Mitochondrial Oxidative Phosphorylation Reserve Is Required for Hormone- and PPAR γ Agonist-Induced Adipogenesis

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Adipocyte differentiation requires the coordinated activities of several nuclear transcription factors. Recently, mitochondria biogenesis was reported to occur during adipocyte differentiation and following treatment with thiazolidinediones *in vitro* and *in vivo*. Crif1 is a translational factor for mitochondrial DNA (mtDNA) and is important for transcription of the mitochondrial oxidative phosphorylation (OXPHOS) complex. To investigate the role of OXPHOS in adipogenesis, we analyzed adipocyte differentiation following disruption of *Crif1* *in vitro* and *in vivo*. The adipose-specific *Crif1* knockout mouse had a lower body weight and less fat mass than wild-type mice. Furthermore, adipocytes were smaller and had a dysplastic morphology in the adipose-specific *Crif1* knockout mouse. 3T3-L1 adipocytes or adipose-derived stem cells (ADSCs) that lacked *Crif1* expressed lower levels of mtDNA-encoded OXPHOS subunits, and adipocyte differentiation was disrupted. Rosiglitazone treatment did not induce adipogenesis or mitochondria biogenesis in *Crif1* knockout ADSCs. These results show that mitochondrial OXPHOS and *Crif1* are required for rosiglitazone- and hormone-induced adipogenesis.

INTRODUCTION

White adipocyte differentiation involves a complex and highly orchestrated program of gene expression that is driven by synergistic interactions between transcription factors including the peroxisome proliferator activated receptor gamma (PPAR γ), CCAAT/enhancer family [CCAAT/enhancer binding protein α , β , δ (C/EBP α , β , δ)], and sterol regulatory element binding protein-1c (SREBP-1c) (Farmer, 2006). These transcription factors regulate several intracellular pathways, processes, and secretory factors that influence adipogenesis. Understanding the

processes responsible for adipogenesis is important because adipocyte dysfunction occurs in various chronic metabolic diseases. Improving adipocyte function by modulating adipogenic processes may help manage such diseases.

Marked mitochondrial biogenesis occurs during adipocyte differentiation (Newton et al., 2011; Wilson-Fritch et al., 2003). The concentration of mitochondrial proteins is increased 20–30-fold in differentiated 3T3-L1 adipocytes in comparison to the concentration in preadipocytes (Newton et al., 2011). Treatment with chemical inhibitors of respiratory chain function, such as rotenone, suppresses adipogenesis by modulating the expression of the key transcription factors C/EBP α , PPAR γ , and SREBP-1c (Lu et al., 2010). The expression of PPAR γ coactivator-1 (PGC-1), which is a key regulator of adipogenesis, correlates with mitochondrial biogenesis and adipogenesis (Gao et al., 2011). Consequently, mitochondria have been proposed to play an important role in adipogenesis programming and the progression of metabolic diseases. However, how sequential changes in the levels of mitochondrial proteins affect adipocyte differentiation and which genes are involved in mitochondrial development remain poorly understood.

Most cellular energy is released by OXPHOS in the electron transport chain in the inner mitochondrial membrane. The OXPHOS system in the mammalian respiratory chain is composed of at least 89 polypeptides that are encoded by nuclear DNA (nDNA) or mitochondria DNA (mtDNA) (Calvo and Mootha, 2010; Wallace, 2007). The human mtDNA genome encodes only 13 OXPHOS proteins, but these proteins provide an essential framework for the assembly of OXPHOS complexes in the inner mitochondrial membrane (Shoubridge, 2001). The mitochondrial ribosome is responsible for intra-mitochondrial synthesis of mtDNA-encoded OXPHOS polypeptides. Depletion of mitochondrial transcription factor A (TFAM) in 3T3-L1 cells impairs translocation of glucose transporter 4, downstream of Akt, while adipogenic gene expression and lipid ac-

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accumulation are not affected. However, mitochondria-related studies involving the knockdown of genes or inactivation of the OXPHOS complex have used cultured cell lines and have not been performed *in vivo*.

To investigate the role of mitochondrial OXPHOS in adipocyte differentiation, we generated adipose-specific *Crif1* loss-of-function transgenic mouse. A primary mitochondrial function, which is important for the translation of the 13 subunits that constitute the mitochondrial OXPHOS complexes I, III, IV, and V *in vitro* and *in vivo*, was disrupted in this mouse model.

MATERIALS AND METHODS

Mice

Floxed *Crif1* (*Crif1^{lox/lox}*) mice were generated as previously described (Kwon et al., 2008). Adiponectin-*Cre* transgenic mice (C57BL/6J) were kindly provided by Dr. Evan D. Rosen. Mice were bred in our facility and housed at a constant temperature (22°C), with 50–60% humidity and a daily 12 h light/12 h dark cycle. All experiments were conducted using male mice housed in individual cages, and body weights were checked at 4 weeks of age. All mouse experiments were performed in the animal facility according to institutional guidelines, and the experimental protocols were approved by the institutional review board of the Korean Research Institute of Biotechnology and Bioscience, and Chungnam National University.

Histological and morphometric analysis

White adipose tissue (WAT) was fixed in 10% neutralized formalin, washed, and then embedded in paraffin. Paraffin tissue sections of 5 µm were deparaffinized, rehydrated, and stained with hematoxylin and eosin. For whole-mount fluorescence staining, epididymal WAT was fixed in 10% neutralized formalin. Formalin was removed with 20% sucrose, and WAT was then incubated with an anti-PECAM antibody (Millipore; MAB1398Z) and incubated for 10 min at room temperature with DAPI and BODIPY.

Cell culture and Oil red O staining

3T3-L1 preadipocytes were purchased from ATCC. Methylisobutylxanthine (IBMX), dexamethasone and insulin were obtained from Sigma-Aldrich. 3T3-L1 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum (Gibco BRL). Lipofectamine® RNAiMAX reagent (Invitrogen) was used to transfect 3T3-L1 fibroblasts with *Crif1* siRNA (GGA GUG CUC GCU UCC AGG AAC UAU U). Cells were cultured for 2 days, after which cells were differentiated by culturing in DMEM containing IBMX (0.5 mM), dexamethasone (1 µM), insulin (10 µg/ml), and 10% fetal bovine serum for a further 2 days. This medium was then replaced with DMEM containing insulin (10 µg/ml), which was exchanged every 2 days (Hemati et al., 1997). ADSCs were cultured from the *Crif1^{lox/lox}* mouse, as previously described (Estes et al., 2010). After treatment with null or *Cre* recombinase adenoviruses, ADSCs were differentiated into adipocytes by culturing in M199 medium (Gibco BRL) containing IBMX (0.5 mM), dexamethasone (1 µM), insulin (10 µg/ml), and rosiglitazone (0.5 µM) and 10% fetal bovine serum. Differentiated 3T3-L1 adipocytes or ADSCs were fixed with 4% paraformaldehyde for 30 min at room temperature and then washed with 1x PBS. Staining was performed with freshly prepared 0.2% Oil red O solution for 30 min followed by three washes with water. For quantification, Oil red O was dissolved in 200 µl isopropanol and the absorbance at 540 nm was measured by ELISA.

Crif1-HA adenovirus

Crif1 DNA was amplified by PCR from mouse genomic DNA extracted from C57BL/6 mouse liver, and HA was fused at the C-terminal of *Crif1*. The *Crif1*-HA adenovirus was generated using the pAdEasy system as previously described (He et al., 1998).

Western blotting

Western blot analysis was performed using 12% acrylamide SDS-PAGE gels. Primary antibodies against OXPHOS complex subunits (ND6, NDUFA9, SDHA, UQCRC2, and ATP5A1) were purchased from Invitrogen. An anti-COX4 antibody (#4844), an anti-VDAC antibody (#4866) and an anti-PPARγ antibody (#2435) were purchased from Cell Signaling. An anti-ND1 antibody (sc-65237) and an anti-*Crif1* antibody (sc-134882) were purchased from Santa Cruz Biotechnology. Secondary antibodies (goat anti-mouse and goat anti-rabbit) were obtained from Cell Signaling.

Blue native PAGE (BN-PAGE)

BN-PAGE and mitochondrial isolation was performed as previously described (Kim et al., 2012). Mitochondrial isolation was performed by suspending pellets of fully differentiated ADSCs in buffer B (210 mM mannitol, 70 mM sucrose, 1 mM EGTA, and 5 mM HEPES, pH 7.2). The mitochondrial fraction was used in the Native PAGE™ Novex® Bis-Tris Gel system (Invitrogen) with 0.5% n-dodecyl-β-D-maltoside to determine the content of the OXPHOS complex. After electrophoresis, the gel was transferred to a PVDF membrane, which was then incubated with an anti-OXPHOS antibody mixture (Invitrogen).

Oxygen consumption rate (OCR)

OCR was measured using an XF-24 analyzer (Seahorse Bioscience). ADSCs were plated on an XF-24 plate, treated with null or *Cre* recombinase adenovirus, and incubated in differentiation media. The day before OCR measurement, the sensor cartridge was calibrated with calibration buffer (Seahorse Bioscience) at 37°C. Fully differentiated ADSCs were washed and cultured in M199 (Gibco BRL) lacking sodium bicarbonate at 37°C. OCR was automatically calculated and recorded by the sensor cartridge and Seahorse XF-24 software.

Real-time PCR and Northern blot

Total RNA was isolated using Trizol (Invitrogen), in accordance with manufacturer's instructions. Complementary DNA (cDNA) was prepared with M-MLV Reverse Transcriptase and oligo-dT primers (Invitrogen). Real-time PCR was performed using cDNA, QuantiTect™ SYBR® Green PCR Master Mix (QIAGEN), and specific primers. Primer sequences were as follows: NDUFA9 F (5'-ACTGT GTTTG GGGCT ACAGG-3'), NDUFA9 R (5'-GATTG ATGAC CACGT TGCTG-3'), SDHA F (5'-ACACA GACCT GGTGG AGACC-3'), SDHA R (5'-GCACA GTCAG CCTCA TTCAA-3'), UQCRC2 F (5'-ATCAA AAGGG GCAAC AACAC-3'), UQCRC2 R (5'-CACTC AGGAA GCCCT CTGAC-3'), COX4 F (5'-TTGGC AAGAG AGCCA TTTCT-3'), COX4 R (5'-GCCCA CAAT GCTCT CCATT-3'), ATP5A1 F (5'-AGGCC TATCC TGGTG ATGTG-3'), ATP5A1 R (5'-CTTCA TGGTA CCTGC CACCT-3'), ND1 F (5'-ACGCA AAATC TTAGG GTACA-3'), ND1 R (5'-GAGTG ATAGG GTAGG TGCAA-3'), ND6 F (5'-CACCC AGCTA CTACC ATCAT-3'), ND6 R (5'-ATTGG GGGTG ATTAT AGAGG-3'), COX1 F (5'-ATTCTG AGCAG AATTA GGTCA-3'), COX1 R (5'-CTCCG ATTAT TAGTG GGACA-3'), ATP6 F (5'-ACGAA AATCT ATTGT CCTCA-3'), ATP6 R (5'-TGGTG TGTGG ATTAG CATTA-3'),

Fig. 1. Perturbation of adipose tissue development in *Crif1*^{fl/adipoq} mice. (A) Western blot analysis of eWAT probing for *Crif1* and the OXPHOS complex I subunits ND1 and NDUF9, which are encoded by nDNA and mtDNA, respectively. The β-actin was used as an internal control. (B) Tissue weight of mice (n = 10 for each group; means and s.d.s are shown; *P < 0.05). mWAT, mesenteric WAT; sWAT, subcutaneous WAT; BAT, brown adipose tissue. (C) Hematoxylin and eosin staining of eWAT. Magnification, × 200. (D) Distribution of adipocyte sizes in eWAT as shown in Fig. 1C. (E) Immunofluorescence image of eWAT stained with BODIPY (green), DAPI (blue) and an anti-PECAM antibody (red). Magnification, × 200.

Crif1 F (5'-GAA CG CTGGG AGAAA ATTCA-3'), *Crif1* R (5'-ATAGT TCCTG GAAGC GAGC A-3'), *Ppargamma* F (5'-ATCTT AACTG CCGGA TCCAC-3'), *Ppargamma* R (5'-TGGTG ATTTG TCCGT TGTCT-3'), *Adiponectin* F (5'-TCTCC TGTTT CTCTT AATCC TGCC-3'), *Adiponectin* R (5'-CATCT CCTTT CTCTC CCTTC TCTCC -3'), *CD36* F (5'-TGCTG GAGCT GTTAT TGGTG-3'), *CD36* R (5'-TGGGT TTGTC ACATC AAAGA-3'), *Fasn* F (5'-CCCTT GATGA AGAGG GATCA-3'), *Fasn* R (5'-ACTCC ACAGG TGGGA ACAAG-3'), *C/ebpa* F (5'-TGGAC AAGAA CAGCA ACGAG-3'), *C/ebpa* R (5'-TCACT GGTCA ACTCC AGCAC-3'), *C/ebpb* F (5'-CAAGC TGAGC GACGA GTACA-3'), *C/ebpb* R (5'-AGCTG CTCCA CCTTC TTCTG-3'), *C/ebpd* F (5'-ATCGA CTTC A GCGCC TACAT-3'), and *C/ebpd* R (5'-GCTTT GTGGT TGCTG TTGAA-3'). Relative expressions were calculated using Rotor-Gene™ 6000 real-time rotary analyzer software (Version 1.7, Corbett Life Science).

RESULTS

Knockout of *Crif1* in adipocytes perturbs adipose tissue development

The mitochondrial protein *Crif1* associates with the large subunit of the mitochondrial ribosome and plays a critical role in the intra-mitochondrial production and insertion of 13 OXPHOS subunits encoded by mtDNA (Kim et al., 2012). *Crif1* is ubiquitously expressed in WAT and brown adipose tissue (BAT) (data not shown). To identify the function of *Crif1* and mitochondrial OXPHOS in adipocytes, we generated adipocyte-specific *Crif1*

knockout mice by crossing floxed *Crif1* mice (Kwon et al., 2008) with adiponectin-*Cre* transgenic mice. The epididymal WAT (eWAT) of *Crif1*^{fl/adipoq} mice exhibited marked depletion of *Crif1* and ND1, a mtDNA-encoded OXPHOS complex I subunit, and a reduction in the level of NDUF9, a nDNA-encoded OXPHOS complex I subunit (Fig. 1A). *Crif1*^{fl/adipoq} mice demonstrated a marked decrease in the weight of mesenteric WAT (mWAT), subcutaneous WAT (sWAT), and BAT (Fig. 1B). Hematoxylin and eosin staining of adipose tissues indicated that the adipocytes of *Crif1*^{fl/adipoq} mice were relatively smaller and irregularly sized in comparison to those of *Crif1*^{+/+,adipoq} mice (Fig. 1C). Distribution analysis of adipocyte size showed that the peak frequency was approximately $2.5 \times 10^3 \mu\text{m}^2$ in *Crif1*^{fl/adipoq} mice and $15 \times 10^3 \mu\text{m}^2$ in *Crif1*^{+/+,adipoq} mice (Fig. 1D). BODIPY staining was reduced in the eWAT of *Crif1*^{fl/adipoq} mice, whereas nuclei (DAPI) and endothelial cell (anti-PECAM antibody) staining was unchanged. This indicates that mitochondrial OXPHOS dysfunction in these mice may perturb the development of adipose tissue (Fig. 1E). Collectively, data from the adipocyte-specific *Crif1* knockout mouse suggests that loss of *Crif1* disrupts adipose tissue development *in vivo*.

Mitochondrial biogenesis is critical for adipocyte differentiation

It has been previously reported that mitochondrial biogenesis is associated with adipocyte differentiation (Luo et al., 2008; Wilson-Fritch et al., 2003). In this study, we studied mitochondrial biogenesis during adipogenesis of 3T3-L1 cells. The mtDNA copy number was measured using real-time PCR. The mtDNA copy

Fig. 2. Mitochondrial biogenesis during 3T3-L1 adipocyte differentiation. (A) The relative amount of mtDNA was analyzed by real-time PCR during 3T3-L1 adipocyte differentiation. (n = 4; means and s.d.s are shown; *P < 0.05). Ctr, control; R, Rosiglitazone; EtBr, ethidium bromide. (B) Western blot analysis of OXPHOS subunits encoded by nDNA (NDUFA9, SDHA, SDHB, UQCRC2, COX4, and ATP5A1) and mtDNA (ND6) during 3T3-L1 adipocyte differentiation. (C) BN-PAGE analysis of assembled OXPHOS complexes in isolated mitochondria during 3T3-L1 adipocyte differentiation. (D) Western blot analysis of Crif1, VDAC and OXPHOS subunits encoded by nDNA (NDUFA9, SDHA, UQCRC2, COX4, and ATP5A1) and mtDNA (ND1) in isolated mitochondria during 3T3-L1 adipocyte differentiation.

number started to increase on day 1 and peaked at day 3 of 3T3-L1 adipocyte differentiation (Fig. 2A). To determine whether this increase in mtDNA copy number was accompanied by an increase in the levels of the OXPHOS complex proteins, Western blotting of various OXPHOS subunits was performed on whole cell lysates obtained from 3T3-L1 adipocytes (Fig. 2B). The levels of various subunits of OXPHOS complexes I, II, III, and IV were induced on the first day of differentiation. The OXPHOS complex V subunit, ATP5A1, was expressed at a relatively high level in all samples (Fig. 2B).

BN-PAGE analysis showed that the level of assembled OXPHOS complexes increased during adipocyte differentiation of 3T3-L1 cells (Fig. 2C). To normalize the expression levels of OXPHOS subunits, Western blotting of various OXPHOS subunits was performed on mitochondria isolated from differentiating 3T3-L1 cells. The expression of OXPHOS subunits in these isolated mitochondria increased as differentiation progressed. Crif1 was expressed in undifferentiated 3T3-L1 cells and its expression steadily increased during differentiation (Fig. 2D). These data indicate that mitochondrial biogenesis, which correlates with an increase in the mtDNA copy number and increased expression of OXPHOS complex subunits, is linked with adipocyte differentiation of 3T3-L1 cells.

Adipose mitochondria function is suppressed in the insulin resistant state and the reversal of insulin resistance by treatment with PPAR γ agonists, such as rosiglitazone, is associated with an increase in adipose mitochondrial biogenesis (Sears et al., 2009; Wilson-Fritch et al., 2003; 2004). Treatment with a low dose of rosiglitazone over a prolonged period stimulated the differentiation of 3T3-L1 cells. Rosiglitazone treatment in-

creased the intensity of Oil red O staining, whereas staining was reduced when cells were treated with ethidium bromide (EtBr), which depletes mtDNA (Fig. 3A). Treatment with rosiglitazone promoted differentiation of 3T3-L1 cells and induced mRNA expression of *Crif1* and *Cox1*, a subunit of OXPHOS complex IV (Fig. 3B). Western blotting revealed that rosiglitazone treatment increased the expression of subunits of OXPHOS complexes I, II, III, IV, and V, whereas treatment with EtBr did not (Fig. 3C). These data indicate that PPAR γ -induced adipogenesis is linked with the biogenesis of mitochondrial OXPHOS complexes.

Crif1 is induced during adipogenesis and is required for the differentiation of adipocytes

Expression of *Crif1* mRNA was induced during the early phase of adipogenesis, in a similar manner to OXPHOS complex subunits (Figs. 2A and 4A). To explore the functional link between mitochondrial OXPHOS function and adipocyte differentiation *in vitro*, we monitored the expression of Crif1 and OXPHOS complex subunits in 3T3-L1 fibroblasts stimulated with various differentiation signals. Crif1 expression was increased by treatment with insulin and/or dexamethasone (Fig. 4B). Treatment with IBMX, dexamethasone and insulin increased the expression of the OXPHOS subunits, ND1, and the expression of PPAR γ , a marker of differentiated adipocytes (Fig. 4B).

The induction of *Crif1* expression with half the dose of dexamethasone promoted 3T3-L1 adipocyte differentiation as indicated by enhanced Oil red O staining and increased expression of *Ppargamma*, *adiponectin*, *Cd36* and *fatty acid synthase (Fasn)* (Figs. 5A and 5B). By contrast, when *Crif1* was depleted in 3T3-

Fig. 3. Modulation of mitochondrial OXPHOS following treatment with rosiglitazone or EtBr. 3T3-L1 cells were treated with 1 μ M rosiglitazone or 100 ng/ml EtBr. (A) Oil red O staining at day 7 of 3T3-L1 adipocyte differentiation. (B) Northern blot analysis of Crif1, Ppargamma, Cox1 and β -actin at days 0, 1, 3, 7 of 3T3-L1 adipocyte differentiation. (C) Western blot analysis of the OXPHOS complex subunits NDUFA9, SDHA, SDHB, UQCRC2, COX4 and ATP5A1, as well as OPA1, TOM40, and β -actin, at days 1, 3 and 7 of 3T3-L1 adipocyte differentiation.

Fig. 4. *Crif1* expression during 3T3-L1 adipocyte differentiation. (A) Northern blot analysis of *Crif1* and *Ppargamma* mRNA during 3T3-L1 adipocyte differentiation (upper). The graph shows the relative densities of *Crif1*/ β -actin or *Ppargamma*/ β -actin mRNA compared with the value at day 0 from the Northern blots. (B) Western blot analysis of 3T3-L1 fibroblasts treated with various combinations of hormones that induce adipocyte differentiation. D, dexamethasone; M, IBMX; I, insulin.

L1 cells using siRNA, the protein levels of the NDUFA9 and COX4, were decreased (Fig. 5D). The mRNA levels of these subunits were unchanged (data not shown). These results suggest that the depletion of *Crif1* inhibits lipid accumulation and decreases the expression of OXPHOS complexes due to defects in mitochondrial translation (Figs. 5C and 5D). Furthermore, depletion of *Crif1* reduced the maximal induction of *Ppargamma*, *Cd36*, and *adiponectin* (Fig. 5F), but did not affect cell viability (Fig. 5E). These observations indicate that *Crif1* plays a crucial role in adipocyte differentiation by regulating the expression of adipogenic genes.

Treatment with rosiglitazone fails to promote adipogenesis in *Crif1* null ADSCs

To identify the role of mitochondrial OXPHOS in rosiglitazone-induced adipogenesis, we studied the ability of rosiglitazone to promote adipogenesis in *Crif1* null ADSCs. These cells were generated by infecting stromal vascular fraction cells isolated from *Crif1*^{flox/flox} mice with an adenovirus expressing *Cre* recombinase. The translation of subunits of OXPHOS complex I (NDUFA9), III (UQCRC2) and IV (COX4) was perturbed in *Crif1* null ADSCs (Fig. 6A), and the assembly of the OXPHOS complex was also disrupted (Fig. 6B). The mRNA levels of these subunits were unchanged (Fig. 6C). This defect in the assembly of the OXPHOS complex led to decreased oxygen consumption by mitochondria (Fig. 6D).

Treatment with rosiglitazone failed to promote adipogenesis in *Crif1* null ADSCs. The defect in mitochondrial OXPHOS in

Crif1 null ADSCs blocked rosiglitazone-induced lipid accumulation (Fig. 7A). As adipogenesis is controlled by the coordinated activities of many transcription factors, we monitored the expression levels of some of these transcription factors in ADSCs on days 1, 3, and 7 of the differentiation time course. The expression levels of *C/ebp β* and *C/ebp δ* , which are involved in 3T3-L1 adipocyte differentiation and are induced at early time-points, were not perturbed in *Crif1* null ADSCs. Furthermore, rosiglitazone treatment increased the expression of these transcription factors. However, the expression levels of *Ppargamma* and *C/ebp α* were decreased in untreated *Crif1* null ADSCs and rosiglitazone treatment did not induce their expression (Fig. 7B). The expression of *adiponectin* was abolished in *Crif1* null ADSCs, irrespective of whether they were treated with rosiglitazone. These data suggest that mitochondrial OXPHOS may play a role in the generation of endogenous PPAR γ ligands.

In summary, these findings indicate that mitochondrial OXPHOS in adipose tissue is crucial for hormone- and rosiglitazone-induced adipogenesis *in vitro* and *in vivo*.

DISCUSSION

In the present study, we showed that mitochondrial OXPHOS affects adipose tissue development *in vivo* and is required for adipogenesis in response to hormones and PPAR γ ligands *in vitro*. The most important function of mitochondria is to produce energy; however, mitochondria may also modulate the expression of genes that control tissue specific functions. Comparative

Fig. 5. Crif1 controls adipogenesis by modulating the expression of various genes. (A) Oil red O staining of 3T3-L1 cells infected with adenoviruses. Null, null adenovirus; Crif1-HA, Ha-tagged Crif1 adenovirus. Magnification, $\times 100$ (upper). (B) Real-time PCR analysis of *Ppargamma*, *adiponectin*, *Cd36* and *Fasn* in 3T3-L1 cells at day 7 of differentiation ($n = 6$; means and s.d.s are shown; $*P < 0.05$). (C) Oil red O staining of 3T3-L1 cells treated with *Crif1* siRNA. Magnification, $\times 100$ (upper). (D) Western blot analysis of differentiating 3T3-L1 cells probing for OXPHOS complex subunits NDUFA9, SDHB, UQCRC2, COX4, and ATP5A1. TOM40 and β -actin were used as internal controls. (E) Viability of 3T3-L1 cells treated with *Crif1* siRNA or control siRNA. Cells were cultured in the presence or absence of differentiation hormones. The relative cell viability was normalized against that of the control siRNA sample that was not treated with hormones ($n = 6$; means and s.d.s are shown; $*P < 0.05$). (F) Real-time PCR analysis of *Crif1*, *Pparg*, *Cd36* and *adiponectin* mRNA levels following treatment with *Crif1* siRNA or control siRNA ($n = 6$; means and s.d.s are shown; $*P < 0.05$).

Fig. 6. Mitochondrial dysfunction in *Crif1* null ADSCs. Control and *Crif1*-deficient ADSCs were generated by infection with null or *Cre* recombinase-containing adenovirus, respectively. Null, control ADSCs; *Cre*, *Crif1*-deficient ADSCs. (A) Western blot analysis of the OXPHOS complex subunits NDUFA9, UQCRC2, COX4, and ATP5A1 in null and *Crif1*-deficient ADSCs. α -tubulin was used as an internal control. (B) BN-PAGE analysis of OXPHOS complexes in control and *Crif1*-deficient ADSCs. (C) mRNA levels of nDNA-encoded and mtDNA-encoded OXPHOS subunits. ($n = 4$; means and s.d.s are shown; $*P < 0.05$). *Nd1*, *Nd6* and *Nd4a9* for OXPHOS complex I; *Sdh* for OXPHOS complex II; *Uqcrc2* for OXPHOS complex III; *Cox1* and *Cox4* for OXPHOS complex IV; *Atp6* and *Atp5a1* for OXPHOS complex V. (D) Basal OCRs were measured using a Seahorse XF-24 flux analyzer ($n = 8$; means and s.d.s are shown; $*P < 0.05$).

Fig. 7. *Crif1*-deficient ADSCs do not differentiate in response to rosiglitazone. (A) Oil red O staining in fully differentiated control (Null) and *Crif1*-deficient (*Cre*) ADSCs. (B) Real-time PCR analysis of *Crif1*, *C/ebpβ*, *C/ebpδ*, *Ppargamma*, *C/ebpα*, and *adiponectin* at day 3 of ADSC differentiation induced with 1 μ M rosiglitazone. Control cells were treated with DMSO for vesicle (n = 4, means and s.d.s are shown; n = 6; * $P < 0.05$).

proteomic studies with the mitochondrial proteome have revealed that tissue specific remodeling controls mitochondrial function in metabolically active tissues (Bugger et al., 2009).

Mitochondria biogenesis during adipocyte differentiation in 3T3-L1 cells has been studied using proteomics and microarray analyses. Thiazolidinediones are PPAR γ agonists that stimulate mitochondrial biogenesis and remodeling (Wilson-Fritch et al., 2003; 2004). To verify the role of mitochondria and mitochondrial biogenesis in adipocyte differentiation, a number of studies have targeted specific genes, such as TFAM (Shi et al., 2008) and apoptosis inducing factor (AIF) (Shen et al., 2011). TFAM is a critical regulator of the transcription of mtDNA-encoded OXPHOS subunits and AIF is also important for the function of the OXPHOS complex. The ablation of these genes in adipocytes perturbs or inhibits adipogenesis. We showed that *Crif1* regulates the biogenesis of the OXPHOS complex by modulating the translation of mtDNA-encoded OXPHOS subunits. Therefore, homozygous and heterozygous ablation of *Crif1* is an appropriate model to study the function of mitochondrial OXPHOS during adipose development *in vivo*. Interestingly, *Crif1* expression transiently increased during adipocyte differentiation *in vitro*. However, *Crif1* expression was not dependent on PPAR γ expression and was not matched with induction of OXPHOS expression when adipocytes were treated with PPAR γ agonists. These observations indicate that *Crif1* expression, which is independent of PPAR γ expression and PPAR γ agonist stimulation, is linked with mitochondrial OXPHOS complex biogenesis and adipocyte differentiation. However, the regulatory pathways that control *Crif1* expression in adipose tissue remain unknown.

In this study, we showed that loss of *Crif1* impairs mitochondrial OXPHOS and adipocyte differentiation *in vitro* and *in vivo*. It remains unclear how the disruption of mitochondrial OXPHOS modulates gene expression to perturb adipocyte differentiation. It has been recently reported that reactive oxygen species generated by the mitochondrial OXPHOS complex promote the differentiation of adipocytes. We did not measure the level of reactive oxygen species in adipose tissue of *Crif1* knockout

mice. However, it is unlikely that changes in the levels of reactive oxygen species are responsible for the defects in the development of adipose tissue observed in *Crif1* knockout mice. PPAR γ and its co-activators control transcriptional networks that govern gene expression patterns in adipocytes and thereby regulate adipogenesis (Hwang et al., 2011). It is unclear how endogenous PPAR γ ligands control adipogenesis. Mitochondrial OXPHOS regulates fatty acid metabolism, and it is possible that changes in the levels of fatty acid metabolites following mitochondrial OXPHOS dysfunction affect the levels or behaviors of PPAR γ ligands. Defects in the expression or activity of endogenous PPAR γ ligands may affect adipogenesis in *Crif1* knockout mice.

Thiazolidinediones induce mitochondrial biogenesis and adipogenesis (Wilson-Fritch et al., 2003; 2004); however, these processes have not been reported to be dependent on each other. We found that rosiglitazone treatment did not promote adipogenesis in *Crif1*-deficient cells. *Crif1* null ADSCs did not differentiate into fully matured adipocytes when treated with rosiglitazone. These results suggest that the mitochondrial OXPHOS capacity of adipose tissue may determine whether cells undergo adipogenesis in response to PPAR γ agonists.

It is interesting to speculate whether the anti-diabetic effects of PPAR γ stimulation are dependent on the mitochondrial OXPHOS capacity of adipose tissue in humans. A significant number of diabetic patients do not respond adequately to treatment with thiazolidinediones. Furthermore, the anti-diabetic effects of thiazolidinediones gradually decrease when patients are treated with these drugs for a prolonged period of time. Our study suggests that the mitochondrial OXPHOS capacity of adipose tissue in diabetic patients is an important factor for determining the anti-diabetic effects of thiazolidinediones.

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