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Mitochondrial intermediate peptidase is a novel regulator of sirtuin-3 activation by caloric restriction

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Sirtuin-3 (SIRT3) regulates mitochondrial quality and is involved in the anti-ageing and pro-longevity actions of caloric restriction (CR). Here, we show that CR upregulates the mature form of SIRT3 and mitochondrial intermediate peptidase (MIPEP), a mitochondrial signal peptidase (MtSPase), in white adipose tissue. We also demonstrate that upregulation of mature SIRT3 is dependent on MIPEP in 3T3-L1 cells, suggesting that MIPEP may contribute to the maintenance of mitochondrial quality during CR *via* activation of SIRT3. This novel mechanism of SIRT3 activation through MIPEP facilitates the elucidation of additional molecular pathways of CR.

Keywords: caloric restriction; mitochondrial intermediate peptidase; sirtuin-3

Caloric restriction (CR) extends lifespan and suppresses age-associated pathophysiology in various animal models, but the molecular mechanisms underpinning these features remain debatable. We recently reported that CR activates mitochondrial biogenesis *via* sterol regulatory element-binding protein 1c (Srebp-1c) in white adipose tissue (WAT), but not other tissues, suggesting specific activation in WAT may play a pivotal role in the beneficial effects of CR [1].

Sirtuins (SIRTs), classified to include seven mammalian homologs of the yeast *SIR2* gene, mediate the effects of CR on life span extension in yeast and *Caenorhabditis elegans*. SIRTs play an important role in adaptive responses to various stresses, including CR and metabolic stress [2]. SIRT3, a member of the SIRT family that localizes within the mitochondrial matrix, regulates mitochondrial dynamics and protein quality control by modulating acetylation levels of metabolic enzymes [3]. Indeed, SIRT3-dependent mitochondrial adaptations may be a central mechanism of the beneficial actions of CR [4]. Thus, to assess the relationship between CR and mitochondrial function, we investigated detailed changes of SIRT3 expression in mice WAT under CR conditions.

Abbreviations
AL, *ad libitum*; COX4, cytochrome *c* oxidase subunit 4; CR, caloric restriction; MDH2, malate dehydrogenase 2; MIPEP, mitochondrial intermediate peptidase; MnSOD, manganese superoxide dismutase; MPP, mitochondrial processing peptidase; MtSPase, mitochondrial signal peptidase; SIRT, sirtuin; Srebp-1c, sterol regulatory element-binding protein 1c; WAT, white adipose tissue.

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Materials and methods

Animals and diet

Animal experiments were approved by the Ethics Review Committee for Animal Experimentation at Tokyo University of Science or the Tsukuba University Animal Care and Use Committee.

Male mice (B6/129S6 background) were maintained under specific-pathogen free (SPF) conditions at 23 °C with 12-h light/dark cycles in the Laboratory Animal Center at the Faculty of Pharmaceutical Sciences, Tokyo University of Science, and provided with water and food *ad libitum* (AL) with a CR-LPF diet (Oriental Yeast, Tokyo, Japan). From 3 months of age, mice were divided into two groups: one fed AL and the other CR (70% of AL energy intake). At 8 months of age, all mice were killed under anaesthesia with isoflurane inhalation (Mylan, Canonsburg, PA, USA). CR and AL groups were further divided into two treatments (fast or fed) as follows: half of CR mice were fasted overnight for approximately 20 h prior to killing, while the other half were killed without removing food; similar to CR mice, half of AL mice were killed 20 h after the removal of food (which occurred when lights were turned off), while the other half were killed without removing food. After mice were killed, epididymal WAT was collected. A subsample of isolated WAT was immediately diced, frozen in liquid nitrogen, and stored at −80 °C. In addition, male *Sirt3* wild-type (+/+) and deficient (−/−) mice were purchased from Jackson Laboratory (Bar Harbor, ME, USA) and maintained under SPF conditions in the Laboratory Animal Resource Center, University of Tsukuba. At 15 weeks of age, all mice were euthanized by decapitation for harvesting of epididymal WAT as described above.

Cells and reagents

3T3-L1 cells purchased from RIKEN Bioresource Center (Ibaraki, Japan) were maintained in low-glucose Dulbecco's modified Eagle medium (Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (Bovogen Biologicals, Essendon, Vic., Australia) and 1% penicillin/streptomycin (Sigma-Aldrich, St. Louis, MO, USA). MG132 was purchased from Wako.

RNA purification and reverse transcription–polymerase chain reaction (RT-PCR)

RNA purification and RT-PCR were performed as previously reported by our laboratory [5]. In brief, total RNA was extracted from cells using RNAiso PLUS (Takara, Shiga, Japan) and purified with a FastPure RNA kit (Takara) according to the manufacturer's protocol. Purified RNA was subjected to reverse transcription with PrimeScript Reverse Transcriptase (Takara) and random hexamers (Takara). Quantitative RT-PCR was performed using an Applied Biosystems 7300 real-time PCR system (Foster City, CA, USA) and SYBR Premix Ex Taq II (Takara) according to the manufacturer's protocol. Sequences of primers used for RT-PCR were as follows: *Mipep* (Forward: 5′-CAA AGG AGA GGT GTG GTG TAA TG-3′, Reverse: 5′-GGA AGA TTC AGC ATG AGA ACG AC-3′), *Cox4* (Forward: 5′-CAT TTC TAC TTC GGT GTG CCT TC-3′, Reverse: 5′-CAC ATC AGG CAA GGG GTA GTC-3′), *Mdh2* (Forward: 5′-AGG TTG ACT TTC CCC AAG ACC-3′, Reverse: 5′-CAT AAG CCA TGG ACA GAG TGG-3′), *Sirt3* (Forward: 5′-CGT TGT GAA ACC CGA CAT TG-3′, Reverse: 5′-TCC CCT AGC TGG ACC ACA TC-3′), *Pmpca* (Forward: 5′-AGT TTG GAC AAT TCT GTA CCG TAG G-3′, Reverse: 5′-TCG TCT TTG CTG TCA AAT CGA G-3′), *Pmpcb* (Forward: 5′-AAA ACT CTG GGC TCT CAA CGT G-3′, Reverse: 5′-ACA GTC TGC TCT CTG GAG GTA TAG G-3′) and *Tbp* (Forward: 5′-CAG TAC AGC AAT CAA CAT CTC AGC-3′, Reverse: 5′-CAA GTT TAC AGC CAA GAT TCA CG-3′).

Protein extraction and immunoblotting

Tissues and cells were lysed with lysis buffer (50 mM Tris/HCl, pH 6.8, 2% sodium dodecyl sulphate and 5% glycerol) and boiled for 5 min and sonicated. Protein concentrations within soluble fractions were determined using a Pierce BCA Protein Assay Kit (Thermo Scientific, Waltham, MA, USA). Extracted protein samples were standardized by the addition of lysis buffer, 2-mercaptoethanol and bromophenol blue to obtain final concentrations of 5% and 0.025%, respectively, before boiling samples for 5 min. Fifteen micrograms of each protein sample was subjected to SDS/PAGE with 12% (high-density) or 8% acrylamide gels before transfer to nitrocellulose membranes. Membranes were blocked with 2.5% skim milk and 0.25% bovine serum albumin in Tris-buffered saline (50 mM Tris, pH 7.4 and 150 mM NaCl) containing 0.1% Tween 20 (TTBS) for 60 min at room temperature, and then probed with appropriate primary antibodies overnight at 4 °C. Primary antibodies against SIRT3 (Cell Signaling Technology (CST), Beverly, MA, USA), MIPEP (Santa Cruz Biotechnology, San Francisco, CA, USA), COX4 (CST), MDH2 (CST), manganese superoxide dismutase (MnSOD) (CST), MnSOD (acetyl K122) (Abcam, Cambridge, UK), FLAG (Sigma), β-ACTIN (Sigma), α-TUBULIN (Sigma) and GAPDH (Santa Cruz) were used. After several washes with TTBS, membranes were incubated with an appropriate secondary antibody (horseradish peroxidase-conjugated F(ab′)2 fragment of goat anti-mouse IgG or anti-rabbit IgG; Jackson ImmunoResearch, West Grove, PA, USA) for 60 min at room temperature. After washing with TTBS, membranes were incubated with ImmunoStar® LD reagent

(Wako). Specific proteins were visualized with LAS3000 (Fujifilm, Tokyo, Japan), and data were analysed using MULTIGAUGE software (Fujifilm).

Vector construction

A retroviral vector expressing FLAG-Sirt3 was constructed by excising cDNA encoding FLAG-mouse Sirt3 from mSIRT3-L (Addgene plasmid 33309) and cloning this fragment into *Eco*RI- and *Apa*I-digested pMXs-AMNN-puro, the details of which were published in our previous study [1].

The pMXs-puro-mU6 vector was constructed as previously reported [5]. A mouse shRNA against the *Mipep* (sh*Mipep*) expression vector was designed using siDirect 2.0 (http://siDirect2.RNAi.jp/). Sequences of oligonucleotides for sh*Mipep* and shGFP (used as a negative control) were as follows: sh*Mipep* (5′-GTG ATG AGA AGT TTA TGG GGT TTC AAG AGA ACT CCA TAA GCT TCT TAT CAC TTT TT-3′) and shGFP (5′-GGC TAT GTC CAG GGG CGC ATC TTC AAG AGA GGT GCG CTC CTG GAC GTA GCC TTT TT-3′).

FLAG-Sirt3 expression and Mipep knockdown in 3T3-L1 cells

Stable FLAG-Sirt3 – expressing and *Mipep*-knockdown cell lines were generated using retroviral infection, as previously reported [5]. The produced vectors (pMXs-AMNN-puro-*Sirt3*, pMXs-puro-mU6-sh*Mipep* or shGFP) were transfected into Plat-E cells with FuGENE 6 (Promega, Madison, WI, USA), according to the manufacturer's protocol. Virus-containing culture supernatants were collected 2 days after transfection and filtered through 0.22-μm filters (Millipore, Billerica, MA, USA). To obtain stable FLAG-Sirt3 – expressing, *Mipep*- or GFP-knockdown cell lines 3T3-L1 cells were incubated with virus-containing medium for 2 days, followed by selection with 2 $\mu g \cdot mL^{-1}$ of puromycin (Sigma) for 5 days.

Statistical analysis

Data are presented as mean ± standard error of the mean (SEM). Statistical analyses were performed using Student's *t*-test or Tukey's test. *P*-values < 0.05 were considered statistically significant.

Results and Discussion

CR upregulates the mature form of SIRT3 protein in WAT

Initially, we examined SIRT3 protein expression levels in the WAT of CR mice. Immunoblotting with an anti-SIRT3 antibody yielded two bands (approximately 37 and 28 kDa, as estimated by molecular weight markers). Interestingly, the lower band was upregulated in CR groups, while, in contrast, the upper band was reduced in these animals (Fig. 1A). To verify the identity of these bands to be SIRT3 proteins, we performed immunoblotting of WAT from

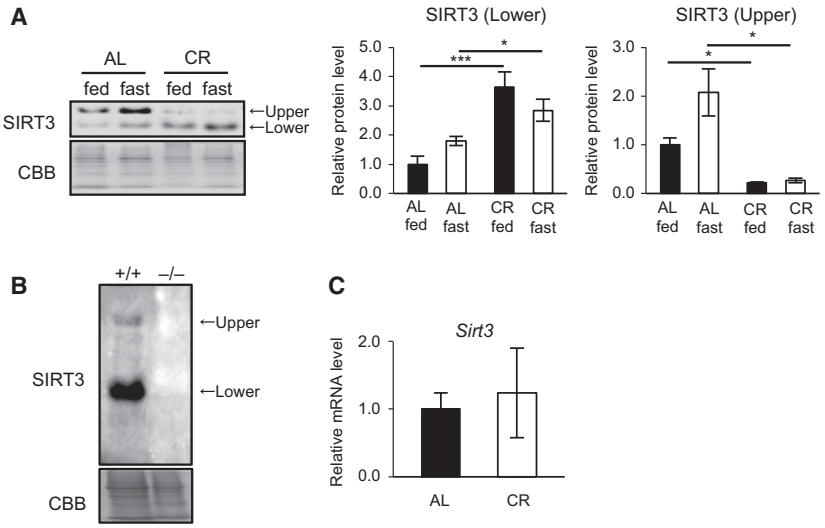


Fig. 1. CR upregulated the mature form of SIRT3 in mice WAT. (A) Protein levels of SIRT3 from the fed and fasted group of both *ad libitum* (AL) and CR mice were obtained by immunoblotting. The left panel is the representative image. The right graphs are quantitative data. (B) SIRT3 protein levels of *Sirt3* wild-type (+/+) and deficient (–/–) mice were obtained by immunoblotting with high-density gels. (C) mRNA levels of *Sirt3* in AL and CR mice WAT were obtained by real-time RT-PCR. Coomassie brilliant blue (CBB) staining was used as a loading control in immunoblotting. *Tbp* was used as an internal control in real-time RT-PCR. Values shown in all panels are means ± SEM ($n = 3$–4 per group). $*P < 0.05$, $***P < 0.001$, analysed by Student's *t*-test or Tukey's test.

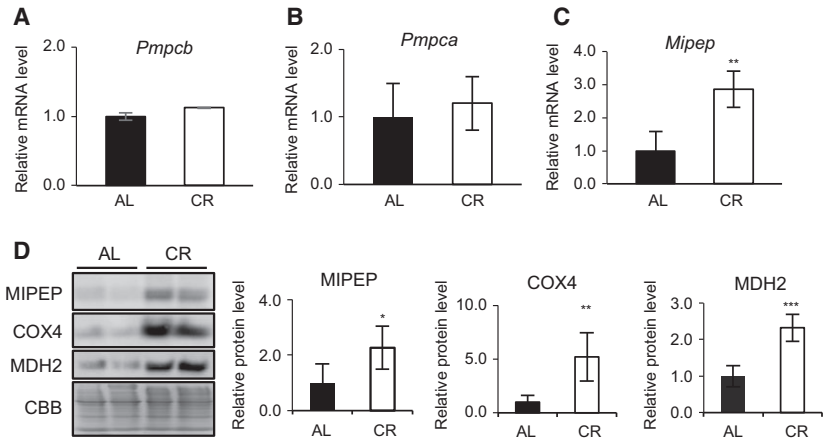


Fig. 2. CR upregulated MIPEP, one of MtSPases in mice WAT. (A–C) mRNA levels of the MtSPases, *Pmpcb* (A), *Pmpca* (B) and *Mipep* (C) in AL and CR mice were obtained by real-time RT-PCR. (D) Protein levels of MIPEP, COX4 and MDH2 in WAT from AL and CR mice were obtained by immunoblotting. *Tbp* was used as an internal control in real-time RT-PCR. Coomassie brilliant blue (CBB) staining was used as a loading control in immunoblotting. Values shown in all panels are means ± SEM (n = 3–4 per group). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, analysed by Student's t-test.

Sirt3-deficient mice using high-density gels. Consequently, no bands were detected around these molecular weights in the WAT of *Sirt3*-deficient mice (Fig. 1B), confirming that both bands were SIRT3 proteins. Notably, we did not observe an elevation of *Sirt3* mRNA in the WAT of CR mice (Fig. 1C), although CR has been reported to upregulate *Sirt3* mRNA expression in WAT [3]. The 28-kDa protein has previously been reported to be the processed product, or activated mature form, of SIRT3 [6]. Therefore, we hypothesized that CR may induce the processing of SIRT3 protein, leading to its activation.

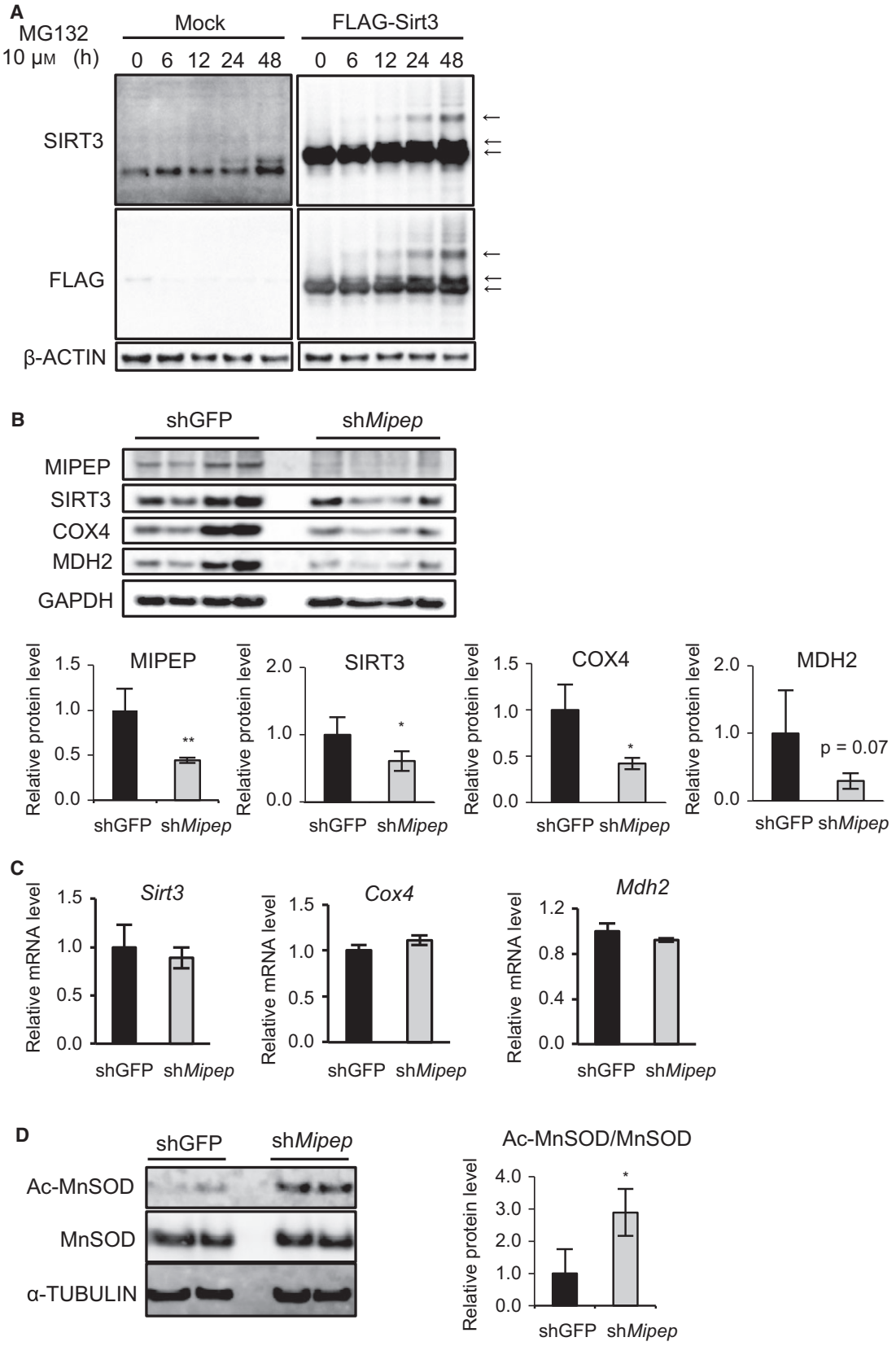
CR upregulates MIPEP in WAT

More than 99% of mitochondrial proteins are synthesized as precursor forms, the majority of which possess an N-terminal signal sequence for mitochondrial targeting. Signal sequences of mitochondrial proteins are proteolytically cleaved and activated by specific mitochondrial signal peptidases (MtSPases) present within mitochondria [7]. A previous report showed that SIRT3 is cleaved by mitochondrial processing peptidase (MPP), a MtSPase [6]. Therefore, we examined mRNA levels of *Pmpca*, which encodes MPPα (recognition unit), and *Pmpcb*, which encodes MPPβ (enzyme unit), in the WAT of CR mice. However, CR did not alter mRNA levels of either *Pmpca* or *Pmpcb* (Fig. 2A,B). To identify the MtSpase upregulated by CR, we reanalysed DNA microarray data from the WAT of CR rats (Gene Expression Omnibus, accession code: GSE30668) [8]. Consequently, we found that CR upregulated mitochondrial intermediate peptidase (MIPEP), a finding that was verified by real-time RT-PCR in the WAT of mice (Fig. 2C). Both cytochrome *c* oxidase subunit 4 (COX4) and malate dehydrogenase 2 (MDH2) are reportedly processed by MIPEP [9]. Indeed, CR upregulated the expression level of MIPEP, COX4 and MDH2 (Fig. 2D), indicating that CR may activate the processing of SIRT3 proteins through upregulation of MIPEP.

MIPEP contributes to SIRT3 activation in adipocytes

MIPEP is known to cleave proteins processed by MPP, namely, contributing to the second of two successive cleavage steps [10]. If SIRT3 protein is cleaved

Fig. 3. Maturation of SIRT3 was dependent on MIPEP. (A) Protein levels of SIRT3 in mock or FLAG-Sirt3 expressing 3T3-L1 cells were obtained by immunoblotting with high-density gel (15%). Cells were treated with or without 10 μM MG132 for 6, 12, 24 and 48 h. β-ACTIN was used as a loading control. (B) Protein levels of MIPEP, SIRT3, COX4 and MDH2 in sh*Mipep* 3T3-L1 cells were obtained by immunoblotting. GAPDH was used as a loading control. The lower panel is the quantitative data. (C) mRNA levels of *Sirt3*, *Cox4* and *Mdh2* in sh*Mipep* 3T3-L1 cells were obtained by real-time RT-PCR. *Rps18* was used as an internal control. (D) Expression levels of Ac-MnSOD (K122 acetylated MnSOD) and total MnSOD proteins in sh*Mipep* 3T3-L1 cells were obtained by immunoblotting. α-TUBULIN was used as a loading control. Values shown in all panels are means ± SEM (n = 4–6). *$P < 0.05$, **$P < 0.01$, analysed by Student's t-test.


A
MG132
10 μM (h)
Mock
FLAG-Sirt3
0 6 12 24 48
0 6 12 24 48
SIRT3
FLAG
β-ACTIN
B
shGFP
shMipep
MIPEP
SIRT3
COX4
MDH2
GAPDH
Relative protein level
MIPEP
SIRT3
COX4
MDH2
p = 0.07
shGFP shMipep
C
Relative mRNA level
Sirt3
Cox4
Mdh2
shGFP shMipep
D
shGFP
shMipep
Ac-MnSOD
MnSOD
α-TUBULIN
Ac-MnSOD/MnSOD
Relative protein level
shGFP shMipep

in such processing steps, immunoblotting for SIRT3 should display at least three different molecular weight forms: precursor, intermediate and mature. However, only two forms, presumed to be the precursor (37 kDa) and mature (28 kDa) forms, were found in mice WAT (Fig. 1A,B). One probable reason for this is that mitochondrial proteins (after initial processing by MPP) may be subject to degradation [9,11]. Therefore, to facilitate the detection of SIRT3 intermediates, and further precursor forms, we treated 3T3-L1 preadipocytes expressing exogenous FLAG-Sirt3 with the proteasome inhibitor MG132. This treatment permitted the observation of three bands, predicted to be precursor, intermediate, and mature forms of SIRT3 present in these cells (Fig. 3A). Moreover, the same band pattern was detected using an anti-FLAG antibody (Fig. 3A). Collectively, these results support the processing of SIRT3 protein by two successive steps.

To evaluate whether MIPEP contributes to SIRT3 activation, we performed knockdown of *Mipep* in 3T3-L1 cells and verified a decrease of mature SIRT3 *in vitro*. We then examined expression of the mature form of SIRT3 in 3T3-L1 cells expressing shRNA against *Mipep*. *Mipep* knockdown diminished COX4 and MDH2 proteins in addition to the mature form of SIRT3 (Fig. 3B), whereas mRNA expression was unaltered (Fig. 3C). Moreover, *Mipep* knockdown increased the acetylation level of K122 on MnSOD, a substrate of SIRT3 [12], reflecting downregulation of SIRT3 deacetylase activity (Fig. 3D). These results suggested that MIPEP positively regulated expression of the mature form of SIRT3, most likely by mediating the processing of an intermediate form.

Conclusions

Herein, we demonstrated that CR increased the mature form of SIRT3 in WAT, likely in a MIPEP-dependent manner, thus presenting the possibility that SIRT3 proteins are processed in two successive steps. Taken together, the induction of MIPEP during CR may enhance the processing of intermediate SIRT3 into its mature form. Given these findings, we propose a novel activation mechanism of SIRT3 by MIPEP, supporting an important role for MIPEP in the maintenance of mitochondrial quality during CR *via* SIRT3 activation.

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Author contributions

YH conceived the idea, designed experiments and wrote the manuscript. KT performed experiments and wrote the manuscript under the leadership of MK, TN, NO, YS, HT, and YM supported the experiments. YN and HS supported the experiments and contributed to the sampling of Sirt3-deficient mice WAT. KN assisted with the experiments.

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