NAT8L (N-Acetyltransferase 8-Like) Accelerates Lipid Turnover and Increases Energy Expenditure in Brown Adipocytes*

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Background: NAT8L (N-acetyltransferase 8-like) synthesizes N-acetylaspartate and is required for myelination in the brain. Its function in other tissues was undefined.

Results: Nat8l is highly expressed in adipose tissues and impacts adipogenic marker gene expression, lipid turnover, and energy metabolism in brown adipocytes.

Conclusion: Nat8l expression influences cellular bioenergetics in adipocytes.

Significance: These findings establish a novel pathway in brown adipocyte metabolism.

NAT8L (N-acetyltransferase 8-like) catalyzes the formation of N-acetylaspartate (NAA) from acetyl-CoA and aspartate. In the brain, NAA delivers the acetate moiety for synthesis of acetyl-CoA that is further used for fatty acid generation. However, its function in other tissues remained elusive. Here, we show for the first time that Nat8l is highly expressed in adipose tissues and murine and human adipogenic cell lines and is localized in the mitochondria of brown adipocytes. Stable overexpression of Nat8l in immortalized brown adipogenic cells strongly increases glucose incorporation into neutral lipids, accompanied by increased lipolysis, indicating an accelerated lipid turnover. Additionally, mitochondrial mass and number as well as oxygen consumption are elevated upon Nat8l overexpression. Concordantly, expression levels of brown marker genes, such as Prdm16, Cidea, Pgc1α, Ppara, and particularly UCP1, are markedly elevated in these cells. Treatment with a PPARα antagonist indicates that the increase in UCP1 expression and oxygen consumption is PPARα-dependent. Nat8l knockdown in brown adipocytes has no impact on cellular triglyceride content, lipogenesis, or oxygen consumption, but lipolysis and brown marker gene expression are increased; the latter is also observed in BAT of Nat8l-KO mice. Interestingly, the expression of ATP-citrate lyase is increased in Nat8l-silenced adipocytes and BAT of Nat8l-KO mice, indicating a compensatory mechanism to sustain the acetyl-CoA pool once Nat8l levels are reduced. Taken together, our data show that Nat8l impacts on the brown adipogenic phenotype and suggests the existence of the NAT8L-driven NAA metabolism as a novel pathway to provide cytosolic acetyl-CoA for lipid synthesis in adipocytes.

Adipose tissue depots are critical organs for the control of energy homeostasis. White adipose tissue (WAT) is the major fat-storing organ in the body, and its adipocytes are characterized by large, unilocular lipid droplets and only few mitochondria. During energy demand, triglycerides (TG)3 are broken down to supply peripheral tissues with fatty acids (1, 2). Brown adipocytes are characterized by multiple, smaller lipid droplets and numerous mitochondria, which contain UCP1, a protein that uncouples oxidative phosphorylation from ATP production to dissipate energy into heat (3). With the discovery of active brown adipose tissue (BAT) in adult humans (4–7) and its association with leanness (5), much attention has been paid to the investigation of BAT development and homeostasis due to its possible role in fighting obesity and its associated disorders (8). Recently, it has been shown that adipose triglyceride lipase-mediated breakdown of TG is required for a distinct brown adipose phenotype in mice (9). Thus, it seems that a

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§§ The abbreviations used are: TG, triglyceride(s); WAT, white adipose tissue; BAT, brown adipose tissue; FFA, free fatty acids; PPAR, peroxisome proliferator-activated receptor; NAA, N-acetylaspartate; iBACs, immortalized brown adipogenic cells; ER, endoplasmic reticulum; BisTris, 2-(bis[2-hydroxyethyl]amino)-2-(hydroxymethyl)propane-1,3-diol; OCR, oxygen consumption rate.
large proportion of fatty acid has first to be stored as TG and thereafter hydrolyzed before they can be used for UCP1 activation (and mitochondrial β-oxidation). Additionally, activated BAT shows high rates of glucose uptake (10), but glucose is suggested to play only a minor role as a direct oxidative substrate (11). Therefore, enzymes involved in de novo lipid synthesis are highly expressed in BAT and further increased upon thermogenic activation (12).

Many of the identified molecular network components controlling white and brown metabolism have been disclosed by the use of novel high throughput technologies. Among others, we performed microarray studies in white and brown adipose tissue of Atg- and Hsl-KO mice (13) and focused on candidate genes that might be of interest in the development and metabolism of adipose tissues. Among these was a gene encoding for the enzyme NAT8L (N-acetyltransferase 8-like).

In the brain, NAT8L was shown to catalyze the formation of N-acetylaspartate (NAA) from acetyl-CoA and L-aspartate (14, 15). NAA then acts as a carrier of acetyl groups between neurons and oligodendrocytes where NAA is catabolized by aspartoacylase into acetate and L-aspartate (16). The acetate moiety is reutilized for acetyl-CoA synthesis and can subsequently be incorporated into lipids (16, 17). The metabolic importance of NAA has been shown in two inborn human neurodegenerative disorders, where defects in NAA biosynthesis (14, 18) as well as catabolism (19) lead to reduced myelin synthesis.

Here, we describe for the first time that Nat8l is highly expressed in adipocytes and that its expression is induced during the differentiation of various mouse and human adipogenic cells. Furthermore, overexpression of Nat8l in an immortalized brown adipogenic cell line influenced lipid turnover, increased mitochondrial mass, and accelerated energy expenditure, most likely by increasing the expression of UCP1 in a PPARα-dependent manner. Our results from Nat8l silencing in brown adipocytes and from examining BAT in Nat8l-KO mice support the hypothesis that the NAT8L/NAA pathway acts as an alternative source to provide acetyl-CoA as a building block for lipid biosynthesis in adipocytes. These data suggest that the NAA pathway exists and is functional in adipose tissue and that modulating this pathway could be a valuable new approach to increase energy dissipation in (brown) adipocytes.

**EXPERIMENTAL PROCEDURES**

**Cell Culture, Differentiation, Lipid Staining, and Quantification**—Immortalized brown adipogenic cells (iBACs) were grown in DMEM containing 10% FBS, 50 μg/ml streptomycin, 50 units/ml penicillin, and 20 μM Heps. C3H-10T1/2 cells were grown and maintained in DMEM containing 10% FBS and penicillin/streptomycin. C3H-10T1/2 cells were induced to differentiate 2 days after confluence with 0.5 mM 3-isobutyl-1-methylxanthine, 1 μM dexamethasone, 2 μg/ml insulin, and 1 μM rosiglitazone (Cayman Chemical). After 3 days, medium was changed to maintenance medium with 2 μg/ml insulin and 1 μM rosiglitazone, and 48 h thereafter, normal growth medium was used until harvest. Simpson-Golabi-Behmel syndrome cells were cultured and differentiated as described by us elsewhere (20). iBACs were induced to differentiate at the day of confluence with 0.5 mM 3-isobutyl-1-methylxanthine, 0.5 μM dexamethasone, 20 nM insulin, 1 nm triiodothyronine, and 125 μM indomethacin. Two days after induction, medium was changed to maintenance medium containing 20 nM insulin and 1 nm triiodothyronine, and cells were kept in this medium until harvest. Cells were fixed (10% formalin in PBS for 30 min), rinsed in PBS, and stained with oil red O (0.25% in 60% isopro- pyl alcohol stock solution diluted 3:2 with distilled H2O for 30 min). To stimulate thermogenesis, cells were incubated with 1 μM isoproterenol for 4 h. iBACs were treated with 10 μM PPARα antagonist GW6471 (Tocris Bioscience) from day 4 until harvest. Cellular triglyceride content was determined in differentiated iBACs using Infinity Triglyceride Reagent (Thermo). Free fatty acid content was measured using the NEFA C test kit (WAKO). Values were corrected by protein content measurement using BCA reagent (Pierce).

**Animal Studies**—Male C57BL/6 and ob/ob mice at the age of 24–26 weeks were used for this study. Before harvesting tissue pads, mice were fasted for 12 h, following refeeding for 1 h. Nat8l-knock-out mice (21) and their controls were used at the age of 3–4 months and fed ad libitum before harvesting tissues. Animals were kept on a 12-h light/dark cycle on a normal chow diet. All animal procedures followed the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals and were approved by the Austrian Ministry for Science and Research and the Committee for Animal Experiments of the University of Toyoa.

**Retroviral Expression of Nat8l in Monoclonal Cell Lines**—Full-length coding sequence of murine Nat8l was amplified by PCR from murine adipose tissue cDNA using Phusion polymerase (Fermentas) and cloned into a murine stem cell virus vector (pMSCV puro, BD Biosciences Clontech) using the restriction sites Xhol/EcoRI. To produce infectious but replication-incompetent recombinant retroviruses expressing Nat8l, PhoenixEco packaging cells (cultured in DMEM with 10% FBS in 5% CO2) were transfected with pMSCV-Nat8l using Metafectene (Biontex Laboratories GmbH). The supernatant containing the viral particles was collected 48 h after transfection. Viral supernatants were supplemented with 8 μg/ml Polybrene and added to iBACs (30–40% confluence) for infections for 18–24 h. Because cells could not be selected with puromycin, single cells were picked under the microscope and expanded as monoclonal populations, and overexpression was controlled by quantitative RT-PCR. Differentiation was induced as described above. As a control for the above described stable cell lines, the empty pMSCV puro was used and underwent the same procedure.

**Silencing of Nat8l Using Short Hairpin RNA (shRNA)-containing Lentiviral Particles**—One control non-targeting shRNA lentivirus and two shRNA lentiviruses directed against Nat8l were purchased from Sigma (MISSION® shRNA lentiviral particles NM_001001985). iBACs were seeded into 6-well plates 12 h before transduction using 3 × 104 cells/well (around 30% confluence). Cells were infected for 16 h with a multiplicity of infection of 10 in complete medium containing 8 μg/ml Polybrene (Sigma). After transduction, the infection medium was replaced with fresh medium, and the cells underwent the same selection process as Nat8l-overexpressing iBACs.
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Site-directed Mutagenesis of Nat8l and Subsequent Stable Transfection of iBACs—For better selectivity, Nat8l coding sequence was transferred into a pMSCV-hygro vector (kind gift from E. D. Rosen). Site-directed mutagenesis was performed by PCR amplification with Phusion polymerase using pMSCV-Nat8l as template with the following primers (base substitution is marked as a lowercase letter): Nat8l

- TGGACGATCATGGAACATGGCGGCC, 5'-AGCCCATGAGTCTCACGACATGAGGAC-3'. This led to a subsequent change from aspartic acid to alanine. The purified PCR product was digested for 1 hr at 37 °C with 20 units of DpnI in order to eliminate the template, and the mutated vector was transformed into Escherichia coli. The complete Nat8l coding region was sequenced to verify the presence of the introduced mutation and the absence of random mutations. iBACs overexpressing Nat8l were generated as described above with pMCSV-hygro as a control. Selection of positive clones was performed with 500 μg/ml hygromycin for at least 7 days.

Mitochondria Isolation and Western Blot Analysis—Mitochondria were isolated from cell pellets of differentiated iBACs with a commercially available kit (Thermo Scientific) using the Dounce homogenizer and 3000 × g to pellet the mitochondria. BAT mitochondria were isolated as described previously (22). Modifications to the protocol were as follows. The tissues were excised from male mice fed ad libitum, washed in ice-cold PBS, and cut into small pieces with a razorblade. Subsequently, they were homogenized using a Dounce homogenizer with about 60 strokes. The centrifugation steps to pellet the mitochondria were carried out at 3000 × g to reduce peroxisomal contamination. Nuclear fraction, mitochondrial fraction, and post-mitochondrial supernatant containing cytosol and ER remnants were lysed in SDS-lys buffer (50 mM Tris-HCl, pH 6.8, 10% glycerol, 2.5% SDS, 1X protease inhibitor mixture, 1 mM PMSF) and used for further analysis. Cytosolic/ER proteins have been precipitated using the trichloroacetic acid (TCA) method. Briefly, cytoplasmic protein lysate was mixed with 50% ice-cold TCA to obtain a concentration of 10% TCA and incubated for 1.5 h on ice. Then it was centrifuged for 10 min at 13,000 rpm and 4 °C. The pellet was washed twice with ice-cold acetone, air-dried, and dissolved in SDS-lys buffer. Control and Nat8l-overexpressing iBACs (at several differentiation time points) were harvested for protein analysis by scraping with SDS-lys buffer. After benzonase digestion, protein concentrations were determined with the BCA protein assay kit (Pierce). 50 μg of sample were subjected to a 12% BisTris gel (NuPAGE, Invitrogen), and gels were blotted to nitrocellulose membranes. The following antibodies were used: anti-NAT8L (1:1000) (Novus Biologicals, catalog no. NP80-06599); anti-UCP1 (1:1000) (Calbiochem); anti-hexokinase (1:1000), anti-histone H3 (1:2000), and anti-protein-disulfide isomerase (1:1000) (all from Cell Signaling Technology); and anti-β-Actin (1:25,000) (Sigma). For chemiluminescent detection, a horseradish peroxidase-conjugated secondary antibody was used (anti-rabbit; 1:5000) (Dako), and ECL (Pierce) served as substrate.

RNA Isolation, Reverse Transcription, and Gene Expression Analysis—Total RNA from cells was isolated using the Total RNA isolation kit (Sigma). Tissue RNA was isolated with TRIzol reagent (Invitrogen). cDNA was generated using SuperScript II reverse transcriptase (Invitrogen). Optimal concentrations of specific inhibitors/accelerators of the electron transport chain were

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**Table 1**

| Target gene | Forward primer (5' → 3') | Reverse primer (5' → 3') |
|-------------|--------------------------|--------------------------|
| Human NAT8L | TGGACGATCATGGAACATGGCGGCC | CGGAGGAGGCTGTTGAGGAT |
| Human β-ActIN | CGCCATGACCCTCTCCCTGC | GACCGGACGCTGTTGAGGAT |
| Marinit Nat8l | TGGACGATCATGGAACATGGCGGCC | CGGAGGAGGCTGTTGAGGAT |
| Tfiib | GTGCTATGTGGCTGCAATGCT | TCAATTACTCGGTCCTCATA |
| Pparγ2 | TGGCTTGGAGACCTTCAAAAGGAAT | CGAAGTTGTCGGGCAGGAA |
| Fabp4/aP2 | CACAGGAGAGGAGCAGATCT | ACCAGCTGTGCGACATCTC |
| Adipoq | TGTCTCTTAACTTACCGCTCGC | CCAACCTGACAACTTTTCTT |
| Fads3 | GTGACCATGGACACAGAAGTG | TGCCTTTTTCTTGTCCTAC |
| Betna | AAGAGAGACCTGGAGCAAGGA | CGAGCTGTGCGACATCTC |
| Psat1 | AGGAGGAGGAGGAGCAGATCT | TACCCTCTTCAAGAAGAAC |
| Pgc1α | CCTGAGACAGGTCTGCAATTTAC | TGGACTGTGCGACATCTC |
| Ppara | CCTGAGACAGGTCTGCAATTTAC | GCGGACTGTGCGACATCTC |
| Prdm16 | TCCAGCAGACGTGAGCAGCA | ATCTGCGTCCTGCAGTCGGC |
| C/ebp | GACGAGCTGCAACCCGAGGA | ACCACCCGCAACACTTTA |
| Cox6b | CGAGTTCAGCTGCTGCTGCT | ACCACCCGCAACACTTTA |
| Cidea | TGACATCTGAGGAGCAGACAG | GCCGACTGTGCGACATCTC |
| Lcep1 | ACCCTTTCCTCAGGAGAAA | TGACGCTGCGACATCTC |
| Dio2 | ACCAGCTCTCATGAGGACAG | CAGCTGCGACATCTC |
| Cox1 | TGGAGCCACCATATTTACAGC | AGGACTGTGCGACATCTC |
| Cpt1b | TGATCCGCGCAACTGAGCAG | TGGACTGTGCGACATCTC |
| Pdk4 | TTCCGCTTCTCAGGCCACAG | GTACAGCAGCTGCTGCTGCT |
| Fabp3 | CCCTGCTGCTATTCAGGATCT | AAAGGAGGAGGAGGAGGAG |
| Actl | AGGAGGAGGAGGAGGAGGAG | GGCAGCTGCGACATCTC |

**Note:** Primer pairs used for quantitative RT-PCR

**All primers were used with murine cDNA except for the indicated human primers.
determined in prior titration experiments, and working concentrations used were 1 μM oligomycin, 2 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone, 2.5 μM antimycin A, and 10 μM norepinephrine.

[14C]Glucose Uptake and Lipid Extraction—iBACs were incubated overnight with DMEM supplemented with 1 μM triiodothyronine, 20 μM insulin, 0.5 g/liter glucose, and 0.1 μCi of [14C(U)]glucose/ml (ARC). After that, cells were washed four times with ice-cold PBS, and neutral lipids were extracted with hexane/isopropyl alcohol (3:2, v/v). Thin layer chromatography was performed with hexane/diethylether/acetic acid (70/29/1, v/v/v) as solvent, and lipids were visualized with iodine vapor and cut out. The incorporated radioactivity was measured by liquid scintillation counting. Total glucose incorporation in each lipid class was calculated, and values were corrected by protein content.

Detection of Mitochondrial Mass by Flow Cytometry—iBACs were grown until day 7 of differentiation, trypsinized, incubated for 20 min in DMEM supplemented with 200 nM MitoTracker™ Green (Invitrogen), washed with PBS, and subjected directly to flow cytometry (BD FACSCalibur, BD Biosciences). 90% of all cells were found in sectors II and IV. Sector II was chosen to flow cytometry (BD FACSCalibur, BD Biosciences). 90% of all cells were found in sectors II and IV. Sector II was chosen to compare fluorescence intensities between control and Nat8l-overexpressing cells.

Transmission Electron Microscopy—Transmission electron microscopy was performed as described (23). In brief, iBACs grown on an Aclar film (Gröpl, Tulln, Austria) were fixed on day 7 in 2.5% (w/v) glutaraldehyde and 2% (w/v) paraformaldehyde in 0.1 M phosphate buffer, pH 7.4, for 1 h, postfixed in 2% (w/v) osmium tetroxide for 1 h at room temperature, dehydrated in a graded series of ethanol, and embedded in a TAAB epoxy resin (Gröpl). Ultrathin sections (75 nm) were cut with a Leica UC 6 Ultramicrotome and stained with lead citrate for 5 min and with uranyl acetate for 15 min. Images were taken using a FEI Tecnai G2 20 transmission electron microscope (FEI Eindhoven) with a Gatan ultrascan 1000 CCD camera. The acceleration voltage was 120 kV.

Measurement of Mitochondrial Membrane Potential—iBACs were plated on 30-mm glass coverslips on day 7. Following overnight incubation, cells were loaded with a 500 nM concentration of the ratiometric indicator JC-1 (Invitrogen) in full medium at 37 °C for 30 min. Cells were then washed, and fluorescence intensities were detected over mitochondrial regions using an array confocal laser-scanning microscope built on an inverse, automatic microscope (Axio Observer.Z1, Zeiss, Germany) equipped with a ×100, 1.45 numerical aperture oil immersion objective (Plan-Fluor, Zeiss), an acousto-optic tunable filter-based laser merge system (Visithron Systems), and a CCD camera (CoolSNAP-HQ, Photometrics). Excitation/emission wavelengths were 488/529 and 535/590 nm for green fluorescent monomers and red fluorescent J-aggregates, respectively. During experiments, cells were perfused with a buffer containing 145 mM NaCl, 5 mM KCl, 2 mM CaCl2, 1 mM MgCl2, 10 mM d-glucose, and 10 mM HEPES, pH 7.4. Basal fluorescence intensity ratios were normalized to corresponding ratios after dissipation of mitochondrial membrane potential using 2 μM carbonyl cyanide p-trifluoromethoxyphenylhydrazone.

Statistical Analysis—If not otherwise stated, results are mean values ± S.D. of at least three independent experiments, or results show one representative experiment of three. Statistical significance was determined using the two-tailed Student’s t test. *, p < 0.05; **, p < 0.01.

RESULTS

Nat8l Is Expressed in Adipocytes and Located in Mitochondria—NAT8L is often referred to as a brain-specific enzyme, and it is still under debate whether it is located in the mitochondria or ER/cytoplasm of neurons (24–26). Fig. 1A shows that Nat8l is expressed to a similar extent in brain and BAT, to a lower extent in WAT, and hardly detectable in skeletal muscle (SM), cardiac muscle (CM), and liver of C57BL/6 mice. In genetically obese mice (ob/ob), the Nat8l mRNA level was unchanged in the brain, whereas it was 50% decreased in BAT and nearly blunted in WAT when compared with WT mice (Fig. 1B). We next determined the expression profile of Nat8l during the differentiation of several adipogenic cell lines.

FIGURE 1. Nat8l is expressed in adipocytes, is strongly decreased in WAT and BAT of genetically obese mice, and localizes in mitochondria in vitro and in vivo. A and B, quantitative real-time PCR analysis of Nat8l mRNA expression in various tissues of male, refed C57BL/6 mice (A), and ob/ob mice (B) (n = 4). BAT, brown adipose tissue; WAT, white adipose tissue; SM, skeletal muscle; CM, cardiac muscle. C–E, Nat8l mRNA in various adipogenic cell lines during differentiation (n ≥ 3). C and D, expression at the start of differentiation is set to 1. C, murine mesenchymal white adipogenic cells C3H/10 T1/2. D, human, white adipogenic Simpson-Golabi-Behmel syndrome (SGBS) cells. E, murine iBACs. All data are presented as means ± S.D. (error bars). F, protein expression of NAT8L in iBACs and C57BL/6 BAT fractionates (cp, crude pellet; mit, mitochondria; nuc, nucleus; cyt/ER, cytosol/endoplasmic reticulum). Shown is one representative blot of n = 3 (left) and n = 2 (right). *, p < 0.05; **, p < 0.01.
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Nat8l mRNA expression increases about 20-fold during white adipogenic differentiation of C3H10 T1/2 cells (Fig. 1C). A similar increase was observed in human Simpson-Golabi-Behmel syndrome adipogenic cells (Fig. 1D). Most relevant for this work, Nat8l mRNA levels highly increased during differentiation of iBACs (Fig. 1E). Next, we investigated the localization of endogenous NAT8L in differentiating iBACs and BAT by subjecting subcellular fractions to Western blot analysis. NAT8L protein could be clearly detected in the mitochondrial fraction, both in vitro (Fig. 1F, left panel) and in vivo (Fig. 1F, right panels), and there was no detectable NAT8L protein in the ER/cytosolic fraction. To control proper fractionation, we included hexokinase 1 (mitochondrial), histone H3 (nuclear), and protein disulfide isomerase (cytosolic) detection. Due to its high expression in BAT, we focused on iBACs as a model system to study the role of Nat8l in adipocyte biology.

Overexpression of Nat8l in Differentiating iBACs Increases Lipid Turnover—In brain, NAT8L has been shown to be required for lipid synthesis, especially for myelination (14, 16). To analyze the influence of Nat8l on lipid metabolism in brown fat cells, we generated iBACs stably overexpressing Nat8l. After clonal expansion, cells were induced to differentiate into brown adipocytes. Two monoclonal populations exhibited substantial overexpression of Nat8l (-fold expression relative to control on day 3 of differentiation: 28.9 ± 8.4 and 12.3 ± 0.5, respectively). To measure neutral lipid synthesis from glucose, iBACs were incubated with 14C-labeled glucose. Nat8l-overexpressing iBACs showed an up to 4-fold increased incorporation of glucose into neutral lipids, such as diglycerides, TG, FFA, and cholesterol ester, on day 3 (Fig. 2A) and day 7 of differentiation (Fig. 2B). However, oil red O staining and TG measurements of Nat8l-overexpressing and control iBACs showed decreased TG levels on day 3 (Fig. 2C; quantified in Fig. 2D), whereas even a slight increase in lipid accumulation could be observed on day 7 of differentiation (Fig. 2C; quantified in Fig. 2E). The fact that, despite higher glucose incorporation into neutral lipids, TG accumulation was delayed in differentiating Nat8l-overexpressing iBACs prompted us to investigate lipolysis. Therefore, we measured glycerol and FFA content in cell lysates and supernatants of Nat8l-overexpressing and control iBACs on day 7. Whereas glycerol release (Fig. 2F) showed a tendency to decrease, FFA release was increased 4-fold in differentiated Nat8l-overexpressing iBACs compared with control cells (Fig. 2F). Additionally, we observed a 3-fold increase in FFA release upon isoproterenol stimulation in Nat8l-overexpressing cells (Fig. 2F). However, FFA content in cell lysates was not changed in any condition (Fig. 2F). Collectively, our data suggest an increased lipid turnover upon Nat8l overexpression in brown adipocytes.

Nat8l Overexpression Increases Mitochondrial Mass, Number, and Cellular Respiration Rate—Because lipolysis is a requirement for a distinct brown adipose phenotype in vivo (9), we asked whether mitochondrial mass, number, and cellular respiration rates are changed in Nat8l-overexpressing iBACs. Transmission electron microscopy clearly demonstrates an increased number of mitochondria in Nat8l-overexpressing cells (Fig. 3A). Counting 14 individual cells from two biological replicates from either control or Nat8l-overexpressing iBACs revealed a doubling of mitochondria/cell upon Nat8l overexpression (Fig. 3B). Furthermore, we incubated cells with MitoTracker Green, which stains mitochondria in a membrane potential-dependent manner (27) and subjected them to flow cytometry. As depicted in Fig. 3C, sector II) and D, Nat8l-overexpressing cells showed increased fluorescence intensity, indicating an increased mitochondrial mass. Nevertheless, others reported that Mitotracker Green changes its abilities with regard to the membrane potential (28). To ascertain that the increase in fluorescence intensity was not due to a membrane potential change, we measured membrane potential via incubation with the mitochondrial stain JC-1. As shown in Fig. 3E, there was no significant change in membrane potential in Nat8l-overexpressing cells when compared with controls. The increased mitochondrial mass led us to hypothesize that Nat8l-overexpressing cells may have an elevated OCR. Therefore, we studied the cells with a Seahorse extracellular flux analyzer. This experiment revealed that Nat8l-overexpressing cells have an increased OCR (Fig. 3F). Specifically, basal respiration and maximal respiration were elevated by at least 40%. Even in an activated state, before preincubation with 10 μM norepinephrine for
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1 h, increased respiration was evident in Nat8l-overexpressing cells compared with control cells (Fig. 3G). In addition, genes that are directly involved in mitochondrial oxidative phosphorylation (Cox1, mitochondrial coded subunit of Cytc) and indirectly in β-oxidation (Cpt1b, carnitine palmityltransferase 1b), Pdk4 (pyruvate dehydrogenase kinase 4), and Fabp3 (fatty acid-binding protein 3) were significantly up-regulated on day 3 and/or day 7 of differentiation of Nat8l-overexpressing cells, respectively (Fig. 3H).

In summary, these data indicate increased energy expenditure in iBACs upon Nat8l overexpression.

**Nat8l Overexpression Augments the Brown Adipose Phenotype**—Although TG accumulation is significantly decreased in Nat8l-overexpressing iBACs on day 3, no significant changes in expression profiles of general adipogenic markers, such as aP2 and adiponectin (AdipoQ), could be observed (Fig. 4A). As an exception, Pparγ2 levels were 3-fold increased on day 3 but reached the level of control cells by day 7. Additionally, three white selective markers (29) were investigated. Whereas the expression of fatty acid desaturase 3 (Fads3) did not change, phosphoserine aminotransferase 1 (Psat1) and resistin (Retn) were significantly decreased in Nat8l-overexpressing cells (Fig. 4A). Notably, the expression of genes that are crucial in the development of brown adipocytes, such as Ppara, Pgc1α (Ppar coactivator 1 α), Prdm16, Cebpβ, Cox8b, Dio2, and Cidea, were all increased in Nat8l-overexpressing iBACs on day 7 (Fig. 4B). Strikingly, Ucp1 mRNA expression was increased up to 17-fold in Nat8l-overexpressing iBACs on day 7 (Fig. 4C), and its expression could be further elevated upon the addition of 10 μM isoproterenol (Fig. 4C), showing that Nat8l-overexpressing cells still have the potential to be activated by β-adrenergic stim-
These changes are mediated by PPARα.

GW6471 treatment of Nat8l-overexpressing cells increased lipogenesis (Fig. 5A) and decreased in basal and isoproterenol-stimulated conditions, as reflected by increased FFA and glycerol release at day 7 when compared with control cells (Fig. 7D). Therefore, we treated Nat8l-overexpressing iBACs with a PPARα antagonist (10 μM GW6471) from day 4 of differentiation until day 7. This treatment did not change differentiation capacity as judged by microscopy and cellular TG content (Fig. 5E). Measuring mRNA levels of marker genes upon PPARα antagonist treatment, we found that Pgc1α and aP2 were unchanged, and Cox1 and Pdk4 were slightly decreased, whereas the direct PPARα target genes Cpt1b (31) and Ucp1 (32) were significantly blunted (Fig. 5A). Importantly, the massive Nat8l-mediated up-regulation in UCP1 protein was nearly reduced to control level (Fig. 5B). In accordance, OCR was reduced close to the level of control cells after GW6471 treatment of Nat8l-overexpressing cells (Fig. 5C). On the other hand, neither the increased lipogenesis (Fig. 5D) nor the elevated basal and isoproterenol-stimulated FFA release evoked by Nat8l overexpression was affected by GW6471 treatment (Fig. 5F). These results underline our hypothesis that Nat8l per se influences lipid turnover, but other effects, such as UCP1 and Cpt1b expression and respiration, require PPARα activation.

Enzymatic Activity Is Required for the Function of NAT8L in Adipocytes—Recently, Tahay et al. (24) investigated which regions of the human NAT8L protein are important for its catalytic activity by introducing several point mutations into the Nat8l gene and subsequent measurement of their enzymatic activities. We generated a mutant of NAT8L, exchanging asparagine at position 165 to alanine, which is described as having no residual enzymatic activity (24), and used this mutated construct to study the effects upon its overexpression. Although we reached a 7-fold increased expression of the mutated enzyme in iBACs (Fig. 6A), we observed no changes in the expression of adipogenic marker genes (Fig. 6A) and no impact on cellular lipid content or glycerol/FFA release (Fig. 6, B and C, unstimulated and isoproterenol-stimulated). Thus, we conclude that the enzymatic activity of NAT8L is required for its function in brown adipocytes.

Nat8l Silencing in iBACs and Knock-out of Nat8l in Mice Lead to Compensatory Up-regulation of ATP-Citrate Lyase—To analyze the influence of Nat8l knockdown on lipid and energy metabolism in brown fat cells, we generated iBACs stably silenced for Nat8l. Although various clonal populations were tested, Nat8l silencing efficiency did not exceed 50% (Fig. 7A and F). Although Nat8l silencing in iBACs did not affect differentiation, as shown by cellular TG content (Fig. 7D) and neutral lipid synthesis (Fig. 7B), lipolysis was significantly increased in basal and isoproterenol-stimulated conditions, as reflected by increased FFA and glycerol release at day 7 when compared with control cells (Fig. 7, D and E). Additionally, the...
FIGURE 5. Nat8l-mediated induction of Ucp1 mRNA and oxygen consumption is PPARα-dependent, whereas lipid turnover is not. IBACs were incubated with a 10 μM concentration of the PPARα antagonist GW6471 from day 4 until day 7 of differentiation. All experiments were performed with day 7 cells. A, mRNA expression of adipogenic marker genes (aP2, Ucp1, and Pgc1α) and genes involved in mitochondrial oxidative phosphorylation (Cox1) and β-oxidation (Cpt1b and Pdk4) (n = 3). B, UCP1 protein expression. One representative blot of n = 3 is shown. Relative band intensity is calculated from n = 3 and normalized to β-ACTIN. C, OCR measured with the Seahorse extracellular flux analyzer (n = 3). D, incorporation of [14C]glucose into neutral lipids (n = 3). Shown are TG content (E) and fatty acid content (F) in cell lysates and supernatants of cells with and without isoproterenol treatment (10 μM for 4 h; +/−) (n = 3). All data are presented as means ± S.D. (error bars), two-tailed Student’s t test. *, p < 0.05; **, p < 0.01; ***, p < 0.001.

FIGURE 6. Enzymatic activity is required for the function of Nat8l in brown adipocytes. IBACs were infected with retroviral particles harboring a Nat8l coding sequence for producing the enzymatically inactive D165A mutant of the NAT8L protein or an empty vector as control. Cells were differentiated, and all experiments were performed with day 7 cells. A, mRNA expression of general (Pparγ2 and aP2) and brown adipogenic genes (Ucp1, Ppara, and Cidea) (n = 3). Shown are TG content (B) and fatty acid content (C) in cell lysates and supernatants of cells with and without isoproterenol treatment (10 μM for 4 h; +/−) (n = 3). All data are presented as means ± S.D. (error bars), two-tailed Student’s t test. **, p < 0.01.
expression of adipogenic marker genes, such as *aP2*, *Ucp1*, *Ppar*/*H9251*, *Pgc1*/*H9251*, and *Cidea*, was significantly increased in Nat8l-silenced iBACs in comparison with control cells (Fig. 7F). However, Nat8l-silenced cells did not show a difference in OCR, neither under basal nor under norepinephrine-stimulated conditions (Fig. 7C). Because we could not reach a very strong...
Nat8l silencing in iBACs, we examined BAT from Nat8l-knock-out mice to evaluate some of our in vitro data. BAT from Nat8l-KO and WT mice were similar in weight (0.13 ± 0.04 g for Nat8l-KO versus 0.12 ± 0.06 g for WT mice, respectively; n = 3) and gross anatomy (Fig. 7G, inset), in agreement with our in vitro data showing that Nat8l silencing does not influence differentiation capacity. Moreover, we used Nat8l-KO mice to investigate the expression of several adipogenic marker genes. As seen for Nat8l-silenced iBACs (Fig. 7F), ap2, Ucp1, Ppara, Pgc1α, and Cidea mRNA expression was also significantly increased in our in vivo model (Fig. 7G).

Our data from Nat8l-overexpressing iBACs strongly suggest that NAA, the product of the enzymatic activity of NAT8L, can be used as an alternative source for lipid synthesis in adipocytes. Due to the fact that Nat8l silencing does not contrarily influence lipogenesis, we wondered whether another acetyl-CoA-producing pathway might be up-regulated to compensate for the decreased NAA-derived acetyl-CoA. Indeed, we found ATP-citrate lyase, the cytosolic enzyme converting citrate to acetyl-CoA and thereby linking cellular glucose catabolism and ATP-citrate lyase, the cytosolic enzyme converting citrate to products are used by NAA. NAA delivers the acetate moiety for synthesis of cytosolic acetyl-CoA that is further used for FFA synthesis. These FFA are readily esterified to prevent lipotoxicity. Thus, an increase in Nat8l expression could drain acetyl-CoA from mitochondria. To maintain the mitochondrial acetyl-CoA pool, acetyl-CoA is mainly provided by decarboxylation of pyruvate via the pyruvate dehydrogenase complex or via β-oxidation of fatty acids. We suppose that glycolysis-derived pyruvate is not sufficient to compensate for acetyl-CoA drained into the NAA pathway because we see Pdk4 up-regulated upon Nat8l overexpression. Pdk4 inactivates the pyruvate dehydrogenase complex, resulting in a switch from glucose to fatty acid oxidation (36). Additionally, upon Nat8l overexpression, we see increased lipolysis providing FFA as substrate for β-oxidation. Concomitantly, the expression of Fabp3 and Cpt1b is elevated. Both proteins have been shown to deliver FFA to mitochondrial β-oxidation in BAT (37, 38).

It is known that fatty acids and their derivatives are crucial for the activation of PPARα and UCP1 (9, 30, 39–41) and that sustained activation of PPARα increases β-oxidation (42). Recently, Ahmadian et al. (9) showed that lipolysis via adipose triglyceride lipase plays an essential role in maintaining adaptive thermogenesis in BAT. Together with the results of Haemmerle et al. (40) in cardiac muscle, this substantiates that lipolytic action is required for PPARα activation. PPARα, together with PGC1α, induces Ucp1 expression (32). Therefore, the massive increase in Ucp1 expression in Nat8l-overexpressing cells could be explained by a prominent PPARα activation by elevated lipolysis-derived FFA. Using the PPARα antagonist GW6471, we found that the Nat8l-induced Ucp1 expression and the concomitant OCR increase were diminished to control cell levels, indicating that it is indeed a PPARα-mediated mechanism. However, increased lipogenesis and FFA release are retained during GW6471 treatment. The fact that Nat8l levels are still high in this condition (Fig. 5A) argues for a dissociation of PPARα-mediated effects and NAA-mediated lipid turnover in adipocytes. Hence, delivery of acetyl-CoA via NAT8L-mediated NAA seems to be upstream of PPARα-related effects. Moreover, our studies using the enzymatically inactive protein
support this concept because the D165A mutant does not increase lipogenesis. PPARα, its coactivator PGC1α (43, 44), and PRDM16 (45) have been shown to be important for mitochondrial biogenesis. Elevated expression of all of these genes upon Nat8l overexpression could explain the increase in mitochondria in these cells. Also, other brown phenotypic marker genes, such as Dio2 (46), C/ebpβ (47), and Cidea (48), are upregulated in Nat8l-overexpressing cells, rendering NAT8L as a factor to enhance “browning.” This is further supported by the fact that white-specific genes, like Retn and Pstat1 (29), are repressed. All of the observations mentioned above are consistent with our measurement of increased basal and norepinephrine-stimulated OCR and explain why these cells have a higher metabolic rate per se.

Knockdown of Nat8l in brown adipocytes had no impact on TG content, lipogenesis, and OCR, whereas lipolysis and the expression of several adipogenic marker genes were still increased. The increased lipolysis observed upon Nat8l knockdown might be a mechanism to generate acetyl-CoA for energy production. Lipolysis provides fatty acids that eventually can become acylated and are further used for β-oxidation, thereby contributing to the acetyl-CoA pool. In parallel, the FFA produced by lipolysis might also activate PPARα (9) and thus increase the expression of PPARα target genes, such as Lcep1 and Cidea (49), as seen in Nat8l-silenced iBACs and Nat8l-KO mice. Most interestingly, ATP-citrate lyase was significantly increased upon Nat8l-silencing in vitro and in BAT of Nat8l-KO mice. ATP-citrate lyase is well described to link glucose catabolism to lipogenesis by catalyzing the production of acetyl-CoA from citrate. Thus, the increase of ATP-citrate lyase mRNA expression might present a compensatory mechanism (as outlined in Fig. 7f) to sustain the acetyl-CoA pool upon Nat8l knockdown.

Although NAT8L has been implicated in many functions in the brain (mainly in the generation of substrates for myelination), its main function in adipose tissue was unknown. We imagine that, under certain circumstances, the NAA pathway could similarly serve as an additional acetyl-CoA-metabolizing mechanism in adipocytes. Our data propose (Fig. 8) that elevating Nat8l expression in brown adipocytes results in increased acetyl-CoA flux via the NAA pathway and concomitant higher cytoplasmic FFA anabolism, resulting in elevated TG synthesis. A parallel increase in lipolysis followed by an activation of β-oxidation can then restore acetyl-CoA back to the mitochondria. This “futile” cycle may result in increased lipid turnover and raise the oxidative potential of the brown fat cell and thereby boost the brown adipogenic phenotype. However, it remains to be seen which physiological stimuli are contributing to the regulation of the NAA pathway.

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