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ATAD3 is a Limiting Factor in Mitochondrial Biogenesis and Adipogenesis of White Adipocyte-like 3T3-L1 Cells

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Abstract

ATAD3 is a vital ATPase of the inner mitochondrial membrane of pluri-cellular eukaryotes, with largely unknown functions. Invalidation of ATAD3 blocks organism development at early stages requiring mitochondrial mass increase. Since ATAD3 knock-down (KD) in *C. elegans* inhibits first of all the development of adipocyte-like intestinal tissue, we used mouse adipocyte model 3T3-L1 cells to analyze ATAD3 functions during adipogenesis and lipogenesis. By stable and transient modulation of ATAD3 expression in adipogenesis-induced 3T3-L1 cells, we show that (i) an increase in ATAD3 is preceding mitochondrial biogenesis; (ii) down-regulation of ATAD3 inhibits adipogenesis, lipogenesis, and impedes overexpression of many mitochondrial proteins; (iii) ATAD3 re-expression rescues the phenotype of ATAD3 KD, and (iv) differentiation and lipogenesis are accelerated by ATAD3 overexpression, but inhibited by expression of a dominant-negative mutant. We further show that the ATAD3 KD phenotype is not due to altered insulin signal, but involves a limitation of mitochondrial biogenesis linked to Drp1. These results demonstrate that ATAD3 is limiting for *in vitro* adipogenesis and lipogenesis.

Keywords: 3T3-L1 Cells; ACC; Adipocyte; AMPK; ATAD3; Drp1; Endoplasmic Reticulum; Fission; Lipogenesis; Mitochondria; Mfn2

Abbreviations

ATAD3 (ATPase family AAA Domain-containing protein 3); ER (endoplasmic reticulum); KO (knock-out); KD (knock--down); Drp1 (dynamin-related protein 1), PPAR (peroxisome proliferator-activated receptor); C-EBP (CCAAT/enhancer-binding protein); TFAM (transcription factor A, mitochondrial); PGC (peroxisome proliferator-activated recep-

tor gamma coactivator); NRF (nuclear respiratory factor); ANT (adenine nucleotide translocator); VDAC (voltage-dependent anion channels) ; ATP5A (ATP synthase F1 complex, subunit 1); UQCRC2 (cytochrome b/c complex, subunit 2); MTCO1 (cytochrome oxydase, subunit 1); SDHB (succinate dehydrogenase) and NDUFB8 (NADH dehydrogenase, subunit 8); WT (wild-type).

Introduction

Mitochondria are at the crossroad of many anabolic and catabolic pathways and much more than a main cellular ATP supplier. Among the many other functions, they are also a key player in lipid metabolism. They are well known for their role in lipolysis via β-oxidation, but they also contribute to lipogenesis and lipid storage in adipocytes and other tissues [1-5] and to biogenesis of membrane phospholipids like phosphatidylethanolamine, phosphatidylcholine or cardiolipin [6-8]. Mitochondria are also essential for the conversion of cholesterol, *e.g.* the synthesis of steroids in endocrine cells [9], and require themselves lipid import for their biogenesis. Within the cell, mitochondria are tethered to the endoplasmic reticulum (ER) [10] thus allowing among others lipid trafficking, local calcium signalling [11-14] and translocation of nuclear-encoded mitochondrial proteins [15]. Finally, mitochondria are dynamic as they are able to divide and fuse, move and dock or die, according to specific mitochondrial and cellular conditions like e.g. cell growth or starvation [16-18]. Consequently, dysregulation of mitochondrial activities gives rise to many otherwise unrelated pathologies such as myopathies, neuropathies, cardiopathies, obesity, diabete and some others like cancers [19,20]. ATAD3 was discovered in 2003 as gene target of c-Myc [21,22]. Although *in silico* analysis reveals an ATPase domain, its true physiological function is still unknown. ATAD3 is present in all pluricellular organisms as a single gene and further evolved in primates into three ATAD3 encoding genes present in hominidae [22]. ATAD3 protein is overexpressed in patients with carcinoma [23,24] and is related to cancer initiation and progression as well as to chemoresistance and apoptosis [23,25-28]. The localization of ATAD3 is mitochondrial [24] and proteomic approaches identified ATAD3 as a protein of the inner membrane [29,30]. More detailed analysis revealed the topology of ATAD3 membrane insertion and its polymeric structure [31-33,22]. ATAD3 presents a C-terminal half, corresponding to the ATPase core domain, which locates inside the mitochondrial matrix, and a specific N-terminal half located in the intermembrane space and potentially interacting with the outer membrane and cytoplasmic partners. No intrinsic ATPase activity could be identified so far, although ATAD3 has been purified as full length protein [34]. Chow and colleagues have shown a possible interaction of ATAD3 with the proteins at contact sites between mitochondria and ER, like Mfn2 and dynamin-related protein 1 [Drp1; 35] and this may reflect a functional role of ATAD3 in biochemical communication between ER and mitochondria [36]. More is known about the role of ATAD3 at the cellular and organ level. ATAD3 is essential for early embryogenesis of *Caenorhabditis elegans* [37-41], *Drosophila melanogaster* [42,36], and *Mus musculus* [43]. Although, ATAD3 is expressed in all tissues and cells studied so far, the knock--down of ATAD3 in *C. elegans* primarily affects the intestinal fat tissue and the gonads at a time when these cells have to initiate mitochondrial biogenesis and lipogenesis [41]. Because in worm the intestinal cells are the functional equivalent of white adipocytes [44], 3T3-L1 murine cells can be used as a well-accepted and extensively studied mammalian model to analyze molecular processes associated with adipocyte differentiation and lipid storage. Upon induction with insulin, dexamethasone and isobutylmethyl-xanthine, these cells undergo a complete differentiation into adipocytes, acquiring the capacity for massive lipogenesis. These processes are characterized by a significant mitochondrial mass increase that just precedes lipid storage [45-47]. Mitochondrial biogenesis-linked lipogenesis has been also observed in humans [48,49]. Mitochondrial biogenesis may be linked to lipid storage by (i) ATP supporting metabolic processes involved in lipid storage and/or (ii) mitochondrial export of acetyl-CoA (or citrate transformed into acetyl-CoA by the ATP citrate lyase), which is then metabolized into malonyl--CoA by the acetyl-CoA carboxylase (ACC), a precursor of triacylglycerol that is ultimately forming lipid droplets [50-53]. Lipid droplets become coated with a monolayer of

phospholipids 94 and a set of proteins (Perilipin) that stabilizes them within the cytoplasm [54]. Electron microscopy studies have detailed the spatial organisation of the cellular organelles during adipogenesis/lipogenesis and have shown that mitochondria, ER and lipid droplets are closely organized [54], and that mitochondria increase in density during differentiation [45]. Here we investigated the role of ATAD3 during adipocyte differentiation of 3T3-L1 cells by transient and stable modifications of the cellular ATAD3 level. We found that ATAD3 is overexpressed early upon induction of 3T3-L1 differentiation, at the beginning of the mitochondrial mass increase. Gradual knock-down of ATAD3 in 3T3-L1 inhibited adipocyte differentiation and lipogenesis in a correlative manner, without affecting the insulin pathway, and this phenotype could be rescued by ATAD3 re-expression. Inhibition of lipogenesis was linked to a lack of ACC overexpression and activation. It appeared to correlate with altered mitochondrial remodeling and reduced mitochondrial biogenesis, and this phenotype could be partially complemented by overexpression the mitochondrial fission protein Drp1. These results suggest an essential role of ATAD3 in mitochondrial biogenesis, necessary for terminal adipocyte differentiation and lipogenesis.

Materials and Methods

Cells, Treatments and Biopsies

The mouse 3T3-L1 cells used in this study were purchased from American Type Culture Collection (ATCC, USA). 3T3-L1 preadipocytes were cultured in a Dulbecco's Modified Eagle's Medium (DMEM, GibcoTM, USA) containing 10% FBS (Biowest, EU) at 37°C and 5% CO2 until confluence and maintained for 48h (day 0). Cells were induced to differentiate using MDI induction medium (DMEM containing 10% FBS and 0.5 mM 3-i-sobutyl-1-methylxanthine, 1 µM dexamethasone, and 1 µM insulin) for 2 days, followed by insulin medium (DMEM containing 10% FBS and 1 µM insulin) for 2 days. The medium was subsequently replaced with fresh culture medium (DMEM with 10% FBS) containing insulin (1 µM) every 2 days until the terminal differentiation. Adipose tissues were taken from C57Bl6 male mice according to ethic rules (animal experimentation certificate 380537). Cell numbers were determined by manual counting of the cells under microscope. Data presented in this work (if not mentioned otherwise) are normalized to cell number, since expression of almost all proteins increased along the differentiation process, including actin, considered constitutive and often used for normalization (*e.g.* Figure 3B).

ShRNA Stable Cell Lines

For the stable ATAD3 knockdown cells, a retrovirus-based siRNA expression system was used. The sequences of ATAD3 were 223, 5'- GGACAAATGGAGCAACTTC; 354, 5'-GAACAGCAGTCCAAGCTCA; 530, 5'- ACAACAGCAACTTCTGAAT; 1084, 5'-GCCATCGCAACAAGAAATA; and 1791, 5'- GCAGAAGATGCAGTGGCTT, and cloned into a pSIREN-Retro-Q Vector and shuttled into Knockout RNAi Systems (Clontech Laboratories Inc., Otsu, Shiga, Japan). A control sequence, 50-AACTAGAGCCACAACTACC-3', was used as control. The retrovirus was produced in HEK293 cell following the manufacturer's protocol and infected the proliferating

3T3-L1 cells. The stable cell lines were selected in medium supplemented 136 with 5 µg/ml of puromycin. Sequences of siRNA are presented in supplementary table 1.

Confocal Microscopy

Cells were seeded in the Lab-TekTM-Chamber slide system (Nunc, Danemark) and induced to differentiate for various time periods. Just before imaging, cells were incubated with 200 nM MitoTraker Green FM (Interchim, France) at 37°C in a 5% CO2 incubator for 30 min and 1µg/ml Hoechst 33342 in the dark at 37°C for 10 min. After staining, cells were washed twice with pre-warmed phosphate-buffered saline (PBS), and fresh DMEM medium was added. Images were collected with a Leica TCS SP2 AOBS inverted laser scanning confocal microscope equipped with a 63X water immersion objective (HCX PL APO 63.0x/ 1,40 W Corr). Laser excitation was 351-364 nm for Hoechst and 488 nm for MitoTracker Green. Fluorescence emissions adjusted with AOBS were 390-470 nm for Hoechst and 498-549 nm for MitoTracker Green. 3D images of mitochondria were acquired by at least ten optical sections of 1µm z-step. Analysis and quantification of mitochondrial skeleton was processed using ImageJ software.

Electron Microscopy

Cells adherents to the coverslip were fixed with 2.5% glutaraldehyde in 0.1 M cacodylate buffer pH 7.4 during 2 hours at room temperature. Cells were then washed with buffer and post-fixed with 2% Osmium tetroxyde in the same buffer during 1 hour at 4°C. After extensive washing with distilled water, cells were stained with 1% uranyl acetate pH 4 during 1 hour at 4°C. Cells were then dehydrated through a graded series of alcohol concentrations (30%-60%-90%-100%-100%-100%) and infiltrated with a mix of 1/1 Epon/alcohol 100% during 1 hour before several baths in fresh Epon (Fluka, USA) during 3 hours. Finally, a capsule full of Epon was deposed on the surface of the cells and the resin

160 was let to polymerise during 72h at 60°C. The polymerised bloc was then detached from the culture cover-slip with hydrofluoric acid 48% during 1 h at room temperature 161 and ultrathin sections of the cell monolayer were cut with an ultramicrotome (Leica, Germany). Sections were post-stained with 4% Uranile acetate and 0.4% lead citrate before being observed in an electron microscope at 80 kV (JEOL 1200EX). Images were acquired with a digital camera (Veleta, SIS, Olympus, Japan) and morphometric analysis was performed with iTEM software (Olympus).

Oil-Red-O Staining

Cells were washed twice with PBS and fixed with 4% paraformaldehyde in PBS for 30 min at room temperature. After fixation, cells were washed two times with distilled water and one time with 60% isopropanol. The plates were dried for 30 min at room temperature and then cells were stained with filtered Oil-Red-O working solution (stock solution: 3 mg/ml in isopropanol; working solution: 60% Oil-Red-O stock solution and 40% distilled water) for 10 min at room temperature, washed 4 times with distilled water, and plates were dried and scanned for images. Oil-Red-O dye was extracted using 100% isopropyl alcohol and measured by spectrophotometry at 500 nm. Plastic well staining background was subtracted, and data normalized to the staining of pre-adipocytes (day 0) set to a value of one.

Western Blot Analysis

The 3T3-L1 wild-type and the three siRNA cell lines were propagated in duplicate in 6-well plates. Proteins were extracted, at various time points, in Laemmli buffer containing 10 mM DTT and 5% 2-mercaptoethanol. Samples (containing same cell amount) were separated by 10% SDS-PAGE and electro-blotted onto nitrocellulose membrane. The nitrocellulose membranes were incubated for 1 h at room temperature with blocking buffer containing TBST (Tris-buffered saline (25 mM Tris), 0.1% Tween 20) complemented with 3% non-fat dry milk powder, further incubated at 4°C overnight with primary antibodies in blocking buffer, washed three times for 10 min each with TBST at room temperature, and finally incubated at room temperature for 1 h with secondary 187 antibodies in blocking buffer. After washing three times for 10 min each in TBST, detection was performed by enhanced chemiluminescence (E-CL Plus, GE Healthcare). Primary rabbit polyclonal antibodies (all from Cell Signalling Technology and used at 1:1000 dilution unless stated otherwise) were directed against: phospho-ACC (Ser79), ACC, phospho-Akt (Thr308), Akt (Santa Cruz), phospho-mTOR (Ser2448), mTOR, 4EBP1, phospho-4EBP1 (Ser65), phospho- S6K (Thr389), S6K, phospho-AMPK∝(Thr172, BD Biosciences), AMPK∝, β-tubulin (Santa Cruz), β-actin (Santa Cruz), and Drp1 (Santa Cruz). Mouse monoclonal antibodies were directed against: Glut4 (Cell Signaling), Leptin (Peprotech), NRF1 (Enogene), Myc (Abcam, to detect myc-tagged Mfn2), ANT 1/2 (Santa Cruz), VDAC 1 (Santa Cruz), AK2 (ABGENT), cytochrome C (BD Biosciences), MTCO2 (Santa Cruz), NDUFA10 (Santa Cruz), and finally (as part of the MitoProfile OXPHOS set, MitoScience) ATP5A (ATP synthase F1 complex, subunit 1), UQCRC2 (cytochrome b/c complex, subunit 2), MTCO1 (cytochrome oxydase, subunit 1), SDHB (succinate deshydrogenase) and NDUFB8 (NADH deshydrogenase, subunit 8). ATAD3 was detected using rabbit polyclonal antibodies raised against a specific sequence of ATAD3 (Anti-Nter: R40PAPKDKWSNFDPT-G53 in ATAD3As; produced by Eurogenetec, Belgium) and used as purifed immunoglobulins. Secondary antibodies were anti-mouse or anti-rabbit IgG-horse radish peroxidase conjugate (GE Healthcare). The membranes were occasionally incubated with TBST blocking buffer again with sodium azide for irreversible inactivation of HRP and re-probed with anti- β-actin and anti- β-tubulin antibodies as a load-

ing control. Exposed films were analysed and quantified using ImageJ (NIH).

Citrate Synthase Activity Measurements

The reduction of 5′,5′-dithiobis(2-nitrobenzoic acid) (DNTB) by citrate synthase (CS) at 412 nm (extinction coefficient 13.6 mM−1cm−1) was followed in a coupled reaction with coenzyme A and oxaloacetate. A reaction 212 mixture of 150 mM Tris-HCl, pH 8.0, 5mM acetyl-coenzyme A, 3 mM DTNB and 20 µl of cells lysates were incubated at 37°C for 5 min. The reaction was initiated by the addition of 0.5 mM oxaloacetate and the absorbance changes were monitored for 5 min. All assays were normalized to cell amount by Western blot quantification of tubulin and compared to tubulin levels at day 0.

Measurement of Mitochondrial Cytochrome aa3 Content

Cell samples were solubilised with 1% of sodium deoxycholate in phosphate buffer (KH2PO4 100mM, pH8). The differential absorbance spectrum of reduced (by dithionite) versus oxidized cytochromes was obtained by scanning from 400 to 650 nm using a Cary50 Bio spectrophotometer (Varian, Palo Alto, USA). Cytochromes of the respiratory chain were reduced by addition of sodium dithionite crystals to 1 ml of suspension (final concentration of 0.25 mg/ml). Absorbance at 605 nm was used for quantification of respiratory chain cytochrome aa3 (constituent of cytochrome c oxydase) with extinction coefficient 24 mM-1cm-1 [55].

Transient Transfection of siRNA and Plasmids

RNA interference was used for transient down-regulation of ATAD3. The sequences of double stranded RNAs for ATAD3 were: Sequence 1: Forward 5'-ACAACAGCAACUUCUGAAUdTdT-3', Reverse 3'-dTdTUGUUGUCGUUGAAGACUUA-5' Sequence 2: Forward 5'-ACAGCAGUCCAAGCUCAAGdTdT-3' Reverse 3'-dTdTUGUCGUCAGGUUCGAGUUC-5' Plasmid encoding mouse ATAD3 is pCDNA3.1+ based; Drp1- and Mfn2-Myc-encoding plasmids are respectively P26048 and P23213 pCDNA3-based from Addgene. For transient transfection, wild-type 3T3-L1 preadipocytes cultured in 6-well plates and induced to differentiate for 2 days were transfected once with 50 nM dsRNAs or twice 237 with 10 µg of plasmid DNA (differentiation days 2 and 3) using Lipofectamine 2000 (Invitrogen, USA) according to the manufacturer's protocol. After 6 hours, the transfection medium was replaced with Dulbecco's Modified Eagle's Medium (D-MEM) containing 10% FBS. The samples for Western blot were collected at day 6 (siRNA) or day 8 (plasmids). The cells prepared for the Oil-Red-O coloration were maintained and replaced with insulin medium (DMEM containing 10% FBS and 1 µM insulin) until terminal differentiation (days 8 to 12). For the stable transfection, wild-type and siRNA knock-down 3T3-L1 preadipocytes cultured in dishes were transfected with 5 µg/ml of plasmids encoding ATAD3, Drp1 and Myc-tagged Mfn2 using Lipofectamine 2000 (Invitrogen). 72 hours post-transfection, cells were selected with antibiotic G418 (750 µg/ml) during 2 to 3 weeks with medium replaced every 2 days. Selected cells were grown as pools. For stable re-expression of ATAD3, we used an untagged ATAD3 expression plasmid because the rescue efficiency of a C-terminally Myc-tagged expression plasmid was very low.

Oxymetry

Wild-type and siRNA 3T3-L1 preadipocytes were isolated by free Ca2+ Mitomed solution (0.5 mM EGTA, 95.21 mM MgCl2, 60 mM K-lactobionate, 20 mM taurine, 3 mM KH2PO4, 20 mM HEPES, 110 mM sucrose, 0.5 mM DTT). A standardized number of cells (106) was permeabilized with saponin (25 µg/mL) and the rates of oxygen consumption (nMole O2/cell/min) were determined with high--resolution respirometry (oxygraph-2K, OROBOROS Instruments, Austria) in Mitomed solution. Measurements were carried out by sequential addition of (final concentrations given): 5 mM glutamate/2 mM malate as complex I substrates (v0 Glu/Mal, state IV respiration), 2 mM ADP for maximal respiration (vmax Glu/Mal, state III respiration), 3 µM rotenone to inhibit complex I, and finally 10 mM succinate as complex II substrate (vmax succinate, state III respiration). All measurements were carried out at 25°C and the solubility of oxygen was taken as 240 nmol. Oxygen consumption is expressed in nM of 02/cell/min and Respiratory Indexes indicated in the text for glutamate/-malate are vmax/v0 ratios.

Quantitative RT-PCR

Total RNA was extracted from 3T3-L1 cells with Trizol (Invitrogen, Eragny, France). First-strand cDNAs were synthesized from 1µg of total RNA in the presence of the PrimeScript RT Enzyme Mix (Takara), using a mixture of random examers and Oligo(dT) primers (Promega, Charbonnières, France). Real-time PCR assays were performed using a Rotor-Gene Q (Qiagen, France). TATA box-binding protein (TBP) mRNA level was used to normalize the data. PCR primer sequences are listed in Supplementary Table 2.

Adenylate Nucleotides

ATP, ADP, and AMP were determined in protein-free extracts prepared as follows. Cell lysis and protein precipitation were performed directly in culture flasks. Cells were washed 3 times with PBS, and ice-cold 0.6 N perchloric acid was added to lyse cells and precipitate proteins. Precipitated material was removed by centrifugation (2 min, 15500×g, 4°C). The supernatant was neutralized with KOMO (KOH 2N, MOPS 0.3M) and precipitate formed during neutralization was removed by centrifugation (10 min, 15 500×g, 4°C). Supernatants were stored at -80°C and used for HPLC-based nucleotide quantification as described [84].

Statistics

Experiments were repeated at least three times and mostly analyzed in different parallels. Resulting data are given as means +/- standard deviation. Differences between data were analyzed by two-tailed, two-sample, unequal--variance Student's t-test with P-levels indicated according to * for P<0,05, ** for P<0,01, *** for P<0,001.

Results

Adipogenesis and Lipogenesis Correlate with Changes in Mitochondrial Mass and Function, and Early ATAD3 Upregulation

Mouse 3T3-L1 pre-adipocyte cells were induced by insulin to differentiate into adipocytes, *i.e.* to undergo adipogenesis and lipogenesis. This activated canonical Akt signaling (Figure 1A) and led to an about 3-fold increase in cellular protein content (Figure 1B). We characterized this differentiation process, lasting until days 10-12, in respect to mitochondrial structure and function, as well as expression of ATAD3 and other mitochondrial proteins. The mitochondrial network of 3T3-L1 cells underwent a strong remodeling process during the course of differentiation (Figure 1C) as observed also by others [54,45,46]. Starting around days 3-4 and until terminal differentiation around days 10-12, mitochondrial mass increased as observed by elevated Mitotracker staining (Figures 1C and S1), mitochondrial citrate synthase activity (12-fold increase, Figure 1D) and cytochrome aa3 absorbance (11-fold increase, Figure S2-A,B). These latter parameters, like most data presented in this work (if not mentioned otherwise) were normalized to cell number. As compared to Mitotracker, these measures are also less likely perturbed by lipid-induced quenching. A similar increase of the mitochondrial mass during 3T3-L1 differentiation was suggested by a 6-fold increase in mtDNA copy number [47]. Tracing of the mitochondrial network during 3T3-L1 differentiation by confocal 3D-analysis (Figure 1C, E) revealed a transient reticulation of the network occurring until day 6, followed by the onset of a fragmentation process until day 12. This was confirmed by electron microscopy, showing in the differentiated state more numerous and less elongated mitochondria (from 1.25µm in mean to 0.9µm) that were located close to ER-associated lipid droplets (Figures 1F and S2-C/F). Triglyceride accumulation as quantified by Oil-Red-O staining showed onset of lipogenesis at around days 2-4 of differentiation (Figure 1G). Thus, mitochondrial remodeling and lipid storage seem to occur concomitantly. However, there is no evidence that the change in mitochondrial ultrastructure is forced by the density of lipid droplets, since these are not yet very abundant at day 8 as compared to later time points of adipocyte differentiation. The effect of mitochondrial mass increase and reorganization on respiration was analyzed by oxymetry in permeabilized 3T3-L1 cells at days 0 and 8. When normalized to cell number, respiratory parameters changed only moderately with time (Figure 2C, black bars). Mitochondrial respiration showed a 2-fold increase of state IV with glutamate/malate and state III with succinate, while state III with glutamate/malate remained unchanged. The resulting respiratory control ratio with glutamate/-malate (RCR, state III/state IV) decreased from 2.1 to only 1.1, indicating some uncoupling in the differentiated state.

However, when respiration was normalized to mitochondrial mass (Figure 2D), all types of respiration were drastically reduced. Taken together, these findings suggest that mitochondrial activity during differentiation is much less devoted to respiration, and rather redirected to other metabolic activities like lipogenesis. Triglyceride accumulation may not require so much respiration *per se* since Antimycin A, an inhibitor of the respiratory complex III, can induce itself lipogenesis (61 and figure S7-A, with a 150% increase of lipogenesis at day 16 with 100nM Antimycin).

The level of mitochondrial proteins as determined by Western blotting increased with mitochondrial mass, but the exact relation depended on the protein (Figure 2A): ATAD3 showed an increase that occurred already early (day 2) and was strong (8-fold). These ATAD3 protein levels were comparable to those of C57Bl6 mouse peritoneal adipose tissue (Figure 2B). Other analyzed proteins increased later and to variable degree. ACC, a rate limiting enzyme in lipid synthesis, is overexpressed along differentiation as later its phosphorylated form.

ATAD3 Knock-down Inhibits Lipogenesis by Blocking ACC, not Insulin-Akt Signaling

To understand whether induction of ATAD3 expression in 3T3-L1 cells is important for the differentiation phenotype, including lipid storage and mitochondrial biogenesis and remodeling, we performed ATAD3 knock-down (KD) by stable lentiviral ATAD3-targeted siRNA expression in 3T3-L1 cells. Two cell lines with graded reduction in ATAD3 expression, intermediate (line 1084) and low (line 530), were selected for further experiments (Figures 3A and S3-A). They revealed that lowered ATAD3 level clearly correlates with reduced triglyceride accumulation, thus indicating inhibition of lipogenesis during adipocyte differentiation. A similar result was obtained in a transient ATAD3 KD model using siRNA in 3T3-L1 cells (Figure S3-B), suggesting that inhibition of lipogenesis is an early consequence of ATAD3 invalidation. Also the opposite effect was observed by transient ATAD3 overexpression (Figure S3-C). This inhibition was not due to altered insulin-Akt signaling, since increased activation of Akt (p-Akt/total Akt ratio) (Figure 3B). Furthermore, inhibition of differentiation in stable ATAD3 KD is neither due to a delayed response to insulin, as forced induction of differentiation using a 4 times higher dose of insulin for 24 days, with or without oleic acid, an inducer of adipogenesis and lipogenesis (56), could not overcome the ATAD3 KD-induced inhibition of lipogenesis (Figure S3-D). Consistent with these findings, the insulin-induced increase in cellular protein content was only moderately inhibited by about 25% in KD lines (Figure 3C), with some proteins even accumulating normally in KD lines, such as actin (Figure 3B). We then asked whether the observed changes in lipogenesis are controlled by acetyl--CoA carboxylase (ACC), a rate-limiting key enzyme in fatty acid synthesis and triglyceride production. ACC expression levels were indeed strongly up-regulated during 3T3-L1 differentiation, to the largest extend observed among all proteins tested (Figure 3B), and this up-regulation was decreased in ATAD3 KD cells. ACC can also be inhibited by phosphorylation via AMP-activated protein kinase (AMPK), a central nutrient and energy stress sensing pathway involved in the regulation of lipogenesis/lipolysis. 3T3-L1 differentiation indeed led to decreased AMPK-dependent ACC phosphorylation (p-ACC/total ACC ratio, figure 3B), i.e. more active ACC, and this effect was again reduced or absent in KD lines.

We therefore examined whether ACC phosphorylation state depended on AMPK activation (Figures 3B), which is often inversely regulated in respect to Akt. Although AMPK phosphorylation state (p-AMPK∝/AMPK∝) decreased upon insulin-induction, this occurred in both, WT and KD cells. Allosteric activation of AMPK via cellular ADP/ATP or AMP/ATP ratios (57, 58) was neither decreased in WT versus KD cells (Figure S3-E). Rather the opposite was observed, namely a transient increase in these ratios in WT cells at differentiation day 4, indicative for energy stress at the onset of massive lipogenesis. In conclusion, stable ATAD3 KD partially blocks ACC overexpression and activation (lowering of phosphorylation state) after insulin-induction, and such a regulation is entirely consistent with observed lipogenesis inhibition (Figure 3A). However, stable ATAD3 KD has no significant effect on AMPK activation, and changes in ACC phosphorylation state may rather be a consequence of the large differences in ACC expression, and/or unknown ACC dephosphorylation steps.

Since cellular KD models suggest ATAD3 as a po-

tent limiting factor in mitochondrial remodeling, adipocyte differentiation and lipogenesis, we investigated the expression of several transcriptional (co)factors involved in these processes by RT-qPCR (Figure S4). ATAD3 KD had no effect on expression of PGC1∝ (a master regulator of mitochondrial biogenesis and adipogenesis), its partners NRF1/2, and the mitochondrial transcriptional factor TFAM, a PGC1∝/NRF1-target gene. Thus downstream insulin signaling was undisturbed at that level, and activation of these signaling pathways during adipogenesis or any effect of ATAD3 KD on them seems to occur rather at the protein level. The situation is different for the key players that control the transcriptional cascade leading to adipocyte differentiation. While mRNA levels of C-EBPβ, an early marker of pre-adipocyte differentiation, was up-regulated at day 2 in both control and KD lines, mRNA levels of EER∝, PGC1β, PPARγ and C-EBP∝, later markers of adipocyte differentiation, increased up to differentiation day 7 only in control cells, but not or much less in KD cells. This indicated a block or delay of the adipogenesis program in ATAD3 KD cells already after the early differentiation steps.

ATAD3 Knock-Down Inhibits Mitochondrial Biogenesis/Remodeling

We next used stable ATAD3 KD lines to analyze functional relationships between ATAD3 level and mitochondrial mass and morphology during insulin-induced adipogenesis. Although mitochondrial mass in ATAD3 KD lines also increased continuously, it remained much lower as in control cells. Citrate synthase activity (3-5-fold; Figure 3D) and cytochrome aa3 contents (2-3-fold; Figure 3E) increased much less than in control cells (12-fold and 11- fold at day 8, respectively). Also expression of dynamin-related protein 1 (Drp1), a key player in mitochondrial fission and ER-mitochondria tethering processes (15), especially in 3T3-L1 cells (46), was unaffected by ATAD3 KD (Figure 3B). Cytosolic markers of adipogenesis were not affected by ATAD3 KD, i.e. insulin induced Glut4 and NRF1 overexpression in all cells as expected. The effect of ATAD3 KD on mitochondrial morphology was again analyzed by confocal microscopy. In constitutive ATAD3 KD lines induced to differentiate, most cells showed only limited increase of Mitotracker staining and a tubular-branched mitochondrial network, similar to undifferentiated cells, persisting throughout the entire remodeling period (days 2-6) (Figures 4A and S5). Electron microscopy confirmed that mitochondria of insulin induced ATAD3 KD cells remain less numerous and tubular, like those of undifferentiated cells (Figure 4B). In KD cells undergoing only slight lipogenesis, we observed also recurrent accumulation of transition vesicles, probably from endoplasmic reticulum (Figure S6-A, B) with abundant enlarged granular ER and some tubular granular ER close to small lipid droplets, like in differentiating cells. Moreover, mitochondria appeared clear, with very few crests (Figure S6-C). To rule out the possibility of increased mitophagy being responsible for reduced mitochondrial mass and lipogenesis in ATAD3 KD lines, we treated the cell lines with two inhibitors of autophagy, chloroquine or bafilomycine, during the time course of insulin induction (Figure S7-B). This reduced lipogenesis to the same extend in ATAD3 KD and control cell lines, thus excluding autophagy as causative for the ATAD3 KD phenotype. Stable ATAD3 KD leading to low ATAD3 levels (line 530) reduced respiratory parameters as compared to control already before onset of differentiation (Day 0, figure 2C). Both state III and state IV respiration (with glutamate/-malate or succinate) were reduced, irrespective whether data were normalized to cell number (Figure 2C) or mitochondrial mass, i.e. citrate synthase activity (Figure 2D). Thus, below a certain threshold level of ATAD3, respiration is impaired, possibly indicating a stronger contribution of the glycolytic pathway. During differentiation (Day 8), respiration of both ATAD3 KD cell lines was clearly affected at the per cell level (Figure 2C), lacking the increase of state III and state IV respirations seen in control cells and linked to the mitochondrial mass increase. When normalized to mitochondrial mass, all respiratory parameters of KD cells decreased drastically until day 8, including the RCR with glutamate/malate (decrease from 2.1 to 1.1), as already seen in controls (Figure 2D). However, ATAD3 KD mitochondria performed now as well as controls (with succinate) or even slightly better (with glutamate/malate).

Rescue of ATAD3 Phenotypes by ATAD3 Overexpression and Complementation by Drp1

To ascertain that the observed phenotype is indeed directly due to loss of ATAD3 we conducted a rescue experiment by stably transfecting the wild-type ATAD3 in-

to the two stable ATAD3 KD cell lines. Re-expression of ATAD3 in both ATAD3 KD cell lines indeed rescued the lipogenenic potential (Figures 5A) and restored mitochondrial biogenesis and remodeling (Figures 5B and S7-C), with the most pronounced effect observed in line 530 which has the lowest initial ATAD3 level. We further tested the effect of stably overexpressing ATAD3 wt along with a dominant negative ATAD3 dead mutant in the original 3T3-L1 cells during differentiation. This mutant contains a non--functional ATPase (K358E mutation in the Walker A motif GPPGTGKT) and is dominant negative because it heteropolymerizes with endogenous ATAD3 wt [33]. While stable expression of ATAD3 wt increased lipogenesis and mitochondrial biogenesis in 3T3-L1 cells induced to differentiate, expression of the dead mutant led to a significant decrease in lipogenesis and mitochondrial remodeling (Figure 5C and 5D). We finally hypothesized that two other mitochondrial proteins, Drp1 and Mfn2, may partially complement ATAD3 deficiency, since they are known as interactors of ATAD3 [35,36,43]. Drp1 and Mfn2, involved in mitochondrial fission and fusion, respectively, where therefore overexpressed in ATAD3 KD cell lines by stable transfection. Indeed, stable overexpression of Drp1, but not of Mfn2, was able to restore lipogenesis to a variable degree in both 530 and 1084 KD cell lines (Figures 5A, B and S-7C).

Discussion

The ubiquitous mitochondrial ATPase ATAD3 is essential during early developmental stages, as shown by knock-down in *C. elegans* (41) and, more recently, in a mouse knock-out model [43], but its precise functions are still largely unknown. Some studies have suggested a role in mitochondrial biogenesis, the maintenance of the mitochondrial network and its interaction with the endoplasmic reticulum [41,36,26]. However, ATAD3 has not yet been implicated in any precise mitochondrial processes. Here we show by using stable and transient ATAD3 knock-down and overexpression in 3T3-L1 cells that ATAD3 plays a vital role in the adipocyte differentiation process, affecting mitochondrial mass, structure and function and lipogenesis. ATAD3 deficiency inhibits mitochondrial biogenesis, impedes changes in mitochondrial network morphology linked to Drp1 function, reduces respiration, and down-regulates lipogenesis in an ACC-regulated manner. Normal insulin-induced adipogenesis in 3T3-L1 was found to involve lipogenesis, mitochondrial biogenesis and mitochondrial network fragmentation, as already established by others [45,46]. Importantly, up-regulation of ATAD3 levels was preceding the increase of most other parameters, suggesting a role in the early differentiation program. The mitochondrial mass increase per cell was between 5-16-fold (abundance of different mitochondrial proteins) and 11-12-fold (based on cytochrome aa3 level and citrate synthase activity), even exceeding numbers in literature, like a 2-6 fold increase in mtDNA reported for a 3T3-L1 model or human adipose tissue [49,47]. In this respect, it is important to note that almost all literature data are normalized to total cellular protein, which increases however itself by at least 3-fold, while we decided to normalize to the only stable reference parameter in this system, the cell number. At this per cell level, also state IV respiration and state III respiration with succinate doubled during differentiation, as also observed earlier [45,59,60]. However, the respiratory control ratio decreased, as did other respiratory parameters when calculated per mitochondrial mass, indicating mitochondrial uncoupling and redirection of mitochondrial metabolic activity from ATP generation towards biosynthetic activities. As seen by Antimycin lipogenic effect, triglyceride accumulation may not require so much respiration *per se*.

Functional and proteomic studies clearly suggest that during adipocyte differentiation a hyperactive TCA cycle driven by various substrates generates citrate and acetyl--CoA as precursors for triglyceride synthesis [52,62-64]. Gradual stable or transient knock-down of ATAD3 in 3T3-L1 cells led to drastic inhibition of insulin-induced adipocyte differentiation as compared to controls. This inhibition was observed at different levels, including lipogenesis, mitochondrial biogenesis and network remodeling. Consistent with these findings, overexpression of ATAD3 in 3T3-L1 cells accelerated adipogenesis, while overexpression of a dominant negative ATAD3 mutant was again inhibitory. Importantly, the 3T3-L1 KD phenotype could be rescued by re-expression of ATAD3. Thus, there exists a very close correlation between the levels of functional ATAD3 and adipogenesis, with ATAD3 representing a true limiting factor in adipogenesis. ATAD3 KD did not affect insulin-induced Akt signalling, gene expression of key transcription regulators of mitochondrial mass increase (PGC1α, NRF1, NRF2,

TFAM) or of early markers of adipogenesis like C-EBP β. We thus conclude that overall insulin signaling is intact, and that ATAD3 interferes with more downstream, mitochondria-related signaling, finally inhibiting late adipogenesis after the early stages as seen by reduced gene expression of adipogenic markers like EER α, PPARγ, PGC1 β and C-EBP α. Some insulin-dependent down-stream signaling is still occurring in ATAD3 KD cells, since expression of Glut4 and NRF1, two markers of adipogenesis and lipogenesis processes, is unaffected. Probably other potential activators of these genes are involved here like MEF2A, GEF, SREBP1 or CEBP β. How ATAD3 KD can down-regulate transcription of adipogenic genes? These down regulations induced by ATAD3 KD probably reflect feed-back mechanisms. Since mitochondrial biogenesis cannot be normally increased, limiting the lipogenesis, it is expected that the adipogenic/lipogenic genetic program could be down-regulated. Also and notably, ATAD3 contains a NES (Nuclear Exporting Signal) and is expressed under several isoforms [22]. Therefore, ATAD3 could be itself involved in gene regulation. Also, and importantly, the adipogenic/lipogenic genetic program is only delayed by ATAD3 KD, but not blocked, thus meaning that ATAD3 is a true limiting factor in terminal adipogenesis and lipogenesis. Lipogenesis in our experimental system seems to be closely related to ACC, a rate limiting enzyme of fatty acid synthesis that switches metabolism between β-oxidation and lipogenesis (for recent reviews see [58,69,70]. In adipocytes, the most predominant ACC isoform is ACC1 [67,68], which we detected, but much less is known on ACC regulation in this tissue. With 3T3-L1 cells, differentiation leads to higher ACC activity due to two effects: (i) strongly increased ACC levels, and (ii) reduced inhibitory ACC1 phosphorylation. This will accelerate lipogenesis in controls, while in ATAD3 KD cells both effects are largely diminished, thus slowing down lipogenesis. A main factor controlling ACC is AMPK, exerting negative control on ACC expression via sterol regulatory-element-binding protein-1c (SREBP-1) and ACC activity via inhibitory phosphorylation. In addition, differentiation of 3T3-L1 cells and cultured preadipocytes is inhibited by pharmacological AMPK activators (AICAR, A-769662 and many others [72-75] or activation of AMPK upstream kinase CamKK β [76]. Such regulation may be present in 3T3-L1 controls, where covalent AMPK activation (via its T172 phosphorylation) decreases, consistent with the observed effects on ACC and lipogenesis. However, the ATAD3 KD does not affect AMPK, neither the covalent activation, nor the allosteric activation [65,66] as deduced from the determined adenylate ratios in control and KD cells. Probably, up-regulation of transcriptional regulators like PPARγ and C-EBPα, which does not occur in KD cells, plays a major role here in controlling ACC expression, and smaller changes in phosphorylation state may be secondary to large changes in expression level. The down-regulated ACC pathway in ATAD3 KD cells could be a decisive factor for decreased lipid droplet formation and reduced adipogenesis in general, although participation of increased lipolysis cannot be excluded. Besides reduced lipogenesis, the major phenotype of ATAD3 KD in 3T3-L1 cells was the inhibition of mitochondrial mass increase and remodeling. Inhibited mitochondrial biogenesis was also observed in intestinal ATAD3 KD cells in *C. elegans* [77], the functional equivalent of vertebrate adipocytes, and in case of embryonically lethal ATAD3 KO in mice, where development is early inhibited at a stage requiring mitochondrial biogenesis and ATP generation [43]. Altered mitochondrial morphology and dynamics were also observed in different other cellular ATAD3 KD models [77,78]. This ATAD3 KD phenotype was not due to induced autophagy, since inhibition of autophagy did not increase lipogenesis as reported for hepatocytes [79], possibly because adipocyte tissue is metabolically very different [80,81]. Importantly, we show here that Drp1, a central element of the mitochondrial fission machinery, is able to complement ATAD3 KD by partially restoring mitochondrial biogenesis and also the lipogenesis that depends on the mitochondrial remodeling. This identifies Drp1 as an efficient co-factor for ATAD3 function, probably by promoting fission-based mitochondrial remodeling [16], consistent with earlier data on a putative interaction between ATAD3 and Drp1 [35]. These data also represent first direct evidence for a role of ATAD3 in mitochondrial remodeling and the role of this mitochondrial process in lipogenesis. ATAD3 may thus contribute to some initial fission-linked processes that trigger remodeling and biogenesis of the mitochondrial network as well as its interaction with lipid droplets (and the associated fatty acid synthesis) and the ER (and the associated local calcium signaling [15], all processes involving also Drp1. In support of such functional inter-

actions, ATAD3 is targeted by calcium signaling protein S100B [33] and incidentally, also, both Drp1 and ATAD3 KD increase the life span of *C. elegans* [82,77]. ATAD3 interacts also with another key player in mitochondrial dynamics, the fusion protein Mfn2, for importing apoptosis inducing factor (AIF), a FAD-dependent NADH oxydase, into mitochondria [35]. Finally, at the protein level, ATAD3 invalidation was reported to specifically impact mitochondrial protein synthesis [83]. How ATAD3 KD can down-regulate abundance of mitochondrial proteins remains an open question, since some crucial transcription factors like PGC1∝, NRF1, NRF2, and TFAM are not affected. Rather than transcriptional control, post-translational processes may be involved, including protein synthesis/turnover, mitochondria/ER interactions and mitochondrial import and maturation of proteins and lipids [85]. Indeed, most recently, ATAD3 has been shown to be involved in, and essential for cholesterol flux between ER and mitochondria compartment, especially for steroidogenesis in Leydig cells [86-89]. All these results shed the light on a possible protein/lipid transport pathway involving ATAD3 and necessary for mitochondrial biogenesis.

Considering all about its major role in mitochondrial biogenesis, it is not surprising therefore that ATAD3 is a putative and strong candidate to be responsible of mutation-inherited diseases such as myopathies and neuropathies [90-93].

In summary, this study revealed the requirement of ATAD3 for adipocyte differentiation. First, ATAD3 is necessary for mitochondrial proliferation and remodeling. This role of ATAD3 probably involves its interaction with Drp1 that can partially compensate loss of ATAD3. Second, ATAD3 and mitochondrial biogenesis are necessary for lipogenesis and lipid droplet formation. ATAD3 seems to maintain proper mitochondrial function, despite some uncoupling and increased biosynthetic activity at the expense of ATP generation, and thus favors fatty acid synthesis via ACC and FAS. Further investigation will be necessary to understand in mechanistic detail how ATAD3 controls mitochondrial biogenesis and structural dynamics during differentiation.

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