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SIRT3, a Mitochondrial Sirtuin Deacetylase, Regulates Mitochondrial Function and Thermogenesis in Brown Adipocytes*

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SIRT3 is one of the seven mammalian sirtuin homologs of the yeast *Sir2* gene, which mediates the effect of caloric restriction on life span extension in yeast and *Caenorhabditis elegans*. Because adipose tissue is essential in energy homeostasis and also plays a role in life span determination, we decided to investigate the function of sirtuin members in fat. We report here that murine SIRT3 is expressed in brown adipose tissue and is localized on the mitochondria inner membrane. Caloric restriction activates SIRT3 expression in both white and brown adipose. Additionally, cold exposure up-regulates SIRT3 expression in brown fat, whereas elevated climate temperature reduces the expression. Enforced expression of SIRT3 in the HIB1B brown adipocytes enhances the expression of the uncoupling protein PGC-1α, UCP1, and a series of mitochondria-related genes. Both ADP-ribosyltransferase and deacetylase activities of SIRT3 are required for this action. Furthermore, the SIRT3 deacetylase mutant exhibits a dominant negative effect by inhibiting UCP1 expression. This inhibitive effect can be abolished by the coexpression of PGC-1α, indicating a major role of PGC-1α in the SIRT3 action. In addition, SIRT3 stimulates CREB phosphorylation, which reportedly activates PGC-1α promoter directly. Functionally, sustained expression of SIRT3 decreases membrane potential and reactive oxygen species production while increasing cellular respiration. Finally, SIRT3, along with genes related to mitochondrial function, is down-regulated in the brown adipose tissue of several genetically obese mice. In summary, our results demonstrate that SIRT3 activates mitochondria functions and plays an important role in adaptive thermogenesis in brown adipose.

Sir2 and related members of the sirtuin gene family are highly conserved in both prokaryotes and eukaryotes (1). The yeast *Sir2* encodes a nuclear protein that is localized mainly in nucleoli and telomeric foci (2). In yeast, *Sir2* maintains the silencing of the heterochromatin at the mating-type loci (3), telomeres (4), and rRNA-encoding DNA repeats (5). Furthermore, *Sir2* is also involved in the repair of DNA double-strand breaks (6), cell cycle progression through anaphase, the meiotic chromosome segregation checkpoint (7), and the suppression of sister-chromatid recombination by recruiting cohesins (8). Most importantly, though, *Sir2* mediates the effect of caloric restriction on life span extension (9). An increased dosage of the *Sir2* gene prolongs life span in yeast (10), *Caenorhabditis elegans* (11), and protozoan parasite *Leishmania* (12), whereas its mutation would shorten life span. Sir2 proteins possess NAD-dependent ribosylation (13) and protein deacetylase activities (14–16). Blocking NAD synthesis in yeast abolishes the life span extension of caloric restriction (17). On the other hand, resveratrol, a natural compound present in grapes and wine, has recently been shown as an activator of sirtuins to lengthen life span in yeast (18), *C. elegans*, and fruit fly (19).

There are seven mammalian *Sir2* homologs (20), all of which maintain the catalytic core domain of *Sir2*. NAD-dependent deacetylase activity has been demonstrated for mammalian SIRT1, SIRT2, and SIRT3 proteins. The presence of NAD-dependent ADP-ribosylase and protein deacetylase activities of sirtuin proteins suggests that they may function as sensors of metabolic or oxidative states of cells and regulate cellular functions accordingly. Mammalian SIRT1, which resides in the nucleus, is the most closely related to yeast *Sir2*. SIRT1 binds and deacetylates p53 (21, 22), NF-κB (23), forkhead transcription factors (24–26), and histones (27). In contrast, SIRT1-deficient cells are p53-hyperacetylated and have elevated p53-dependent apoptosis, whereas SIRT1 knock-out mice exhibit developmental defects (28). SIRT1 also suppresses muscle differentiation in response to the redox state (29). SIRT2, on the other hand, is a cytoplasmic protein, which colocalizes with microtubules and deacetylates α-tubulins (30). SIRT2 abundance increases during mitosis, suggesting that the protein plays a role in cell cycle regulation (31). Unlike SIRT2, human SIRT3 is a mitochondria protein, with its N-terminal 25 amino acid residues responsible for its mitochondrial localization (32, 33). Synthesized as an enzymatically inactive protein, human SIRT3 is activated by matrix-processing peptidase (33). Compared with human SIRT3, however, murine SIRT3 lacks the N-terminal 142 amino acid residues necessary for the mitochondria localization for the human version. Nevertheless, the murine SIRT3 was shown to maintain a paranuclear localization, consistent with a mitochondrial distribution pattern (34).

Similar to yeast *Sir2*, mammalian sirtuin genes may also mediate caloric restriction effects on metabolism. It has been reported recently that calorie restriction induces SIRT1 expression in fat and other tissues to promote mammalian cell survival (35). SIRT1 also suppresses adipocyte differentiation and activates fat mobilization (36). In agreement with this line of reasoning, mice lacking the insulin receptor in their adipose tissues live longer (37). Because adipose tissue, an essential player in energy homeostasis, responds to caloric restriction (38), we decided to focus our investigation on sirtuin function in the mammalian adipose tissue.

Adipose tissue not only plays a part in energy storage, but it

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is also a critical endocrine organ, producing hormones such as leptin and adiponectin. There are two types of adipose, white adipose tissue and brown adipose tissue. White adipocytes store energy as triglycerides in large lipid droplets and mobilize the lipid in support of the energy need of the body. Brown adipocytes are morphologically different from white adipocytes as they harbor abundant mitochondria and store lipid in multilocular lipid droplets. The physiological role of brown adipose tissue also differs from that of white adipose tissue as it serves primarily to dissipate energy in the form of heat. The uncoupling protein UCP1,[1] which resides on the mitochondrial inner membrane, mediates this process of adaptive thermogenesis. Because UCP1 induces proton leakage, it generates heat instead of ATP. As a result, UCP1 knock-out mice exhibit cold sensitivity (39), whereas transgenic mice, which express UCP1 in their white adipose tissues, resist obesity (40).

In this report, we investigate the expression of mouse sirtuin family members in adipose tissue. We then focus our studies on SIRT3, which displays a high expression in brown fat but maintains a low level in white fat. Moreover, the expression of SIRT3 in brown adipose tissue responds to the changes of environmental temperature and dietary restriction. Additionally, we determined that murine SIRT3 proteins localize in the mitochondrial inner membrane, and the expression of SIRT3 correlates with the expressions of genes related to mitochondrial function, including UCP1, through the activation of PGC-1α. SIRT3 also influences mitochondrial function by reducing membrane potential and reactive oxygen species (ROS) production and by increasing oxygen consumption.

EXPERIMENTAL PROCEDURES

Animals and Caloric Restriction—C57BL/6 male mice were used for the experiments. For caloric restriction, starting from 8 weeks of age, mice were caged singly. The *ad libitum* mice were fed NIH-31 standard feed (Harlan Teklad) *ad libitum*, whereas the caloric restricted mice were fed with NIH-31/NIA fortified diet (Harlan Teklad) with a daily food allotment at 90% of the amount eaten by the *ad libitum* mice at the 1st week, then 70% at the 2nd and 3rd weeks. From the 4th week, daily food allotment was kept at 60% for the caloric restricted mice. Three months after the onset of caloric restriction, tissues were harvested to examine SIRT3 gene expression by Northern blot analysis.

Cell Culture and Antibodies—HIB1B preadipocytes and NIH3T3 and BOSC23 cells were cultured in Dulbecco's modified Eagle's medium containing 10% bovine calf serum. For HIB1B differentiation, at confluence, cells were incubated in Dulbecco's modified Eagle's medium with 10% cosmic calf serum (Hyclone), supplemented with 5 μg/ml insulin (Sigma), 0.5 mM isobutylmethylxanthine (Sigma), 1 μM dexamethasone (Sigma), and 1 nM triiodothyronine (Sigma) for 3 days. They were then refed every 2 days with 10% cosmic calf serum in Dulbecco's modified Eagle's medium, containing only insulin and triiodothyronine at the concentrations mentioned above. On the 8th day of differentiation, cells were stimulated with 1 μM isoproterenol (Sigma) for 6 h prior to harvest. The antibodies used for Western blot analysis included anti-FLAG (M2, Sigma), anti-cytochrome *c* (clone 7H8.2C12, BD Pharmingen), and anti-cytochrome *c* oxidase subunit IV (clone 20E8-C12, Molecular Probes). Anti-CREB antibody and anti-phosphor-CREB (Ser-133) antibody were from Cell Signaling.

Plasmid Construction—The murine SIRT3 cDNA in the pSport-CMV vector was obtained from Open Biosystem. It was excised with EcoRI and XhoI and ligated into the EcoRI and SalI sites of pBabe-puro to generate pBabe-mSIRT3. For the construction of mSIRT3-FLAG, full-length mSIRT3 with a FLAG-tagged C terminus was generated by PCR using the primers 5′-GCAGTGGGTGGTCATG-3′ and 5′-ATTACTTGTCGTCATCGTCTTTGTAGTCTCTGTCCTGTCCATCCAG-3′. The PCR fragment was cloned into pCR-Blunt II-TOPO (Invitrogen) and then excised out with HindIII and XhoI and ligated into pCDNA3.1. Site-directed mutagenesis was performed based on a reported method (41). Amino acid residue Gly-11(G11A) or Asn-87 (N87A) of SIRT3 was replaced by Ala to disrupt the ADP-ribosyltransferase or the deacetylase activities, respectively. The primers for these two mutants are CATCAGCACACCCAGTGCCATCCCGGACTTCAGATCC (G11A) and GCGGCTCTATACACAGGCAATCGACGGGCTTGAGA (N87A). The SIRT3 mutants were confirmed by sequencing and subcloned into the pBabe-puro vector (42).

Retroviral Infection—For retroviral infection, pBabe-puro or pBabe-hygro constructs were used to transfect BOSC23 cells by the calcium phosphate method. Two days after transfection, supernatants containing viral particles were harvested and used to infect HIB1B cells. The cells were then selected by 4 μg/ml puromycin or 200 μg/ml hygromycin, respectively.

Northern Blot Analysis—Total RNA was isolated using TRIzol (Invitrogen) according to the manufacturer's instructions. Approximately 10 μg of RNA was separated through 1% agarose-formaldehyde gel and transferred to a nylon membrane (Schleicher & Schuell) in 20× SSC. The membrane was hybridized overnight at 65 °C with a probe labeled with [α-^{32}P]dCTP (MP Biomedicals) by random primed DNA labeling (Promega). The autoradiography was obtained by a Storm 860 PhosphorImager and ImageQuant software (Molecular Dynamics).

Confocal Microscopy—The mSIRT3-FLAG construct was used to transfect NIH3T3 cells, grown on coverslips, using Lipofectamine (Invitrogen) reagent. 48 h after transfection, cells were incubated for 45 min with 100 nM MitoTracker Orange (Molecular Probes) in Dulbecco's modified Eagle's medium at 37 °C. Cells on coverslips were rinsed in PBS and then fixed in 3.7% formaldehyde and PBS for 30 min. After blocking with 10% goat serum, cells were incubated with the M2 anti-FLAG antibody (1:500, Sigma) and then with a goat anti-mouse IgG antibody, conjugated with Alexa Fluor 488 (1:500, Molecular Probes). The coverslips were mounted and observed under a laser scanning confocal microscope (Olympus Fluoview).

Preparation of Mitochondria Fractions—NIH3T3 cells, transfected with mSIRT3-FLAG, were harvested in an isotonic mitochondrial buffer (MB) (210 mM mannitol, 70 mM sucrose, 1 mM EDTA, 10 mM HEPES, pH 7.5), supplemented with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 50 μg/ml leupeptin), and then homogenized in a Dounce homogenizer (Wheaton) on ice. The suspension was centrifuged at 400 × *g* on a microcentrifuge (ThermoForma) at 4 °C. This procedure was repeated twice, and supernatants from each step were combined and centrifuged at 10,000 × *g* at 4 °C for 10 min to pellet mitochondria. The mitochondria were fractionated further by either alkaline or digitonin treatment. For digitonin treatment, 100 μg of purified mitochondria was dissolved in 100 μl of digitonin solution (1.2 mg/ml). After a 25-min incubation on ice, the mixture was centrifuged at 10,000 × *g* for 10 min to generate mitoplasts, which consist of the inner membranes and the mitochondria matrix. The supernatant included the intermembranous space fraction and outer membrane. For alkaline treatment, mitochondrial pellets were washed and resuspended in freshly prepared 0.1 M sodium carbonate, pH 11.5. The suspension was then incubated at 0 °C for 30 min. Mitochondrial membranes and supernatant fractions were recovered by centrifugation at 100,000 × *g* for 30 min at 4 °C. Trichloracetate precipitation was used to concentrate the proteins in the supernatant. The pellet and the concentrated proteins were resuspended in SDS-PAGE sample buffer and separated by SDS-PAGE and then analyzed by Western blotting.

Measurement of Respiration in Whole Cells—Oxygen consumption of HIB1B fibroblasts was measured by using a Clark-type oxygen electrode. Each sample was analyzed by incubating 2×10^6 cells with PBS solution in a magnetically stirred chamber, thermostated to 37 °C. After recording the basal respiration, 500 nM cyanide *p*-trifluoromethoxyphenylhydrazone (FCCP) was added to the chamber to measure the maximum respiration, and 2.5 μg/ml oligomycin was added to determine the uncoupled respiration. The results are presented as the means ± S.D. of three independent experiments.

Determination of Mitochondrial Membrane Potential ($\Delta\psi_m$)—The fluorescent probe 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide (JC-1; Molecular Probes) was used to measure the $\Delta\psi_m$ of HIB1B cells. Briefly, 1×10^6 HIB1B cells were harvested by trypsinization. After washing with PBS, cells were incubated with 10 μg/ml JC-1 at 37 °C for 15 min. Cells were then washed in PBS, and 10,000 cells were analyzed for each sample by flow cytometry, using Moflo Cell Sorter (DakoCytomation). Data were presented as a ratio of relative red to green (aggregate to monomer) fluorescence intensity

[1] The abbreviations used are: UCP1, uncoupling protein 1; COX, cyclooxygenase; CREB, cAMP-response element-binding protein; DCFHDA, 2′,7′-dichlorodihydrofluorescein diacetate; $\Delta\psi_m$, mitochondrial membrane potential; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; JC-1, 5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazole carbocyanide iodide; MAP, mitogen-activated protein kinase; mSIRT3, murine SIRT3; PBS, phosphate-buffered saline; ROS, reactive oxygen species; PGC-1α, peroxisome proliferator-activated receptor α coactivator-1α.

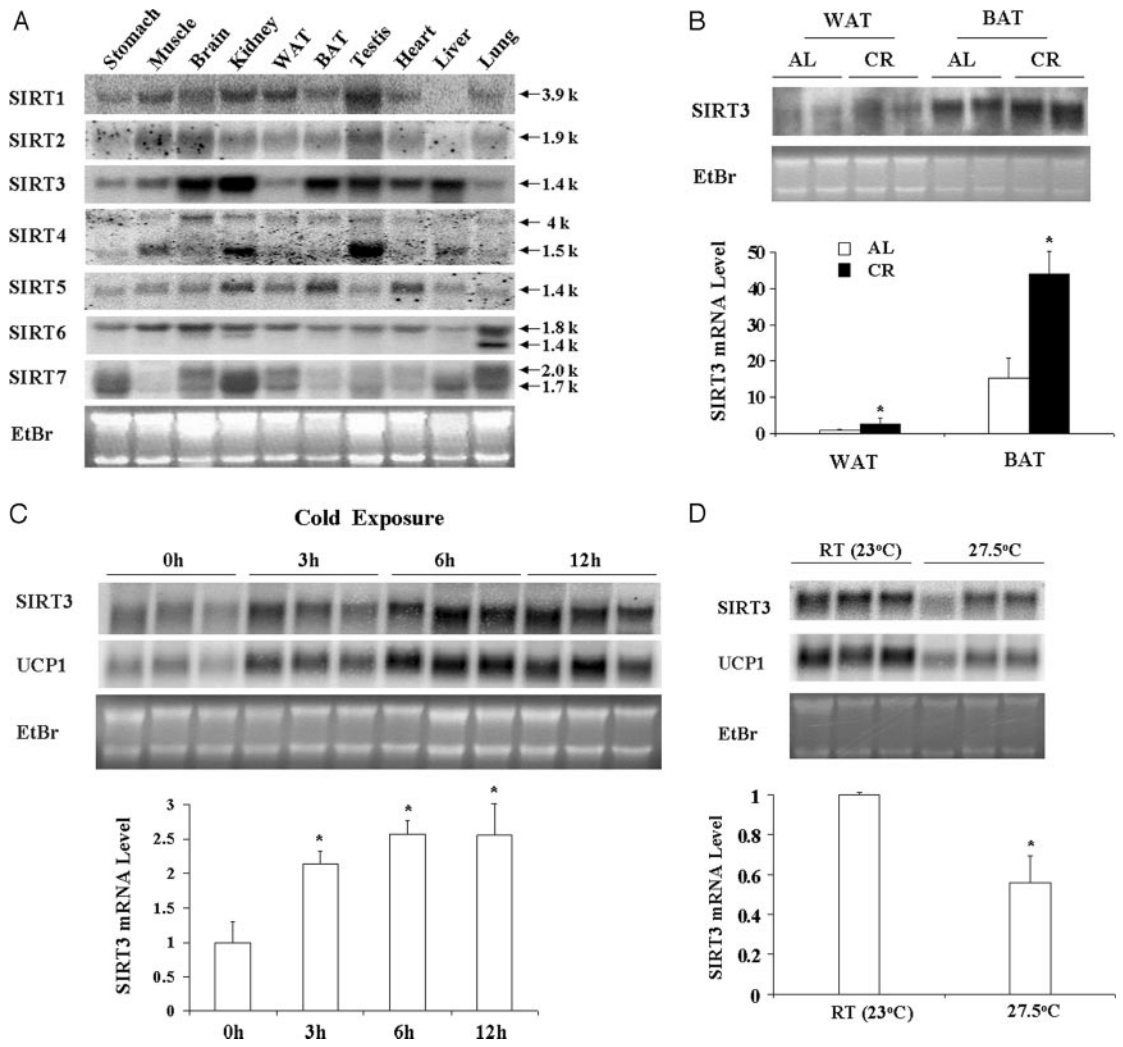

FIG. 1. *A*, RNA expression of sirtuins in mouse tissues. Total RNA was isolated from the indicated tissues, and 10 μg was loaded in each *lane*. Northern blot analysis was performed by hybridization with mouse cDNA probes for SIRT1 to SIRT7. The size of transcripts was estimated and indicated. *WAT*, white adipose tissue; *BAT*, brown adipose tissue. *B*, SIRT3 mRNA expression in white and brown adipose of mice after 3 months of caloric restriction. *AL*, *ad libitum*; *CR*, caloric restricted. Three mice of each group were used. *C*, expression of SIRT3 upon exposure of mice to cold. Mice were exposed to 5 °C for 3, 6, or 12 h, and the control mice were kept at 23 °C. Total RNA was isolated from the brown adipose, and Northern blot analysis was used to detect SIRT3 expression. Three mice from each group were used. *D*, expression of SIRT3 upon exposure of mice to 27.5 °C. Mice were exposed to 27.5 °C for 16 h, and control mice were kept at 23 °C. Three mice from each group were used. EtBr staining of the RNA is shown for the loading and the integrity of the samples. *, $p < 0.05$.

values. The results are presented as the means ± S.D. of three independent experiments.

Measurement of ROS—The ROS level was measured by using the probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFHDA) (Sigma), which is intracellularly esterified and becomes a fluorescent product, 2′,7′-dichlorodihydrofluorescein, when oxidized (43, 44). HIB1B cells were trypsinized and washed once in PBS. Then the cells were incubated with 10 μM DCFHDA for 10 min at 37 °C. 10,000 cells of each sample were then analyzed by flow cytometry. The results are presented as the means ± S.D. of three independent experiments.

Statistical Analysis—Statistical significance was determined by Student's *t* test. Differences between groups were considered statistically significant if $p < 0.05$.

RESULTS

Mammalian Sirtuins Are Expressed in Adipose Tissue—We first examined the expression pattern of seven mouse sirtuin members in various mouse tissues by Northern blot analysis. All seven mammalian sirtuin members are expressed in adipose tissues as well as a broad range of other tissues (Fig. 1*A*). Although SIRT2, SIRT4, and SIRT6 reside in both the white and brown adipose at comparable levels, SIRT1 and SIRT7 maintain a higher expression in white fat, whereas SIRT3 and SIRT5 are preferentially expressed in brown adipose *versus* white adipose. SIRT4, SIRT6, and SIRT7 display multiple mRNA species, probably because of alternative splicing.

SIRT3 Expression in Adipose Is Responsive to Caloric Restriction and Environmental Temperature—Among all of the sirtuin members, SIRT3 exhibits an interesting expression pattern with a high expression in the brown adipose *versus* white adipose. It was known that *Sir2* gene mediates caloric restriction in yeast and worms (9, 17). Recently, it was reported that mammalian SIRT1 is activated by caloric restriction in white fat, liver, brain, and kidney (35). Therefore, we decided to determine whether SIRT3 expression in adipose tissue is altered by caloric restriction. The SIRT3 mRNA level in both white adipose tissue and brown adipose tissue is up-regulated by 3 months of caloric restriction (Fig. 1*B*). Because rodent brown adipose is responsible for cold-stimulated nonshivering thermogenesis, we also examined whether SIRT3 expression is regulated by cold exposure. As shown in Fig. 1*C*, the RNA levels of both SIRT3 and UCP1 exhibit a similar pattern of increase in brown adipose during a 12-h period of cold exposure. When we subject mice to a thermoneutral temperature at 27.5 °C for 16 h, we found that SIRT3 expression was down-

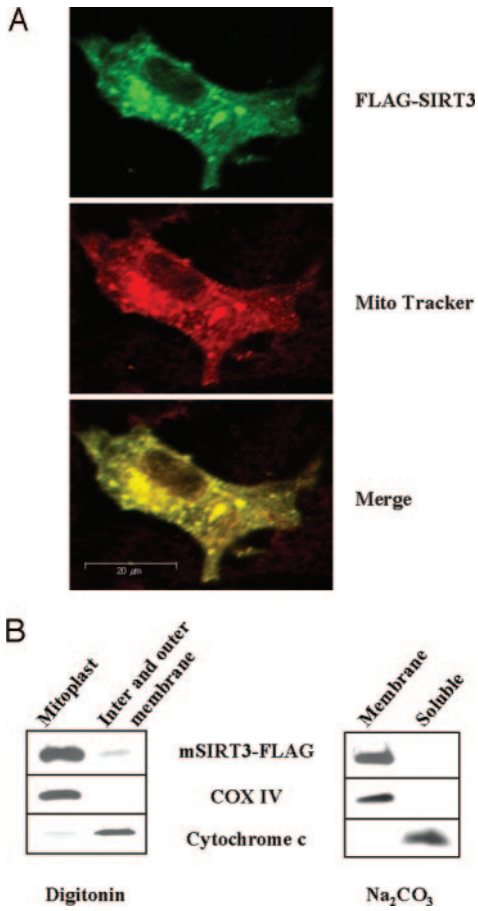

FIG. 2. *A*, subcellular localization of murine SIRT3 in mitochondria. Mouse SIRT3 with a FLAG tag at the C terminus was expressed in NIH3T3 cells by transient transfection. The mitochondria in the cells were stained with 100 nM MitoTracker Orange, and the FLAG-tagged SIRT3 was detected by an anti-FLAG antibody and an Alexa Fluor 488-conjugated secondary antibody. The result was observed using a laser scanning confocal microscope. Anti-FLAG immunofluorescence of the mSIRT3-FLAG fusion protein, fluorescence from the MitoTracker-stained mitochondria in the same focal plane, and a merged image demonstrating overlap of the two staining patterns are shown. *B*, murine SIRT3 is localized on the mitochondria inner membrane. Mitochondria were isolated from NIH3T3 cells transfected with mSIRT3-FLAG and treated with digitonin, which selectively disrupts the outer membrane (*left panel*). Isolated mitochondria were also resuspended in Na_2CO_3 buffer, which disrupts both the outer and inner membranes (*right panel*). The resulting mitochondrial fractions were analyzed by Western blot with anti-FLAG, COX IV, and cytochrome *c* antibodies.

regulated compared with the mice kept at room temperature of 23 °C (Fig. 1*D*). This result raised the possibility that SIRT3 may play a role in regulating adaptive thermogenesis.

Murine SIRT3 Protein Is Localized on the Mitochondrial Inner Membrane—The human SIRT3 protein was shown to be located in the mitochondrial matrix (32, 33), and the N-terminal 25 amino acid residues are critical for its mitochondrial localization (33). Because mouse SIRT3 lacks the N-terminal 142 amino acid residues of human SIRT3, the mitochondrial localization of mouse SIRT3 is in question. To determine the subcellular distribution of mouse SIRT3, it was expressed in a C-terminal FLAG-tagged form in the NIH3T3 cells by transient transfection. The mSIRT3-FLAG protein was detected by immunofluorescence and was observed using a laser scanning confocal microscope. As shown in Fig. 2*A*, the subcellular distribution of FLAG-tagged mouse SIRT3 protein shares a distribution pattern similar to that of the mitochondria stained by the MitoTracker dye. This finding indicates that the majority of mouse SIRT3 proteins are situated in the mitochondria.

To define more precisely the exact localization of mouse SIRT3 in the mitochondria, mitochondria from NIH3T3 cells transiently transfected with mSIRT3-FLAG were isolated and incubated with digitonin, which ruptures the mitochondria outer membrane and releases soluble proteins from the intermembranous space. Immunoblotting against the interstitial protein cytochrome *c*, which appeared in the supernatant fraction, was used to confirm the rupture of the outer membrane by digitonin (Fig. 2*B*, *left panel*). The inner membrane protein cytochrome *c* oxidase subunit IV (COX IV) remained in the mitoplast fraction, unaffected by the rupture of the outer mitochondrial membrane. The mouse SIRT3 proteins were detected in the mitoplast fraction, indicating that mouse SIRT3 resides on either the inner mitochondrial membrane or in the mitochondria matrix. To distinguish between these two possibilities, we treated mitochondria with sodium carbonate under an alkaline condition. Under this treatment, soluble and intermembranous proteins were released into the supernatant after ultracentrifugation, whereas membranes with integral membrane proteins sedimented into a pellet. The murine SIRT3 proteins were found in the pellet (Fig. 2*B*, *right panel*), indicating that it associates with the inner membrane. Taken together, we concluded that murine SIRT3 proteins localize on the mitochondria inner membrane.

Constitutive Expression of SIRT3 Promotes the Expression of Mitochondria-related Genes—To investigate the role that SIRT3 plays in brown adipocytes, we constitutively expressed mouse SIRT2 and SIRT3 in the HIB1B brown preadipocyte cell line using a retroviral system (42). After selection with puromycin, these cells were induced for differentiation for 8 days and stimulated with isoproterenol for 6 h before harvest. We found that in HIB1B brown adipocytes expressing SIRT2 and SIRT3, the expression of common adipocyte markers such as adipsin and aP2 shows little difference compared with the vector control (Fig. 3*A*), indicating that the expression of neither SIRT2 nor SIRT3 affects the general adipocyte differentiation process. However, the expression of SIRT3 but not SIRT2 up-regulates the expression of mitochondria-uncoupling protein UCP1. Similarly, the expression of PGC-1α and other mitochondria-related genes such as cytochrome *c* oxidase subunit II and IV (COX II and COX IV) and ATP synthetase is also activated specifically in the SIRT3-expressing cells.

Deacetylase and ADP-ribosyltransferase Activities Are Required for SIRT3 Action—Sirtuin proteins possess deacetylase and ADP-ribosyltransferase activities. To determine whether either enzymatic activities are required for SIRT3 action in brown adipocytes, SIRT3 mutants with either defective deacetylase or ADP-ribosyltransferase activity were generated by site-directed mutagenesis. The amino acid residue Asn-229 of human SIRT3 was demonstrated to be essential for the deacetylase activity (33). Accordingly, we generated an equivalent deacetylase mutant by replacing Asn-87 of murine SIRT3 with alanine. Based on the work on SIRT1 (14), we also generated an ADP-ribosylase mutant by replacing Gly-11 of SIRT3 with alanine. These mutants were then constitutively expressed in HIB1B cells using the retroviral system. As shown in Fig. 3*B*, although the G11A mutation eliminates the effect of SIRT3 on both UCP1 and PGC-1α expression, the N87A mutant of SIRT3 represses UCP1 and PGC-1α expression to a level even lower than that of the vector control, indicating a potential dominant negative effect. This suggests that both the deacetylase and ADP-ribosylase activities of SIRT3 play a role in its regulation of UCP1 expression.

PGC-1α Mediates the Action of SIRT3 on UCP1 Expression—It was reported previously that PGC-1α activates UCP1 in response to cold exposure (45) and also promotes mitochondria

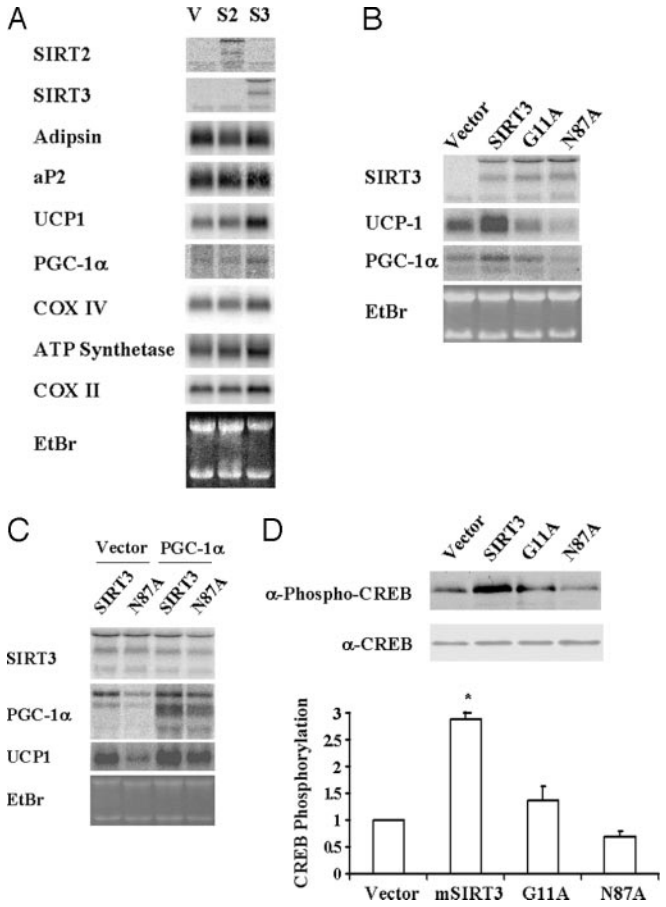

FIG. 3. *A*, constitutive expression of SIRT3 promotes the expression of UCP1, PGC-1α, and other mitochondria-related genes. HIB1B cells expressing murine SIRT2 or SIRT3 or a control vector were induced to differentiate. On the 8th day, cells were stimulated with 1 μM isoproterenol for 6 h prior to harvest. Total RNA (10 μg) was used for Northern blot analysis. *B*, enzymatic activity of SIRT3 is required for its action. Murine SIRT3 and its mutants, G11A and N87A, which disrupt the ADP-ribosylase and deacetylase activity, respectively, were expressed in the HIB1B cells. Cells were differentiated and stimulated as described above. Total RNA was isolated and analyzed by Northern blot analysis. *C*, PGC-1α rescues the down-regulation of UCP1 by the dominant negative SIRT3 N87A mutant. PGC-1α was coexpressed in the SIRT3- or N87A-expressing HIB1B cells with a retroviral system. Total RNA was isolated from differentiated and stimulated cells, and UCP1 expression was detected by Northern blot. EtBr staining of the RNA is also shown as a control for the loading and the integrity of the samples. *D*, constitutive expression of SIRT3 promotes activation of CREB. HIB1B cells constitutively expressed with murine SIRT3 and its mutants, G11A and N87A, were differentiated and stimulated as described above. Cells were harvested, and phospho-CREB (Ser-133) and total CREB were detected by immunoblot analysis. Data shown are the means ± S.D. of three independent experiments. *, $p < 0.01$.

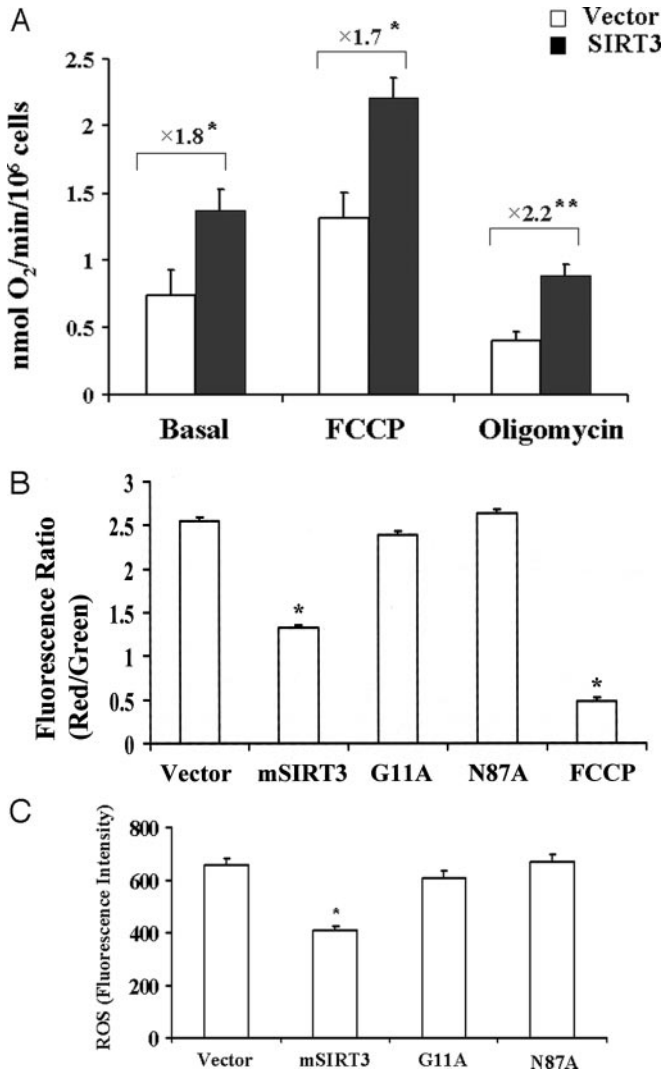

FIG. 4. *A*, constitutive expression of SIRT3 increases oxygen consumption. Oxygen consumption of HIB1B cells expressing mouse SIRT3 or the empty vector was analyzed using a Clark-type oxygen electrode. The chemical uncoupler FCCP (500 nM) and ATP synthetase inhibitor oligomycin (2.5 μg/ml) were used. *B*, constitutive expression of SIRT3 decreases $\Delta\psi_m$. HIB1B cells expressing SIRT3, G11A, or N87A SIRT3 mutants or vector alone were differentiated. The cells were then stained with 10 μg/ml JC-1 for 15 min and analyzed by flow cytometry. Cells treated with 10 μM FCCP were used as a control. The relative aggregate/monomer (red/green) fluorescence intensity values were used as an indication for $\Delta\psi_m$. *C*, constitutive expression of SIRT3 reduces cellular ROS level. Differentiated HIB1B cells were stained with 10 μM DCF-HDA. The cells were analyzed by flow cytometry. All data are the means ± S.D. of three independent experiments. *, $p < 0.01$; **, $p = 0.001$.

biogenesis (46). We next investigated whether PGC-1α mediates the action of SIRT3 on UCP1 expression by utilizing the dominant negative N87A deacetylase mutant of SIRT3, which decreases UCP1 and PGC-1α expression. When we coexpressed PGC-1α together with the SIRT3 N87A mutant in HIB1B cells, the expression of UCP1 was restored (Fig. 3*C*), suggesting that PGC-1α does mediate SIRT3 action on UCP1 expression.

Constitutive Expression of SIRT3 Promotes Activation of CREB—We then tried to elucidate the signaling pathway employed by SIRT3 to activate PGC-1α. It has been reported that CREB can activate PGC-1α gene expression (47, 48) and that p38 MAP kinase can directly phosphorylate and stabilize PGC-1α (49). We examined CREB and p38 phosphorylation, which indicates their activation, in HIB1B cells expressing SIRT3 and its mutants by immunoblot analysis. Although there is no evidence of increase of p38 phosphorylation in SIRT3-expressed cells (data not shown), SIRT3 enhances CREB phosphorylation compared with control (Fig. 3*D*). The G11A ADP-ribosylase mutant abolishes this effect partially and the N87A deacetylase mutant further suppresses CREB phosphorylation (Fig. 3*D*). The results indicate that SIRT3 activates PGC-1α through CREB but not p38 MAP kinase.

Constitutive Expression of SIRT3 Increases Oxygen Consumption—Because SIRT3 promotes the expression of PGC-1α, which in turn activates mitochondria respiration (46), we measured oxygen consumption of SIRT3-expressing cells. As shown in Fig. 4*A*, the basal oxygen consumption of the SIRT3-expressing HIB1B cell is 80% higher than the level of control cells. The chemical uncoupler FCCP can completely uncouple mitochondria and raise the respiratory capacity to the maximal

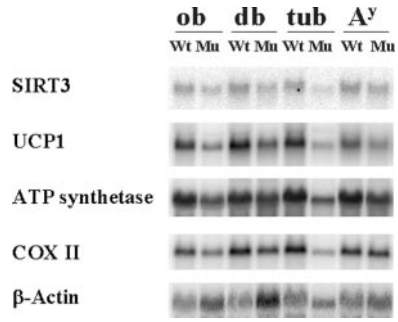

FIG. 5. **The expressions of SIRT3 and mitochondria-related genes are reduced in the brown adipose of obese mice.** Total RNA (10 μg) isolated from the brown fat pads of mice with different forms of genetic obesity along with lean littermates, matched by identical genetic background and sex, was subjected to Northern blot analysis. *Wt*, wild-type; *Mu*, mutant; *tub*, tubby; and A^y, yellow agouti. A β-actin probe was used as a control.

level. Under FCCP stimulation, oxygen consumption in the SIRT3-expressing cells is still about 70% higher than that of the control cells, indicating that these cells have a higher mitochondrial electron transport activity. Oligomycin inhibits F_1/F_0 ATP synthetase to suppress all oxidative phosphorylation-associated respiration. As a result, only the uncoupling mediates the residual respiration. SIRT3-expressing cells maintain 120% higher oxygen consumption than the control consumption under the oligomycin treatment, reflecting an increase in uncoupling capacity. Combining these results, we can conclude that constitutive expression of SIRT3 stimulates both mitochondria electron transport and uncoupling.

Constitutive Expression of SIRT3 Reduces $\Delta\psi_m$ and ROS Production—Because the uncoupling protein reduces $\Delta\psi_m$ and SIRT3 increases UCP-1 expression, we expected a decrease of $\Delta\psi_m$ in SIRT3-expressing cells. The $\Delta\psi_m$ levels were analyzed by JC-1 staining of cells followed by flow cytometry. JC-1 exists as a monomer when $\Delta\psi_m$ is low and emits a green fluorescence. In contrast, it aggregates at high $\Delta\psi_m$ to produce a red fluorescence. Thus, a ratio of red to green fluorescence reflects the level of $\Delta\psi_m$. In differentiated HIB1B cells, SIRT3 expression significantly reduces the $\Delta\psi_m$ (Fig. 4*B*). This effect, however, is fully abolished by the N87A mutation, which disrupts the deacetylase activity of SIRT3, whereas the mutation of the ADP-ribosyltransferase activity partially mitigates the action of SIRT3. The ROS levels in those cells were also determined by staining the cells with DCFHDA followed by flow cytometry analysis. As a result, we found that the ROS production decreases in SIRT3-expressing HIB1B cells (Fig. 4*C*) and that SIRT3 requires both the deacetylase and the ADP-ribosyltransferase activities for this action.

The Expression of SIRT3 and Mitochondria-related Genes Is Reduced in the Brown Adipose Tissue of Obese Mice—Because SIRT3 activates PGC-1α and UCP1 expression and increases respiration, the decrease of SIRT3 functions may reduce thermogenesis and energy expenditure. Because brown adipose tissue thermogenesis is defective in obese mice (50, 51), and obesity results from a combined effect of increasing energy intake and/or reducing energy expenditure, we decided to analyze whether SIRT3 expression has any correlation with obesity. Four different mouse models of genetic obesity (ob/ob, db/db, tub/tub, and KK) were used. The expression of SIRT3 was determined by Northern blot analysis, which manifests the down-regulation of SIRT3 expression in the brown adipose of obese mice compared with their lean wild-type littermates (Fig. 5). Furthermore, the expressions of UCP1 and several mitochondria-related genes, such as COX II and ATP synthetase, are also decreased in the brown adipose of obese mice in coordination with the down-regulation of SIRT3. The RNA level of SIRT3 in the white adipose does not change in obese mice (data not shown).

DISCUSSION

Sirtuin mediates the effect of caloric restriction on life span extension in yeast and *C. elegans*. In mammals, caloric restriction not only delays aging but also improves metabolism. Whether or not mammalian sirtuin homologs play a role in aging or metabolic regulation is an important question. We focused our research on sirtuin function in the mammalian adipose tissue. In this study, we revealed that all seven mammalian sirtuin members are expressed in adipose tissues. Although most of them reside in both white and brown adipose, SIRT3 is expressed preferentially in brown *versus* white adipose.

The expression of SIRT3 in adipose is physiologically regulated. Caloric restriction activates SIRT3 expression in both white and brown adipose. In addition, we demonstrated that SIRT3 expression in brown adipose tissue is elevated in response to cold while reduced in a thermoneutral temperature. Constitutive expression of SIRT3 in brown adipocytes not only increases the expression of genes related to mitochondria function and thermogenesis, such as PGC-1α and UCP1, but also functionally reduces $\Delta\psi_m$ and increases oxygen consumption. This action appears to be relatively specific for SIRT3 because SIRT2 has no such effect when expressed in the HIB1B cells. In addition, the dominant negative SIRT3 down-regulates the expression of PGC-1α and UCP1. Moreover, reduced expressions of SIRT3 and related mitochondrial genes in brown adipose correlate with obesity, consistent with previous findings that demonstrated the defectiveness of genetically obese mice in cold and diet-induced adaptive thermogenesis (50, 51). The reduced thermogenesis may contribute to the development of obesity in those mice.

We further demonstrated that murine SIRT3 proteins mainly situate on the mitochondrial inner membrane. However, it was reported that the human SIRT3 protein is localized on both the inner membrane and in the matrix (33). The difference might be explained by the facts that murine SIRT3 is shorter than the human version, and different cells were used to investigate human or murine SIRT3 submitochondrial localization. Murine SIRT3 resides predominantly on the inner membrane. It was reported that human SIRT3 protein is in an enzymatically inactive state until it is processed by matrix-processing peptidase (33). It is not clear if the same is true in mice. It is also possible that the murine SIRT3 protein may be processed and released into the mitochondrial matrix under particular physiological conditions.

The intriguing question is, with its mitochondria localization, how does SIRT3 activate nuclear genes such as PGC-1α? It was demonstrated previously that CREB stimulates the expression of PGC-1α by binding to its promoter and that p38 MAP kinase activates PGC-1α through direct phosphorylation of PGC-1α. Yet, our results reveal that SIRT3 is not able to activate p38 MAP kinase but is able to activate CREB phosphorylation. However, many signaling pathways, such as the cAMP-protein kinase A pathway (52), can activate CREB. The exact nature of the pathway that SIRT3 protein utilizes to activate CREB from the mitochondria is under investigation at this moment.

As a critical regulator of brown adipocyte thermogenesis (45) and mitochondria biogenesis (46), PGC-1α not only induces the expression of UCP1 but also increases oxygen consumption by functioning as a coactivator of transcription factors, such as the nuclear hormone receptor peroxisomal proliferator-activated receptor γ and the nuclear respiration factor NRF-1 (46). Because the reduction of UCP1 expression under the dominant

negative SIRT3 can be rescued by the coexpression of PGC-1α, we demonstrate that SIRT3 affects UCP1 expression through PGC-1α. The effects of SIRT3 on $\Delta\psi_m$ and ROS production can be explained by the activation of UCP1 by SIRT3 because it was demonstrated previously that uncoupling proteins cause the reductions of both the membrane potential and ROS level (53, 54). It is also possible that SIRT3 protein on the mitochondrial inner membrane affects mitochondrial respiration, membrane potential, and ROS generation directly, although we failed to detect a direct protein-protein interaction between SIRT3 and UCP1 using a coimmunoprecipitation method (data not shown).

The fact that SIRT3 is a mitochondria inner membrane protein and affects mitochondria uncoupling and respiration suggests a possible link between SIRT3 and metabolic regulation. Dysfunction of mitochondria is linked with diabetes and insulin resistance. Recent studies associated the reduction of muscle mitochondrial oxidative phosphorylation activity with insulin resistance in young (55) and aged patients (56). Moreover, in the muscle of diabetic patients, the expression of PGC-1α-related oxidative phosphorylation genes is decreased (57, 58). Because SIRT3 is expressed in muscle and liver, the question of whether SIRT3 regulates mitochondria function or plays any role in insulin resistance in those tissues awaits further experimental exploration.

Mitochondria also play a key role in caloric restriction and aging. It was demonstrated that caloric restriction lengthens yeast life span and increases mitochondrial respiration (9), although studies on *C. elegans* show that suppression of mitochondria function prolongs life (59, 60). A recent finding indicates that long lived mice tend to have higher metabolic rate, higher mitochondria uncoupling, and increased oxygen consumption (61). The mitochondria are the major sites for ROS production, which causes oxidative damage. Oxidative stress is closely linked with aging and life span (62). Caloric restriction reduces the production of ROS (63) and oxidative damage (64, 65). As our results reveal the effect of SIRT3 on mitochondria function and ROS production in brown adipose, we conjecture that SIRT3 may also exert a similar effect in other tissues, such as the brain, heart, and kidney, in which it is expressed. SIRT3 may therefore function to reduce free radicals and delay aging. Moreover, genetic variations of human SIRT3 gene have been linked to life span in humans (66).

Overall, we have provided evidence to show that one sirtuin member, SIRT3, is not only expressed in adipose tissue in a physiologically relevant fashion but also regulates adaptive thermogenesis by increasing mitochondrial respiration. Because SIRT3 also reduces free radical levels, it will be very interesting to see whether SIRT3 plays any role in life span extension.

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