genes need to be identified and the underlining molecular mechanisms studied for understanding human obesity and related diseases. In this study, we used suppression subtractive hybridization (SSH) (Larose et al. 2001; Steppan et al. 2001) to identify adipose-specific genes that are overexpressed in obese patients. The results showed that genes encoding many previously known factors related to adipogenesis, lipid metabolism, and diabetes-relevant signaling molecules were identified as differentially expressed in obese versus normal patients' omental adipose tissues, e.g. LIPA, ADFP, SCD, ALDH3A2, IGF1, ZFP36L1, etc. In addition, uncharacterized human factors, including putative membrane bound receptors, secreted proteins, and transcription regulators, were identified in this screen, as we have previously reported (Qiu et al. 2007). Here we present results from our molecular studies on one of the novel factors, C10orf116, which is overexpressed in obesity and shows an expression pattern that is clearly enriched in human adipose tissues. It appears to be a nuclear protein, and may help transcriptionally activate PPARγ and C/EBPα in the early events of adipogenesis. Therefore, understanding regulation and function of C10orf116 may help fight obesity.

Materials/subjects and methods

Ethics statement

All research involving human samples was approved by the Ethics Committee of the Nanjing Medical University. Consent statements from surgery patients were recorded in standard written form.

Adipose tissues from obese and non-obese human subjects

Obese subjects (BMI≥30 kg/m^2) and non-obese "control" (25 kg/m^2≥BMI≥18 kg/m^2) male patients undergoing emergency abdominal surgery for appendicitis at the Department of General Surgery, First Affiliated Hospital of Nanjing Medical University, were recruited with patients consent to donating small fat tissues for biomedical research according to institutional ethics rules (Ethics Committee of the Nanjing Medical University). The full medical records of each patient were then used for screening suitable subjects for the adipogenesis factor isolation project. Special attention was given to the selection procedure to make sure that besides body weight index (BMI), the two groups are comparable in all aspects. This was done by diagnosis of diabetes mellitus, cardiovascular, endocrine, and chronic inflammation diseases by commonly used criteria, as already reported elsewhere (Qiu et al. 2007). All of the selected patients were recorded to have been on a normal diet.

SSH

The differentially expressed genes in omental adipose tissue were screened by SSH, as we have published previously with full experimental details (Qiu et al. 2007). Briefly, A PCR-Select cDNA Subtractive Kit manufactured by Clontech (Palo Alto, USA) was used, according to accompanying product literature. The two directional subtractions were carried out, one for analysis of genes expressed at higher levels in omental tissues from obese patients and the other for analysis of genes highly expressed in omental tissues from normal patients but not obese patients.

Polymerase chain reaction (PCR)

Total RNAs of each sample were extracted using TRIzol reagent (Invitrogen). Reverse transcription was performed using an AMV Reverse Transcriptase kit (Promega, Madison, WI, USA) using a random hexamer primer.

Real-time PCR was performed using an Applied Biosystems 7300 Sequence Detection System (ABI 7300 SDS; Foster City, CA, USA) by following the manufacturer's protocols. RPL27 RNA (transcript encoding the 18S ribosomal protein) was used as a control (Selvey et al. 2001). The sequences of the primers (Allele Biotech & Pharmaceuticals, San Diego, USA) are shown in Supplemental Table 1A. For cDNA cloning, PCR was performed with Advantage™ 2 PCR Kit (Clontech). For generating probes for Northern blots, cDNA fragments were prepared by PCR (Supplement Table 1B) and purified with QIAquick PCR Purification Kit (Qiagen, Germany).

Northern blot analysis

A human 12-lane Multiple Tissue Northern (MTN) blot membrane (Clontech) was probed for analysis of tissue-specific gene expression in Fig. 2a and b. For Fig. 2c, total RNA was extracted from human omental adipose tissue, subcutaneous adipose tissue, and heart (from stored tissue samples provided by the Nanjing Medical University, according to institutional regulations set forth by the Ethics Committee of the Nanjing Medical University) using TRIZOL reagent (Stratagene, San Diego, USA). Ten micrograms total RNA from each sample was subsequently fractionated by electrophoresis on a formaldehyde agarose gel (1.5 % agarose, 6 % formaldehyde, 1×MOPS buffer), transferred to Hybond® N + nylon membrane (GE, USA) using a vacuum blotting apparatus and cross-linked to the nylon membrane with a UVC 500 Ultraviolet Crosslinker (GE, USA). Probes from cDNA templates used in Northern blot analysis were radioactively labeled with (α-^{32}P)-dCTP using the Prime-a-Gene® Labeling System (Promega). After labeling, unincorporated (α-^{32}P)-dCTP was removed using QIAquick Nucleotide Removal Kit (Qiagen). Hybridization was done in ExpressHyb Hybridization solution (Clontech) and washed twice with 2×SSC (0.3 M NaCl, 0.03 M sodium citrate, pH 7.2) and 0.05 % SDS, and once with 0.1× SSC and 0.1 % SDS. The signal of RPL27 transcript was used for normalization of the Northern Blot analysis. Total RNAs of the omental adipose tissues from 8 obese subjects and 8 normal subjects were extracted and Northern blot analysis similarly performed.

Plasmid construct

C10orf116 (also referred to as AFRO, see text for explanation) cDNA was cloned into EGFP-N2 and EGFP-C2 vectors (Clontech) for subcellular localization analysis; or PET-28(a) (Novagen/Merck KGaA, Germany) and pGEX-6P-1 (GE) for protein expression in *E. coli*; or pcDNA.3.1/myc-His(−)A vector (Invitrogen, Carlsbad, USA) for over-expression analysis in tissue culture cells.

Antibody preparation

GST-AFRO was expressed from pGEX6P-1-C10orf116 plasmid in BL21 *E. coli* strains. The recombinant protein and control GST alone protein were purified on Glutathione Sepharose 4B columns (GE Biosciences, Shanghai, China) and used for generating polyclonal antibodies. The AFRO-His fusion protein was expressed using the pET28(a)-C10orf116 plasmid in BL21(DE3) *E. coli* strains and used, without any purification, to independently confirm the specificity of the polyclonal antibodies generated against GST-AFRO.

Cell culture and transfection

Hela, COS-7, 293T, CHO, NIH3T3, and 3T3-L1 cells were purchased from ATCC (Manassas, USA). Human preadipocytes were provided by Allele Biotechnology & Pharmaceuticals (San Diego, USA). For transient transfection, 3T3-L1 and HEK-293T cells were plated at a density of 1×10^5 cells per well in 6-well plates and grown overnight to 80 % confluence, then transfected with pEGFP-N2-C10orf116, pEGFP-C2-C10orf116, or D2EGFP plasmids using Fugene 6 (Roche, Switzerland). The expression of the green fluorescent protein was observed 48 h post-transfection by confocal laser scanning microscopy. For stable transfection, 3T3-L1 cells were plated at a density of 8×10^4 cells per well in 24-well plates and transfected with pcDNA.3.1/myc-His(−)A-C10orf116, pcDNA.3.1/myc-His(−)A pEGFP-N2-C10orf116, pEGFP-C2-C10orf116, or D2EGFP plasmids, as described above. G418 was used at a final concentration of 800 μg/ml 24 h post-transfection. After 14–21 days, G418-resistant populations were enriched and cells maintained in DMEM with 10 % FBS, supplemented with 200 μg/mlG418.

Western blot analysis

Whole cell lysates of adipose tissues and 3T3-L1 cells were prepared by homogenization in modified RIPA buffer (50 mM Tris–HCl, pH 7.4, 1 % Triton X-100, 0.2 % sodium deoxycholate, 0.2 % SDS). Nuclear fraction lysates of 3T3-L1 were prepared by homogenization in Nuclear Protein Extraction Reagent (Pierce/Thermo, Rockford, USA). Fifteen microgram proteins of each sample were separated by electrophoresis on 12 % SDS-polyacrylamide gels and transferred to 0.2 μm nitrocellulose membranes (Schleicher & Schuell/Sigma-Aldrich, USA). Immunological detection was performed by using mouse monoclonal anti-GFP antibody (Clontech), mouse monoclonal anti-6xHis antibody (Clontech), anti-GST-HRP conjugate (GE Biosciences), or the rabbit anti-GST-AFRO antibodies at a 1:500–1:2,000 dilution and corresponding secondary antibodies conjugated

to horseradish peroxidase at a dilution of 1:1,000-1:4,000 (Santa Cruz, USA).

Results

C10orf116 transcript levels are elevated in adipose tissues from obese patients

From a SSH procedure that was carried out to screen for genes that are differentially expressed in omental adipose tissues, 216 genes were found to be expressed at higher levels from obese patients than from normal controls. Several genes known to be related to obesity, such as ACR30, Glut4, IGF1, LPL, and aP2, were among the selected 216 genes (Gomez-Ambrosi et al. 2004). Sequencing of about 1,000 individual cDNA clones generated by SSH indicated that the search for non-redundant genes reached near saturation. Among the 216 obesity up-regulated genes, most were identified in single colonies; more than 20 were represented by 2–3 colonies; 5 genes, including the highly adipocyte-specific adiponectin, were found in 4–5 colonies; only one transcript, C10orf116 (GenBank accession number NM_006829), was found 8 times, the highest abundance in obese white adipose tissue, and always higher than the control white adipose tissue (Supplement Fig. 1).

C10orf116 was first deposited in the GenBank as Adipose Most Abundant Gene Transcript 2 (ApM2, after ApM1). ApM1 was later renamed as adiponectin based solely on DNA sequencing; however C10orf116 does not have any appreciable homology to ApM1/adiponectin1 at the protein or structure levels. Functional studies on this gene could not be found in the literature; even the existence of C10orf116-encoded protein(s) has not been reported previously. Therefore, we considered this protein as a novel factor.

The longest C10orf116 transcript isolated from this screen was 672 bases, containing a 231 nt ORF (Fig. 1a), which is capable of encoding a polypeptide of 76 amino acids, with a predicted molecular weight of ~7.5 kD.

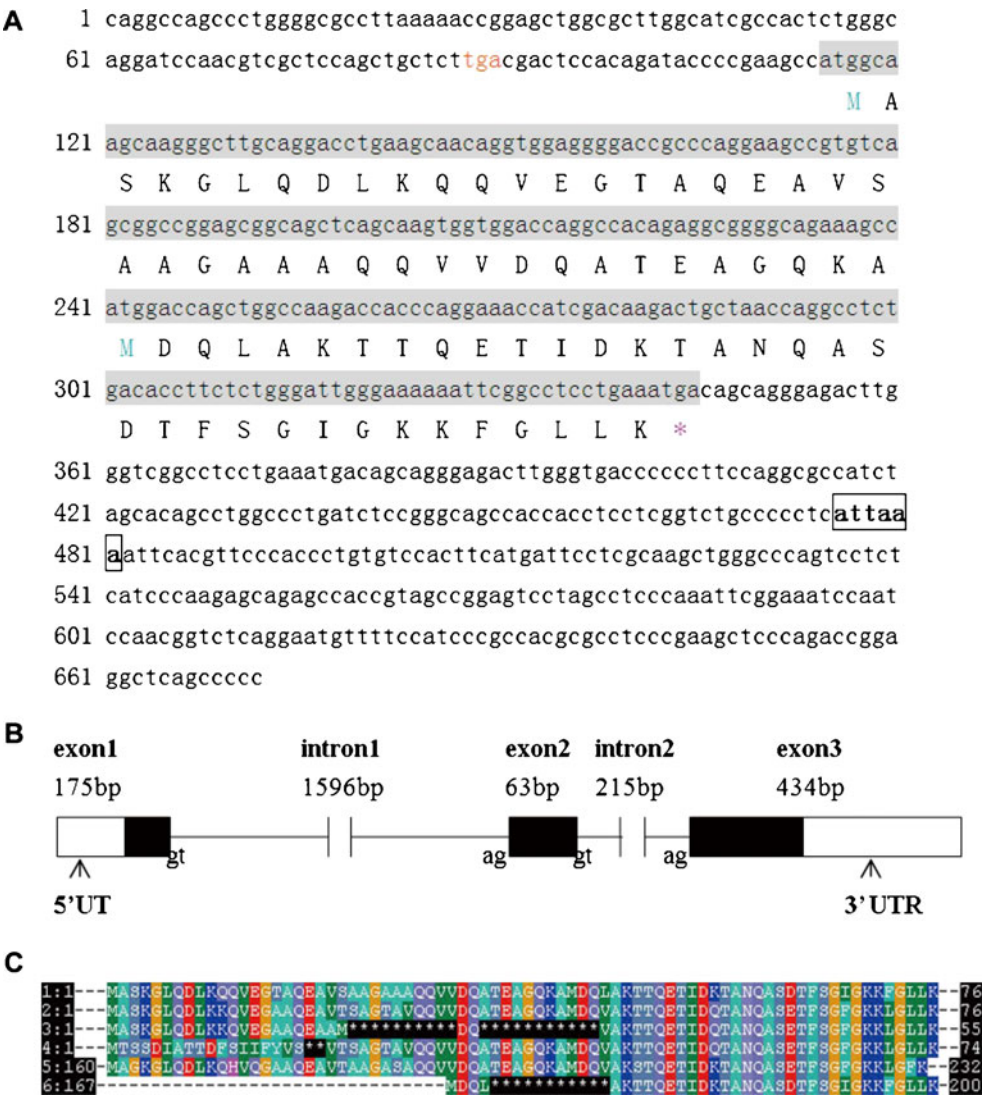

Fig. 1 Gene C10orf116 encodes a conserved protein. **a** Nucleotide sequence of human C10orf116 gene (Genbank accession number: NM_006829) and its deduced amino acid sequence (Genbank accession number: NP_006820). A stop codon, TGA (*red*), is upstream and in frame of the C10orf116 open reading frame (*shaded*), indicating that the ORF is likely complete; a putative polyadenylation signal is also shown (*boxed*). **b** Pre-mRNA splicing of human C10orf116 transcripts. Exons are shown as blocks (*Black*: coding regions). **c** Homology analysis of C10orf116 and its potential homologues in various species, indicated by a number to the left: 1: NP_006820.1, (Homo sapiens), 76aa; 2: XP_870109.1, (Bos Taurus), 76aa; 3: XP_879873.1, (Bos Taurus), 55aa; 4: XP_880037.1, (Bos Taurus), 74aa; 5: XP_853773.1, (Canis familiaris), 234aa; 6: XP_507892.1, (Pan troglodytes), 200aa

C10orf116 is located on chromosome 10, composed of 3 exons with typical GU-AG exon/intron junctions (Fig. 1b). There are a number of isolated EST's in this chromosomal region and at least one alternative splice form that misses exon 2 (data not shown). The predicted protein encoded by this ORF displays homology to a number of hypothetical mammalian polypeptides, suggesting that it might be evolutionarily conserved (Fig. 1c). Somewhat to our surprise, there is no close homologue found in mouse. A thorough search of published protein database failed to identify an isolated protein from this ORF. We refer to the protein encoded by C10orf116 as Adipogenesis Factor Rich in Obesity (AFRO).

We first performed Northern blotting using commercially available blots to confirm the presence of using radioactively labeled cDNA as a probe. Figure 2a showed that, on pre-prepared multiple tissue Northern (MTN) blots (Clontech), up to 6 species of mRNA were hybridized, with uneven distributions in different organs. Upon more stringent washes, however, only one major band of about 700 bases remained (Fig. 2b), in agreement in size with the cloned cDNA. The weak transcripts of bigger sizes might be generated by alternative use of promoters and/or alternative splicing. Among the tested organs, heart had the highest expression level. We note that the MTN blots are manufactured by using RNAs from different organs, each of which contains different cell types, e.g. muscle cells, fat cells. We wanted to see if C10orf116 is enriched in adipose tissues as the SSH screening process had suggested, which were not represented in the MTN blots. Therefore, we isolated RNA samples from human heart tissue,

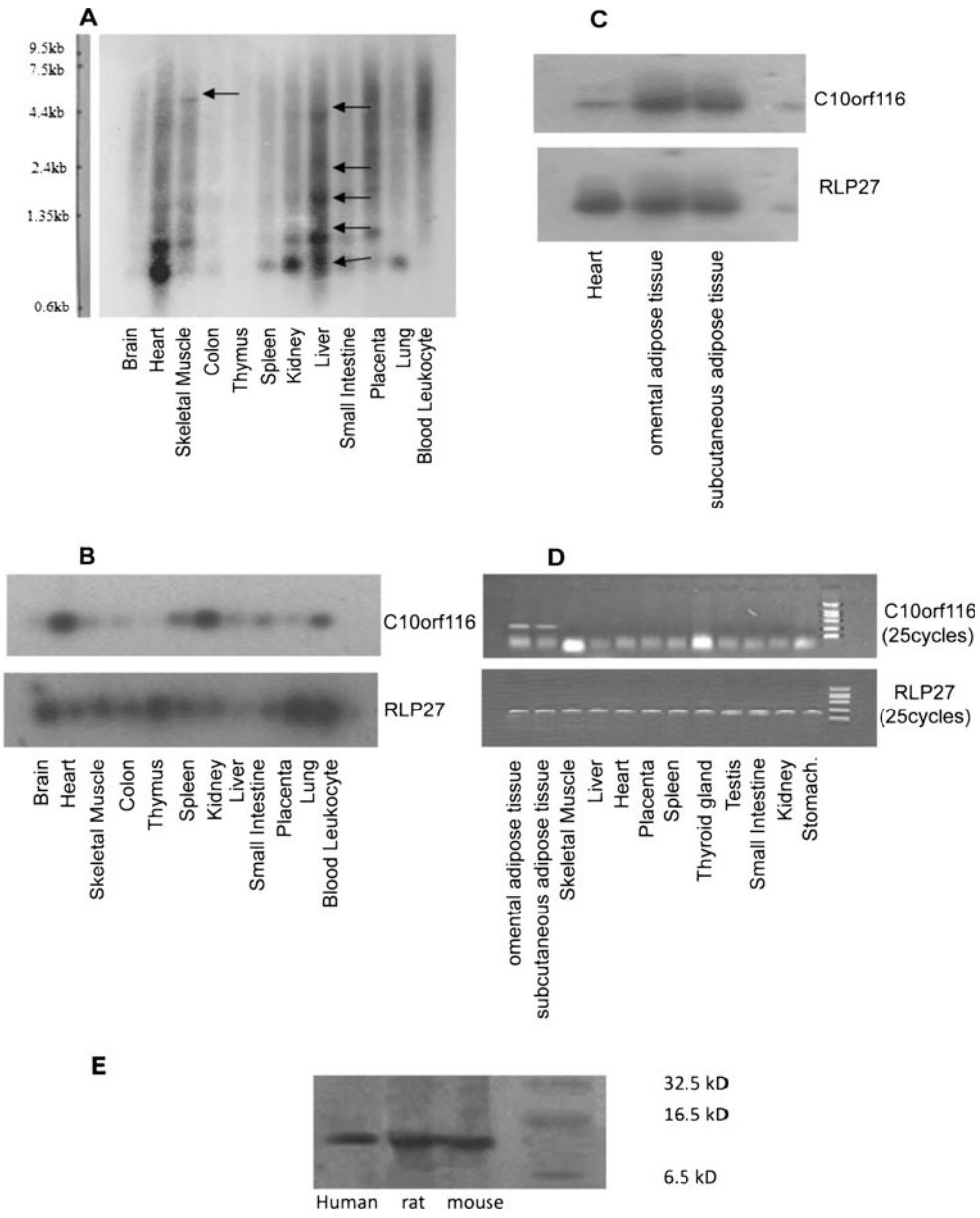

Fig. 2 C10orf116 is highly expressed in adipose tissues. **a** A MTN blot hybridized with α-^{32}P labeled C10orf116 cDNA probes and washed twice with 2×SSC/0.05 % SDS. Arrows indicate possible alternative splicing transcripts, especially in liver and skeletal muscle. **b** The same MTN blot was washed one more time with 0.1×SSC/0.1 % SDS, a more stringent condition, after which signals corresponding only to the main transcript were left. RPL27 cDNA probes were used as controls (bottom). **c** Northern blot of heart muscle, omental adipose tissue, and subcutaneous adipose tissue was hybridized with α-^{32}P labeled C10orf116 cDNA probes (or RLP27 as control, bottom) and washed twice with 0.1×SSC/0.1 % SDS. **d** Presence of C10orf116 in different types of human tissues by PCR analysis revealed that at cycle 25, C10orf116 could be detected only in omental adipose tissue and subcutaneous adipose tissue. **e** Western blot analysis using polyclonal antibodies against GST-AFRO was performed with total proteins of adipose tissues from human, rat and mouse

which showed the highest expression in the MTN blots, and omental adipose tissue and subcutaneous adipose tissue (tissues were provided by the institutional tissue repository under rules set forth by the Ethics Committee of the Nanjing Medical University) was performed under the stringent wash conditions. The results showed that C10orf116 is clearly expressed at higher levels in fat cells than in heart tissue cells (Fig. 2c). This conclusion was independently tested by PCR using RNA samples that we isolated from human omental and subcutaneous adipose tissues, muscles, liver, heart, placenta, spleen, thyroid gland, testis, small intestine, kidney and stomach (tissue samples from the same institutional resource). At lower cycles of the PCR reaction, the C10orf116 signal was visible only in omental adipose tissue and subcutaneous adipose samples (Fig. 2d), supporting the conclusion that it is highly enriched in adipose tissues.

Next, we wanted to analyze expression at the protein level. Because there had not been any report of C10orf116-encoded protein nor antibodies against it, we generated polyclonal antibodies against a GST-fusion protein of AFRO encoded by the C10orf116. The polyclonal antibodies recognized the correct-sized recombinant protein produced in E. coli as a His-tag fusion, but not the GST protein alone encoded by the empty vector (data not shown). As predicted, a protein was identified in human adipose tissues on a Western blot (Fig. 2e). Furthermore, proteins similar in size were recognized by this anti-serum in both mouse and rat adipose tissues (even though a BLAST homology search did not identify a homologue in mouse, possibly because that amino acid variations among the short sequence are not tolerated well by the software filters used in BLAST, Fig. 1c). More quantitative protein analysis awaits the availability of monoclonal antibodies or other reagents.

AFRO promotes adipogenic differentiation when exogenously expressed in preadipocytes

Mouse preadipocytes 3T3-L1 cells were used to investigate whether AFRO is functionally relevant in adipocyte biogenesis, as its expression profile suggested. 3T3-L1 cell lines that stably integrated either a pcDNA3.1/myc-His-C10orf116 plasmid or the empty vector pcDNA3.1/myc-His were established. Both cells were then plated and grown to confluence (day 0). Strikingly, on day 2 after confluence, without any induction, AFRO-over-expressing cells started to show many small lipid droplets when stained with oil red O while the control cells did not (Fig. 3a, day 2). This phenomenon was repeatedly observed only with overexpression of AFRO, among all factors that we have tested during this study. Adipogenesis induction was performed on day 2 with the standard MIX, dexamethasone, and insulin combination. On day 4, the difference between AFRO-over-expressing cells and vector control cells drastically increased: 50–60 % of the AFRO-over-expressing cells had many lipid droplets whilst the control cells only started to have lipid droplets in small numbers. Induction reagent mixtures were removed by reverting back to regular growth medium on day 4 and the cells were allowed to continue differentiation. From pictures taken on day 8, it was clear that the difference between the pcDNA3.1/myc-His-C10orf116 and the control cells was maintained throughout the course of adipogenesis (Fig. 3a). To eliminate well-to-well variations during adipogenesis, this experiment was repeated 6 times and the same results were consistently observed (data not shown).

Concurrently, triglyceride and glycerol assays were conducted to provide a more quantitative comparison of the adipogenesis progress between the two cell lines. As an indicator for adipogenesis, triglycerides were detected in day 2 only in AFRO-over-expression cells and their levels increased dramatically from day 2 to day 4 (Fig. 3b, left panel, $p<0.05$, data from 6 repeats). The average triglyceride levels in AFRO-over-expressing cells were 3–4 times those of the control cells on day 4, when the latter had just started to have detectable amount of triglycerides. The difference decreased in day 6 and day 8, when the majority of control cells also differentiated. To eliminate the possibility that the difference of triglyceride levels merely reflected the difference in growth states instead of adipogenesis between the two cell lines, glycerol contents, commonly used as a lipolysis indicator, were analyzed simultaneously. Although the AFRO-over-expressing cells had higher glycerol levels throughout the course of differentiation than control cells, the differences at the early time points were not as significant as those of triglycerides, and the ratio remained constant in later days (Fig. 3b, right panel, $p<$ 0.05). Lipolysis occurs in both differentiated and undifferentiated cells, the higher level of lipid metabolism in AFPO-over-expressing cells correlated with higher amounts of oil droplets.

AFRO stimulates PPARγ and C/EBPα transcription at early stages of preadipocyte differentiation

From the above data, it appears that AFRO may play a role in the early stages of adipogenesis. We then focused on the relationships between AFRO and other known early acting adipogenesis genes. C/EBPβ and C/EBPδ are the earliest acting genes during adipogenesis, which can turn on the master regulator of adipogenesis, PPARγ. However, it is not clear through what mechanism PPARγ transcription activation is achieved in cells that have received signals to differentiate into adipocytes. We decided to investigate whether AFRO has any effects on the early-acting genes in adipogenesis at the transcriptional level. qPCR reactions using RNA samples from either the pcDNA3.1/myc-His-C10orf116 stable cells or control cells were performed to demonstrate the transcription levels of genes Pref-1, an

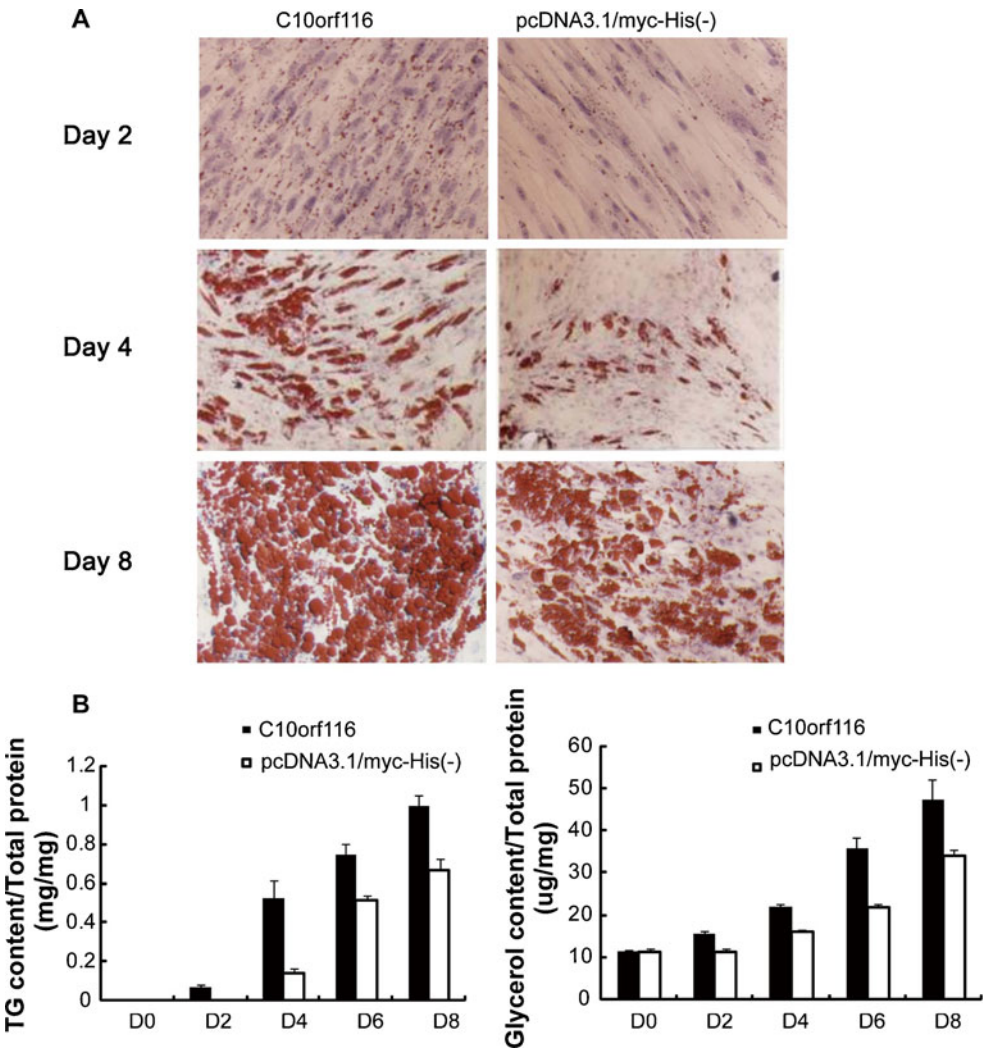

Fig. 3 Overexpression of C10orf116 stimulates adipogenesis. **a** 3T3-L1 cells stably transfected with plasmid C10orf116-pcDNA3.1/myc-His (−) or control plasmid pcDNA3.1/myc-His(−) were induced to differentiate on Day 2. Oil Red O staining revealed that the overexpression of C10orf116 greatly promoted the adipogenic differentiation even before induction (Day 2) and the difference remained clear throughout (Day 4 to Day 8). The process was repeated multiple times and the results remained consistent (not shown). **b** Triglyceride assays were performed in order to qualitatively illustrate the degree of differentiation of 3T3-L1 cells with or without transgenic C10orf116 expression (left). Glycerol contents were analyzed as lipolysis indicator, of which the difference at the early time points was not as significant as that of triglycerides, and the ratio remained constant in later days (right), data from 6 repeats. All values are presented as means ± standard deviation (SD), also in following figures. Statistical analyses were performed using one-way ANOVA or Student's t test. The threshold of statistical significance was defined as $P<0.05$

adipogenesis repressing factor that is turned off transcriptionally right after differentiation induction; C/EBPβ and C/EBPδ, early acting genes that have the capability of activating PPARγ and thereby initiating the process of adipogenesis; ADD/SREBP1c, a lipogenesis factor with a similar expression profile to PPARγ and the capability of promoting PPARγ; PPARγ, and C/EBPα, the two most critical genes required for adipogenesis. As a result, AFRO overexpression specifically up-regulated the mRNA levels of PPARγ and C/EBPα while it did not seem to have any influence on Pref-1, C/EBPβ, C/EBPδ, ADD/SREBP1c, or the unrelated control gene RPL-27. The increase was about 2 fold in the case of PPARγ, and 3–4 fold in case of C/EBPα (Fig. 4, $p<0.05$ data from 6 repeats).

From the above results, we reasoned that AFRO probably acts as a positive factor during adipogenesis downstream of C/EBPβ and C/EBPδ, but upstream of PPARγ and C/EBPα. In a simplified model, if this pathway functioned purely at the transcriptional level, one would expect that the mRNA of the endogenous C10orf116 gene would be present no later than those of PPARγ and CEBPα after induction. In order to find out whether this was the case, we induced differentiation of human preadipocytes and monitored the expression profile of C10orf116 in relation to adipogenesis-relevant genes. Figure 5 showed that AFRO is indeed expressed at approximately the same time as ADD/SREBP1c, but earlier than PPARγ and C/EBPα, in agreement with the results obtained using 3T3-L1 mouse cells. Although the expression profiles of PPARγ and C/EBPα are variable among results reported by different groups, our results are generally agreeable with other publications (Ntambi et al. 2000).

Nuclear localization of AFRO protein

If AFRO plays a role in controlling transcriptional levels of the adipogenesis master genes, it should be expected to locate in the nucleus. Plasmid constructs of both N-terminal and C-terminal fusion proteins of AFRO to D2EGFP were generated and transiently transfected into 3T3-L1, Hela, COS-7, 293T,

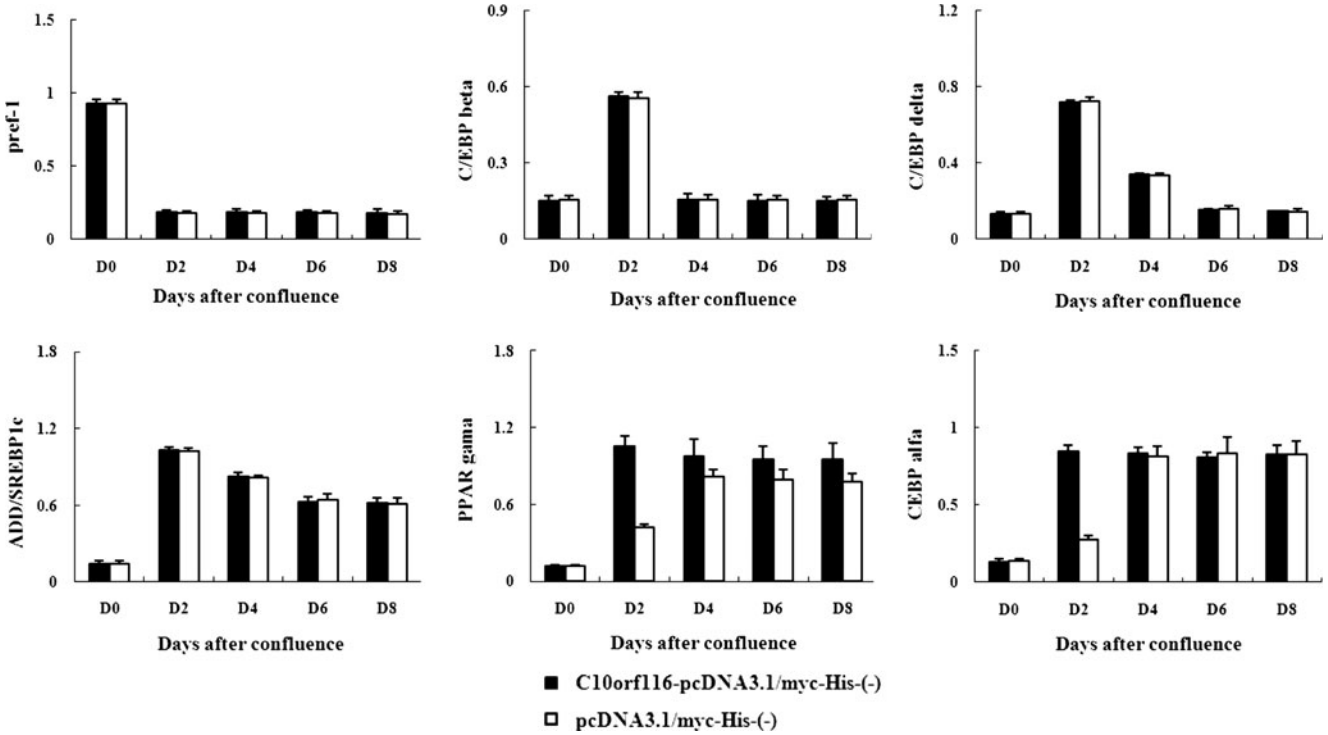

Fig. 4 Effects of C10orf116 overexpression on known adipogenesis-relevant genes. qPCR for analysis of representative adipogenesis genes were performed with RNAs from C10orf116 or control plasmid transfected 3T3-L1 cells. The data obtained from qPCR were normalized to the expression level of RPL27 (18S rRNA) and expressed as the relative mRNA level, $P<0.05$, data from 6 repeats. The result revealed that over-expression of the C10orf116 gene could up-regulate the expression of PPARγ and C/EBPα, but did not affect the expression of Pref-1, C/EBPβ, C/EBPδ, or ADD/SREBP1

CHO, and NIH3T3 cells. While the unfused D2EGFP was present throughout the cells when analyzed with a confocal laser scanning microscope, D2EGFP fused to AFRO at either the N- or C-terminus invariably concentrated, albeit not exclusively localized, in the nuclei in all cells tested. Figure 6a shows representative results in 3T3-L1 and 293T cells.

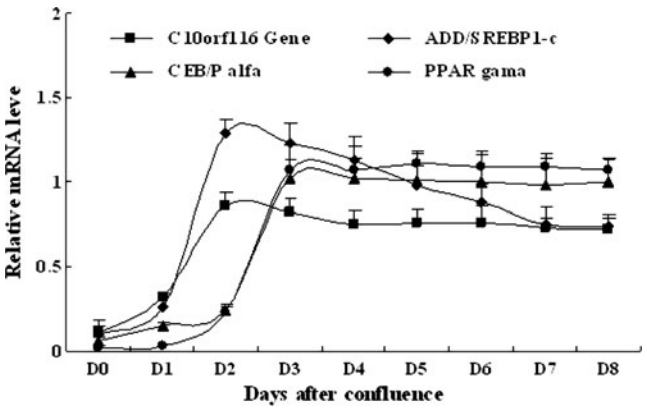

Fig. 5 Relevant gene expression during human preadipocyte differentiation. The data obtained from qPCR were normalized to the expression level of RPL27 and the relative mRNA levels were graphed in the chart, data from 6 repeats. The results revealed that the expression of ADD/SREBP1-c and C10orf116 gene were up-regulated after Day2, while the expression of C/EBPα, PPARγ after Day3

Proteins of molecular weight below 25 kD may shuttle freely between the nucleus and the cytoplasm. While AFRO showed nuclear localization when fused to GFP (~26 kD), how the natural 7.5 kD AFRO protein localizes is unknown at this point. It is possible that AFRO forms oligomers or complexes with other proteins to colocalize to the nucleus.

Immunohistochemical staining with the polyclonal antibodies against the AFRO-GST fusion protein was performed. However, the background was too high to obtain any useful data. In order to further examine the nucleus localization and the ratio of nucleus- versus cytoplasm-localized AFRO-D2EGFP fusion proteins, we established 3T3-L1 cells that stably expressed either the fusion protein or the control D2EGFP, and isolated total proteins, nuclear proteins, and non-nuclear proteins from these cells. The protein samples were then analyzed on a Western blot probed with a mouse anti-EGFP antibody. The results revealed that the majority of the AFRO fusion proteins co-fractioned with nuclear proteins; D2EGFP itself was evenly distributed in nuclear and non-nuclear fractions (Fig. 6b), in agreement with the microscopy data (Fig. 6a). These results suggested that AFRO is likely a nuclear protein and the nuclear localization supports the possibility that AFRO is directly involved in transcription activation. When suitable antibodies become available, staining of endogenous AFRO should provide further proof.

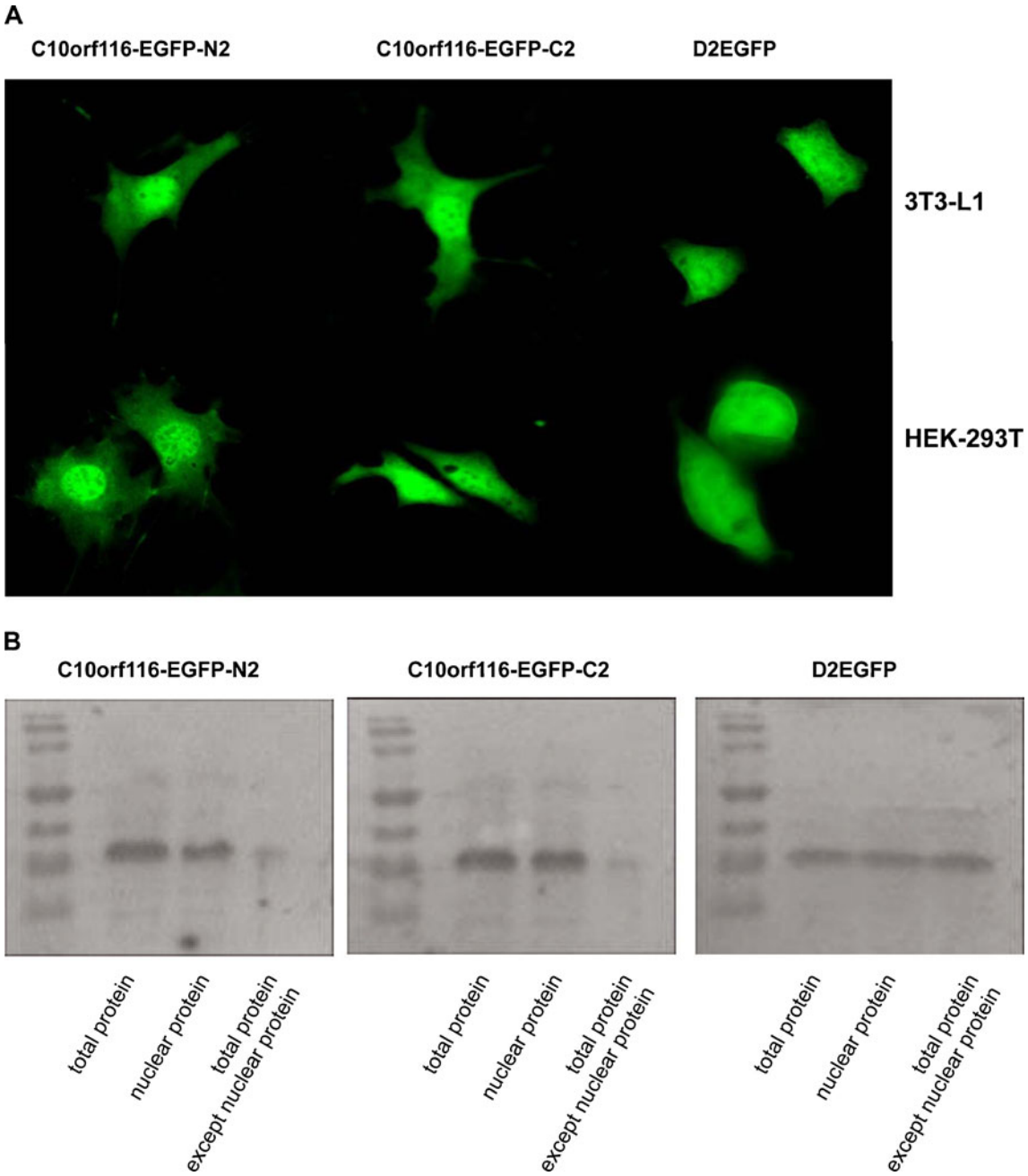

Fig. 6 Nuclear localization of C10orf116-EGFP fusion protein. **a** C10orf116-EGFP-N2, C10orf116-EGFP-C2, or D2EGFP transfected 3T3-L1 or HEK-293T cells were observed with confocal laser scanning microscope. The result revealed that C10orf116-EGFP-N2 and C10orf116-EGFP-C2 fusion protein concentrated in the nucleus, while D2EGFP did not. **b** Total protein, nuclear protein, or total protein except nuclear protein was isolated from C10orf116-EGFP-N2, C10orf116-EGFP-C2, or D2EGFP stably transfected 3T3-L1 cells, and analyzed on Western blots with anti-EGFP monoclonal antibody

Discussion

Using the technique of SSH, we identified AFRO as a previously uncharacterized nuclear factor that can promote adipogenesis, possibly by helping the initiation of transcription of master adipogenesis genes in the early phase. We hypothesized that it is functionally related to obesity and adipose tissue development because it is expressed at higher levels in adipose tissues from obese subjects; thus, we used mouse 3T3-L1 cells and human preadipocytes as *in vitro* systems and studied AFRO's functions. These studies demonstrated that 1) over-expression of AFRO had no effects on Pref-1, C/EBPβ, C/EBPδ, or ADD/SREBP1, but significantly promoted the expression of PPARγ and C/EBPα; 2) AFRO mRNA expression is highly induced by the differentiation signals; its expression precedes those of PPARγ and

C/EBPα, more or less coinciding with ADD/SREBP1-c. It is therefore likely that the position for AFRO in the cascade of gene activation is between C/EBPβ-C/EBPδ and PPARγ, and its presence apparently helps activate PPARγ.

One would expect that any factor that could activate PPARγ and C/EBPα should promote the differentiation of preadipocytes. Indeed, we have observed that over-expression of AFRO clearly accelerated adipogenesis as illustrated by earlier appearance of fat cell morphology (Fig. 3). Our D2EGFP fusion transfection experiments showed that AFRO is very likely a nuclear protein. While appearing to have no significant homology to any other known human proteins or functional domains, AFRO does show a low level similarity to some small proteins within the HMG family (not shown). HMGs affect chromatin structure, and some are known to function particularly in adipocyte development (Esposito et al. 2009; Melillo et al. 2001). Understanding how AFRO affects the expression of PPAGγ and C/EBPα will require more detailed molecular biology studies, including identifying its potential interacting proteins and/or DNA binding capabilities if any, through various immunoprecipitation or array analysis methods.

Among all of the well-studied factors involved in adipogenesis, only PPARγ2 is specific to adipose tissues. PPARγ1 and other PPAR family members participate in differentiation in other cell types, such as in osteoblasts. Most of the signals that influence fat cell development, as mentioned above, are also involved in processes other than adipogenesis. While it is possible that a combination of signals could be sufficient to transcriptionally activate PPARγ in a preadipocyte-specific manner, it is also plausible that factors specific to preadepocyte help to activate PPARγ only in preadipocytes in response to differentiation signals. The fact that over-expression of AFRO does not by itself initiate adipogenesis (even though it resulted in quick appearances of small oil droplets in 3T3-L1 cells, Fig. 3a) indicates that it is not a determinant gene in the process; rather, it is likely to be a helper that can "fine tune" the process, a term previously used to describe a few other factors that were shown to influence master adipogenesis factors.

Strategies of isolating preadipocyte determination genes based on differential expression profiling, somewhat similar to ours, have resulted in a number of recent reports of novel adipogenesis factors. For instance, by comparing expressed genes in preadipose fibroblasts and non-adipogenic fibroblasts, the Spiegelman lab reported in *Nature* that zinc finger protein Zfp423 is a regulator of PPARγ (Melillo et al. 2001). Zfp467 was also identified as an activator of PPARγ after it was first observed to be down-regulated in mesenchymal stem cell progenitors when they commit to osteoblasts, an alternative to adipocytes (Quach et al. 2011). By selecting proteins based on both their domain composition and their expression levels at the early stages of adipogenesis, ZFN638 was identified as an adipogenesis factor (Meruvu et al. 2011). What distinguishes AFRO from a group of such modulators is what we have demonstrated here as its clear enrichment in adipose tissues, particularly abundance in obese patients, which should make it a good candidate gene that contributes to preadipocyte specificity of the adipogenesis transcription regulation cascade. It would also be interesting to find out any potential relationships between AFRO and Zfp423, Zfp467, and ZFN638, etc. Further studies of this factor should help illustrate the events of gene expression during adipogenesis, and potentially provide targets for obesity intervention.

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