SUMO-specific Protease 1 Regulates Mitochondrial Biogenesis through PGC-1α*

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Background: It is unknown whether SUMOylation could affect the function of PGC-1α in mitochondrial biogenesis.

Results: SENP1 de-SUMOylates PGC-1α and regulates the expression of mitochondrial genes and mitochondrial biogenesis.

Conclusion: SENP1 regulates mitochondrial biogenesis and functions.

Significance: These findins help to better understand how PGC-1α SUMOylation and function are regulated.

Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) is a master regulator of mitochondrial biogenesis in response to changes in the cellular environment, physiological or pathological status of mammals. PGC-1α is known to be modified by SUMO (Small Ubiquitin-like Modifier). However, it is not known whether SUMOylation could affect the function of PGC-1α in mitochondrial biogenesis and that how PGC-1α SUMOylation is regulated. In this study, we have identified the role of Sentrin/SUMO-specific protease 1 (SENP1) as a specific SUMO protease to regulate SUMOylation status of PGC-1α. More importantly, we have also found that SENP1 promotes PGC-1α transcription activity, which is essential for the expression of mitochondrial genes and subsequently mitochondrial biogenesis. Thus, we reveal that the SUMOylation of PGC-1α controlled by SENP1 plays an important role in mitochondrial biogenesis and function.

Mitochondrion is a dynamic and highly branched organelle that undergoes changing the number and morphology according to the cell cycle, the cellular differentiation status, the metabolic activity and the energy needs (1–5). A controlled regulation of mitochondrial mass through either the biogenesis or the degradation of this organelle represents a crucial step for proper mitochondrial and cell function (1, 2, 5). Indeed, alterations of both mitochondrial mass and function are associated with a wide panel of human pathologies, ranging from neuro-muscular degenerative diseases to heart failure, unhealthy aging, and metabolic diseases (1, 4).

Peroxisome proliferator-activated receptor γ (PPARγ) coactivator 1α (PGC-1α) is a master regulator of mitochondrial biogenesis and function in many tissues (6–12). PGC-1α stimulates the expression of transcription factors such as nuclear respiratory factor 1 (NRF-1), estrogen-related receptor α (ERRα), and peroxisome proliferator-activated receptor γ (PPARγ). It also coactivates these transcriptional factors to regulate the expression of nuclear and mitochondrial genes that encode mitochondrial proteins (12–15). Loss-of-function studies show that the mutation of PGC-1α reduces mitochondrial function and oxidative capacity in a cell. However, the overexpression of PGC-1α significantly promotes mitochondrial biogenesis and cellular respiration.

PGC-1α is highly responsive to changes in the cellular environment, and physiological or pathological status of mammals. Cold exposure, exercise, or fasting can induce PGC-1α expression in brown fat tissue, muscle, or liver. The cardiac PGC-1α is induced after birth as the heart turns toward mitochondrial fatty acid oxidation as its main energy source. Besides the regulation of PGC-1α expression, PGC-1α activity has also been shown to be regulated by several signaling pathways through protein modification such as acetylation, methylation, or phosphorylation (12, 16–21). For example, the activation of PGC-1α protein can be induced by SirT1 through deacetylation and by p38 MAP kinase through phosphorylation. The inhibition of PGC-1α can occur through protein phosphorylation by AKT and CLK2 or protein acetylation by GCN5.

SUMO (also called Sentrin) is a novel ubiquitin-like protein that can covalently modify a large number of proteins (22, 23). SUMO modification has now emerged as an important regula-

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ty mechanism in many signaling pathways through alternation of the function of target proteins (22, 24, 25). SUMOylation is catalyzed by activating (E1), conjugating (E2), and ligation (E3) enzymes. It is reversed by a family of Sentrin/SUMO-specific proteases (SENPs) (22, 25). In mammalian cells, six SENPs are identified. These six SENPs have substrate specificity and different cellular localization and tissue distribution (22, 25). More and more evidence shows that the de-conjugation induced by SENPs plays a crucial role in determining the protein SUMOylation status and activity (22, 26–28).

Recently, PGC-1α has been reported as a SUMOylated protein (29). However, it has been unknown whether SUMOylation could affect the function of PGC-1α in mitochondrial biogenesis. In this study, we identified SENP1’s role as a specific SUMO protease in regulating the SUMOylation status of PGC-1α. By analyzing Senp1+/−/− cells, we found that SUMOylation greatly reduced the activation of PGC-1α in mitochondrial biogenesis and function. Thus, we revealed the essential role of SENP1 in PGC-1α transcriptional activity and also in mitochondrial biogenesis.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Antibodies**—Flag-PGC-1α and Gal4-PGC-1α were purchased from Addgene. Gal4-PGC-1αK183R mutant and Flag-PGC-1αK183R mutant were generated by using standard cloning procedures and PCR-based mutagenesis. His-SUMO1 and Flag-SENP1 were previously described. We used antibodies against His (Qiagen) and PGC-1α (Santa Cruz Biotechnology).

**Cell Culture**—The generation of Senp1+/+ and −/− MEF cells were previously described (26). MEF and 293 T cells were cultured in DMEM (HyClone) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin).

**Cell Transfection and Luciferase Assays**—293T cells in a 24-well plate were transiently transfected with expression plasmids by Lipofectamine 2000 (Invitrogen). The cells were incubated for 24 h before luciferase was assayed using Dual-Luciferase Reporter Assay System (Promega). Renilla luciferase activity was used as an internal control.

**Recombinant AAV Production and C2C12 Infection**—AAV-SENP1 and AAV-SENP1m were produced using plasmids pAOV-CMV-SENP1-EGFP or pAOV-CMV-SENP1m-EGFP by NeuronBiotech (Shanghai, P.R. China). C2C12 cells were plated in 6-well plate at about 75% confluence and infected with AAV9-SENP1 or AAV-SENP1m at a MOI of 5.

**TALON Resin Precipitation**—The His-tagged SUMO-1-conjugated proteins were precipitated with TALON resin. Cells were lysed with guanidine-HCl denaturing buffer (50 mm Na₂HPO₄, 6 M guanidine-HCl, 300 mm NaCl at pH 7.8). The cell lysate was sheared with a 29-gauge needle to break DNA and centrifuged at 100,000 × g at 15 °C for 30 min. The supernatant was incubated with TALON resin for 1 h at room temperature. The beads were washed twice with washing buffer (50 mm Na₂HPO₄, 8 M urea, 100 mm NaCl at pH 8.0) and subsequently twice with PBS. The pelleted beads were resuspended in SDS loading buffer, incubated at 55 °C for 30 min and subjected to SDS-PAGE and Western blotting.

**Real-time Quantitative PCR**—Total RNA was isolated by Trizol kit (Invitrogen). RNA was treated with DNase (Promega, Madison, WI). Complementary DNA was synthesized using the cDNA synthesis kit (Takara) according to the manufacturer’s instructions. Fluorescence real-time RT-PCR was performed with the double-stranded DNA dye SYBR Green PCR Core Reagents (PE Biosystems, Warrington, UK) using the ABI PRISM 7300 system (Perkin-Elmer, Torrance, CA). PCR was done in triplicate and standard deviations representing experimental errors were calculated. All data were analyzed by ABI PRISM SDS 2.0 software (Perkin-Elmer). Pairs of PCR primers used to amplification of the target genes were as follows: Coxvb, forward-5’-CTATGGCAAGGACACCCATCTC-3’; reverse-5’-GAAGGCGACCATCAAACAGA-3’; Ccycs, forward-5’-CTAGGATGATTTGGAG-3’; reverse-5’-CTATTAAGTCTGCCCTT TTCT-3’; Atpl5, forward-5’-CTGAGATCAAGG GCTGGTGGTGGTCGGAG-3’. Ccycs, forward-5’-CTGAGATCAAGG GCTGGTGGTGGTCGGAG-3’. Ccycs, forward-5’-CTGAGATCAAGG GCTGGTGGTGGTCGGAG-3’. Ccycs, forward-5’-CTGAGATCAAGG GCTGGTGGTGGTCGGAG-3’; Ccycs, forward-5’-CTGAGATCAAGG GCTGGTGGTGGTCGGAG-3’; Ccycs, forward-5’-CTGAGATCAAGG GCTGGTGGTGGTCGGAG-3’; Ccycs, forward-5’-CTGAGATCAAGG GCTGGTGGTGGTCGGAG-3’; Ccycs, forward-5’-CTGAGATCAAGG GCTGGTGGTGGTCGGAG-3’; Ccycs, forward-5’-CTGAGATCAAGG

![FIGURE 1. SENP1 is a specific protease for PGCG-1α de-SUMOylation.](image)

A, more accumulation of SUMOylated PGC-1α in Senp1+/+ than that in Senp1−/+ MEF cells. Senp1+/+ or −/− MEF cells were transfected with His-SUMO1 as indicated. SUMO1-conjugated PGC-1α proteins were pulled down by Talon beads from these cell lysates. Bound proteins were blotted with anti-HA antibody (top panel) or anti-Flag antibody (second panel). Cell lysate (Input) was immunoblotted (IB) with anti-HA antibody (third panel) or anti-RGS antibody (bottom panel). C, 293 cells were transfected with Flag-PGC-1α, HA-SUMO1, RGS-SENP1, or RGS-SENP1m as indicated. Flag-PGC-1α proteins were pulled down by M2 beads from these cell lysates. Bound proteins were blotted with anti-HA (top panel) or anti-Flag antibody (second panel). Cell lysate (Input) was immunoblotted (IB) with anti-HA antibody (third panel) or anti-RGS antibody (bottom panel). D, 293 cells were transfected with Gal4-luciferase and Gal4-PGC-1α WT or Gal4-PGC-1α K183R plus titrated Flag-SENP1 plasmids as indicated. The luciferase activity was measured at 24 h after transfection. The data are presented as means ± S.D. of three independent experiments.
SLEN1 Regulates Mitochondrial Biogenesis

FIGURE 2. SENP1 is essential for expression of nuclear mitochondrial genes. A and B, mRNA levels of nuclear genes that encode mitochondrial proteins (A) and transcription factors (B) in Senp1+/+ (WT) or Senp1−/− (Mut) MEF cells were measured by real-time PCR. The data are presented as means ± S.D. of three independent experiments. Differences between Senp1+/+ and Senp1−/− MEF cells were significant (p < 0.005, t test). C, mRNA levels of nuclear mitochondrial genes in Senp1+/+ (WT) or Senp1−/− MEF cells transfected with GFP (Mut-GFP), SENP1 (Mut-SENP1), or SENP1 mutant (Mut-SENP1m) were measured by real-time PCR. The data are presented as means ± S.D. of three independent experiments. Differences between Mut-GFP and Mut-SENP1, or between Mut-SENP1 and Mut-SENP1m cells were significant (p < 0.005, t test). D, mRNA levels of nuclear mitochondrial genes in Senp1+/+ (WT) or Senp1−/− MEF cells transfected with GFP (Mut-GFP), PGC-1α (Mut-PGC1α), or PGC-1α mutant (Mut-PGC1αm) were measured by real-time PCR. The data are presented as means ± S.D. of three independent experiments. Differences between Mut-GFP and Mut-PGC1α, or Mut-PGC1αm cells were significant (p < 0.005, t test). E, PGC-1α mRNA in Senp1+/+ or Senp1−/− MEF cells were measured by real-time PCR. The data are presented as means ± S.D. of three independent experiments.

TGCCAAAGACCATTTCA-3′, reverse-5′-AGCCCATCT-TGTTCTTATCC-3′; Mfn-2, forward-5′-CCTGTAAGTCTA- TGAGCGACTG-3′, reverse-5′-AATTCGCAAGAACTCGTGGGT-3′; Drp-1, forward-5′-ATTTGCCAAGGCT-3′, reverse-5′-AGCCCATCTGATCC-3′; NRF1, forward-5′-AGCAGTCATCATATAAGAATGTTC-3′; NRF2b, forward-5′-TGGGAACTTCTCCACTTCATCT-3′, reverse-5′-TGGGAACTTCTCCACTTCATCT-3′; COXI, forward-5′-CTTCTGTGACTG-3′, reverse-5′-TGGGAACTTCTCCACTTCATCT-3′; ERRα, forward-5′-CTTCACATAGCTTTTCGTCTT-3′, reverse-5′-CTTCACATAGCTTTTCGTCTT-3′; NRF1b, forward-5′-CTTCACATAGCTTTTCGTCTT-3′, reverse-5′-CTTCACATAGCTTTTCGTCTT-3′; Mfn-2, forward-5′-AGGAGGCTTCAATCGTGT-3′, reverse-5′-AGGAGGCTTCAATCGTGT-3′; PPARδ, forward-5′-AGGAGGCTTCAATCGTGT-3′, reverse-5′-AGGAGGCTTCAATCGTGT-3′.

Mitochondrial Biogenesis Analysis—Mito-Tracker Green FM (Molecular Probes) staining was performed according to manufacturer’s instructions. mDNA/nDNA ratio was determined by quantitative real-time PCR on total DNA template isolated from MEF cells. Pairs of PCR primers used to amplify the target genes were as follows: COXI (for mitochondrial DNA), forward-5′-ATGACAATAAGCCGTTCCGATGCTGTG-3′, reverse-5′-CTTCACATAGCTTTTCGTCTC-3′ and SENP1 (for nuclear DNA), forward-5′-GACATCTCTCAGGGTCTTGCCTG-3′, reverse-5′-GACATCTCTCAGGGTCTTGCCTG-3′.

Mitochondrial Function Assays—The cell oxygen consumption was determined by using XF24 Extracellular Flux Analyzer (Seahorse Bioscience) according to manufacturer’s instructions. ATP levels were measured by using ATP assay kit (Roche). For intracellular ROS production assay, the cells were stained with dichloro dihydro fluorescein diacetate (1 mM, Molecular Probes) at 37 °C for 15 min. Stained cells were analyzed immediately in a FACS (Becton Dickinson).

Statistical Analysis—Results were presented as means ± S.E. The Student’s t test was used to compare the difference between two different groups.
RESULTS AND DISCUSSION

SENP1 is a Specific De-SUMOylation Protease of PGC-1α—
Rytinki and Palvimo reported PGC-1α as a SUMOylated protein (29). Although they showed that overexpression of both SENP1 or SENP2 can de-conjugate SUMOylated PGC-1α (29), it is still important to determine which SENP really regulates the SUMOylation status of endogenous PGC-1α in physiological condition. To do so, we performed an assay by detecting whether SUMOylated PGC-1α proteins were accumulated when SENP was mutated. As it was not available for a good anti-PGC-1α antibody to IP endogenous mouse PGC-1α, we used an approach to purify SUMO1-conjugated endogenous PGC-1α by Talon beads under denaturing conditions. His-SUMO-conjugated proteins were pulled down by Talon beads from His-tagged SUMO1-transfected Senp1+/+ or Senp1−/− MEF cells, and SUMOylated PGC-1α was detected by using anti-PGC-1α antibody. As shown is Fig. 1A, SUMOylated PGC-1α proteins in Senp1−/− MEF cells were significantly more than those in Senp1+/+ cells. The accumulation of SUMOylated PGC-1α in Senp1−/− MEF cells suggests SENP1 action as a specific protease to de-conjugate SUMOylated PGC-1α. We further observed that overexpression of SENP1, not SENP1 mutant, could de-conjugate SUMOylated PGC-1α (Fig. 1B). As SUMOylation attenuates the transcription activity of PGC-1α, we speculated that SENP1 would enhance PGC-1α activity through de-SUMOylation. To test it, we determined the role of SENP1 on transcription activity of PGC-1α by using Gal4-luciferase (G4-luc) reporter system. As expected, the titrated SENP1 expression was able to increase wild type PGC-1α activity, but could not promote any more activity of PGC-1α K183R mutant (29) (Fig. 1, C and D). This suggests that the mutation of SUMOylation site can saturate PGC-1α activity. All these results reveal that SENP1 is the specific de-SUMOylation protease, which promotes PGC-1α activity by regulating the SUMOylation status of PGC-1α.

SENP1 Is Essential for the Expression of Nuclear Mitochondrial Genes—PGC-1α is a master regulator for mitochondrial biogenesis and function (5, 12). It has been shown that PGC-1α is not only a co-activator for transcriptional factors NRF, ERR, and PPAR, which control the transcription of nuclear mitochondrial genes, but also a transcription factor to regulate the expression of these transcription factors such as NRF and ERR (5, 12). Therefore, we speculated that SENP1 would be a regulator for the expressions of these genes through targeting PGC-1α. We first measured the mRNA level of some nuclear mitochondrial genes in Senp1+/+ and Senp1−/− MEF cells by using real-time PCR. As shown in Fig. 2A, mutation of SENP1 remarkably reduced the mRNA levels of these tested genes. We also observed the reduction of NRF and ERRa andb PPAR, in Senp1−/− MEF cells in comparison with that in Senp1+/+ cells (Fig. 2B), which would contribute to the down-regulation of mitochondrial genes.

To test whether the mutation of SENP1 is responsible for the reduction of the expression of these genes, we transfected wild type SENP1 plasmid into Senp1−/− MEF cells to determine whether SENP1 could rescue the phenotypes in Senp1−/− MEF cells. As shown in Fig. 2C, the expression of SENP1 in Senp1−/− MEF cells almost restored the mRNA expressions of mitochondrial genes to the level of wild type cells. More interestingly, the catalytic domain mutant of SENP1 did not show effects in the same system, suggesting that SENP1 and its de-SUMOylation activity are essential for the expressions of mitochondrial genes. We further investigated whether SENP1-controlled expression of mitochondrial genes was dependent on PGC-1α SUMOylation. To test that, PGC-1α wild type or PGC-1α SUMOylation mutant (29) plasmids was transfected into Senp1−/− MEF cells. This resulted in an increase of mitochondrial genes in Senp1−/− cells. More interestingly, PGC-1α mutant showed greater ability to recover the expression of mitochondrial genes than PGC-1α wild type did in Senp1−/− cells (Fig. 2D). We also observed there was no reduction of PGC-1α expression in Senp1−/− cells (Fig. 2E). These results suggest that SENP1 regulates the expression of mitochondrial genes mostly through de-SUMOylation of PGC-1α.

Overexpression of SENP1 Promotes the Expression of Mitochondrial Genes—To further determine SENP1 as a positive regulator in the expression of mitochondrial genes, we expressed exogenous SENP1 into C2C12 cells by infection of AAV-SENP1. As shown in Fig. 3, the expression of exogenous SENP1 significantly increased the expression of mitochondrial genes. However, the SENP1 mutant had no effects on the expression of these genes. These results suggest that SENP1 can promote the expression of mitochondrial genes through de-SUMOylation.

Deficiency of Mitochondrial Biogenesis in Senp1−/− Cells—
The above results revealed the crucial role of SENP1 in the expression of nuclear mitochondrial genes that encode mitochondrial proteins. Thus we speculated that SENP1 would regulate the mitochondrial biogenesis. To test it, we first compared the mitochondria mass in Senp1−/− with that in Senp1+/+.
MEF cells by using Mito-Tracker staining. As shown in Fig. 4A, Senp1−/− MEF cell had much less mitochondria than Senp1+/+ cells. This observation was confirmed by further analysis of mtDNA copies. It showed that the ratio of mtDNA/nDNA in Senp1−/− cells was lower than that in Senp1+/+ cells (Fig. 4B). Morphological analysis by electron microscopy further demonstrated smaller mitochondria and less cristae inside mitochondria in Senp1−/− MEF cells in comparison with that of Senp1+/+ cells (Fig. 4C). These results suggest that mutation of SENP1, like the mutation of PGC-1α, can cause defects in mitochondrial biogenesis.

**SENP1 Regulates Mitochondrial Functions**—Since SENP1 plays an important role in mitochondrial biogenesis, we should expect that SENP1 would be essential for mitochondrial function too. To prove it, oxygen consumption, a mitochondrial respiration marker, was analyzed in Senp1+/+ and Senp1−/− MEF cells. As shown in Fig. 5A, intact Senp1+/+ MEF cells showed normal response to Oligomycin (an inhibitor of ATP synthase), FCCP (a mitochondrial protonophore), or Rotenone (a mitochondrial NADH dehydrogenase inhibitor) treatment associated with the decrease or increase of mitochondrial oxygen consumption. On the other hand, in Senp1−/− MEF cells, not only the responses to these treatments were very mild, but also the basal mitochondrial oxygen consumption and FCCP-induced mitochondrial oxidative capacity remarkably decreased in Senp1−/− cells, indicating there was mitochondrial dysfunction in Senp1−/− cells. In the meantime, a lower levels of ATP (Fig. 5B) and ROS production (Fig. 5C) were observed in Senp1−/− MEF cells in normoxia in comparison with the wild

**FIGURE 4.** The deficiency of mitochondrial biogenesis in Senp1−/− cells. A, Mito-Tracker Green FM (Molecular Probes) staining shows decrease in mitochondrial mass in Senp1−/− MEF cells. B, mtDNA/nDNA ratio was determined by quantitative real-time PCR of Cox1 gene (represent mitochondrial DNA) and Senp1 gene (represent nuclear DNA) on total DNA template isolated from MEF cells. The data are presented as means ± S.D. of three independent experiments (p < 0.05, t test). C, morphological analysis by electron microscopy shows small mitochondria and less cristae inside of mitochondria in Senp1−/− MEF cells as compared with that of Senp1+/+ cells.

**FIGURE 5.** The dysfunction of mitochondria in Senp1−/− cells. A, cell oxygen consumption was determine in Senp1+/+ or Senp1−/− MEF cells after treatment of DMSO, Oligomycin, FCCP, or Rotenone. B, ATP levels were measured in Senp1+/+ and Senp1−/− MEF cells. The data are presented as means ± S.D. of three independent experiments (p < 0.005, t test). C, intracellular ROS production was analyzed in Senp1+/+ and Senp1−/− MEF cells. D, ATP level was measured in the same Senp1+/+ or Senp1−/− MEF cells as shown in Fig. 2D. E, the ATP level was measured in the same C2C12 cells shown in Fig. 3.
type as the result of decreased mitochondrial oxygen consumption and oxidative phosphorylation.

To test whether SENP1-controlled mitochondrial function was dependent on PGC-1α SUMOylation, we transfected PGC-1α wild type or PGC-1α SUMOylation site mutant (29) plasmids into Senp1−/− MEF cells. As shown in Fig. 5D, PGC-1α SUMOylation site mutant could recover the ATP production more than PGC-1α wild type did in Senp1−/− MEF cells. We also determined the ATP production in exogenous SENP1-expressed C2C12 cells and showed that overexpression of SENP1 increased more ATP production than control or SENP1 mutant-expressed cells (Fig. 5E). These data reveal the crucial role of SENP1 in mitochondrial function.

In summary, we have identified SENP1 as a regulator of PGC-1α activity through de-SUMOylation. By testing in Senp1−/− MEF cells and SENP1 overexpressed C2C12 cells, we have found that SENP1 is essential for the expression of nuclear mitochondrial genes through the de-SUMOylation of PGC-1α. More importantly, we have also demonstrated that SENP1 is involved in the regulation of mitochondrial biogenesis and its function.

Our results reveal that PGC-1α is a major target regulated by SENP1 in regulation of the expression of mitochondrial genes (Fig. 2D). However, we still could not exclude other SENP1 targets involved in this process. For example, we detected the reduction of expression of PGC-1β, a high homologous and related protein of PGC-1α, in addition to the accumulation of SUMOylated PGC-1α in Senp1−/− MEF cells. PGC-1β is shown to be able of activating the expression of mitochondrial genes (30–33). Thus PGC-1β may also contribute to the regulation of SENP1 in mitochondrial biogenesis, although we do not know how SENP1 regulates PGC-1β expression. Zunino et al. have shown that SENP5 can also modulate mitochondrial morphology and function (34, 35). Different from SENP1, SENP5 is located in the cytosolic pool and it modulates mitochondrial fission during mitosis through de-SUMOylating the dynamin-related fission GTPase DRP1(34, 35).

Since the change in mitochondrial mass is crucial for cell adaptation under various conditions, and that the altered mitochondrial biogenesis leads to dysfunction and development of pathologies, to decipher regulation signaling during mitochondrial biogenesis will not only help us to better understand the development of such pathologies but also help us to discover potential targets for therapy.

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