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Localization of mouse mitochondrial SIRT proteins: Shift of SIRT3 to nucleus by co-expression with SIRT5

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Abstract

Yeast silent information regulator 2 (SIR2) is involved in extension of yeast longevity by calorie restriction, and SIRT3, SIRT4, and SIRT5 are mammalian homologs of SIR2 localized in mitochondria. We have investigated the localization of these three SIRT proteins of mouse. SIRT3, SIRT4, and SIRT5 proteins were localized in different compartments of the mitochondria. When SIRT3 and SIRT5 were co-expressed in the cell, localization of SIRT3 protein changed from mitochondria to nucleus. These results suggest that the SIRT3, SIRT4, and SIRT5 proteins exert distinct functions in mitochondria. In addition, the SIRT3 protein might function in nucleus.

Keywords: SIRT3; SIRT4; SIRT5; Intramitochondrial localization; Localization shift

Silent information regulator 2 (SIR2) is the enzyme that catalyzes NAD^+-dependent protein deacetylation and produces nicotinamide and *O*-acetyl-ADP-ribose [1,2], and is localized in nucleus [3]. In yeast, lifespan is prolonged in low glucose condition [4,5], but such lifespan extension is abolished by SIR2 gene disruption [6], suggesting that SIR2 plays an important role in determining yeast longevity. In mammals, there are seven SIR2 homologs, SIRT1–7 [7]. However, it remains unclear whether SIRTs mediate lifespan extension by calorie restriction.

Human SIRT3, SIRT4, and SIRT5 proteins are known to be localized in mitochondria [7,8]. Mouse SIRT4 was previously shown *in vivo* to ADP-ribosylate glutamate dehydrogenase and down-regulate its activity in pancreatic islets, inhibiting amino acid-stimulated insulin secretion [9]. On the other hand, recent studies have demonstrated that human SIRT3 deacetylates acetyl-CoA synthetase 2 in mitochondrial matrix *in vitro* [10,11]. Human SIRT5 has been shown to have weak deacetylase activity *in vitro* [12]. However, the function of SIRT3 and SIRT5 *in vivo* is still unknown.

Although human SIRT4 and SIRT5 proteins have been reported to localize in mitochondria, precise localization of these proteins is unknown. Regarding SIRT3, localization of SIRT3 protein has been reported to differ in mouse and human, in mitochondrial inner membrane in mouse [13] and in mitochondrial matrix in human [14]. Furthermore, there has been no report on the interaction of the three SIRT proteins known to be localized in mitochondria.

In the present study, we determined localization of mouse SIRT3, SIRT4, and SIRT5 proteins in COS7 cells in different compartments of mitochondria: inner membrane, matrix, and intermembrane space. In addition, we demonstrate that localization of SIRT3

Abbreviations: SIR2, silent information regulator 2; MTS, mitochondrial targeting signal; NLS, nuclear localization signal; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PNS, post-nuclear supernatant; PHB, prohibitin; ER, estrogen receptor.

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protein in COS7 cells changes from mitochondria to nucleus when co-expressed with SIRT5. We also address the regulatory mechanism of SIRT3 localization by a mutagenesis study of the putative mitochondrial targeting signal (MTS) and nuclear localization signal (NLS).

Materials and methods

Antibodies. The antibodies used for confocal microscopic analysis and Western blot analysis included anti-myc (Santa Cruz Biotechnology), anti-FLAG (Sigma), anti-hsp60 (BD Biosciences), anti-calnexin (Stressgen), anti-glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (Santa Cruz Biotechnology), anti-cytochrome *c* (Cell Signaling), and anti-laminA/C (Cell Signaling).

Plasmid construction. The expression vector for SIRT3-myc, SIRT4-myc, SIRT4-FLAG, and SIRT5-FLAG was constructed as follows. The coding region of SIRT cDNAs was cloned by PCR using mouse liver cDNA. The PCR fragments were subcloned into the pcDNA3.1/myc-His A expression vector (Invitrogen) or the pFLAG-CMV-5a expression vector (Sigma). SIRT3nu and SIRT3mt mutants were constructed by overlap extension PCR method [15] using pcDNA3.1/myc-His-SIRT3 plasmid as the template.

Cell culture and transfection. COS7 cells were maintained in Dulbecco's modified Eagle's medium (Sigma) supplemented with 10% fetal bovine serum (Gibco) in an atmosphere of 5% CO_2 at 37 °C. DNA transfection was performed according to the manufacturer's instructions using FuGene6 Transfection Reagent (Roche). One microgram of plasmid DNA was transfected to COS7 cells in a 3.5-cm dish.

Confocal microscopy. Fluorescence microscopic analysis was performed as described previously [16]. Briefly, COS7 cells transfected with SIRT expression plasmids were fixed and labeled with anti-myc monoclonal IgG and Alexa488-conjugated anti-mouse IgG (Molecular Probes) or anti-FLAG polyclonal IgG and Cy3-conjugated anti-rabbit IgG (Sigma). Fluorescent images were taken and analyzed using a confocal laser microscope (LSM510 META; Carl Zeiss).

Fractionation of post-nuclear supernatant. Fractionation of post-nuclear supernatant was performed as described previously [17]. Twenty micrograms of plasmid DNA was transfected to COS7 cells in a 10-cm dish. The transfected cells were harvested and disrupted in isotonic buffer (PBS containing 0.2 M mannitol, 0.07 M sucrose, and 1 mM EDTA) containing protease inhibitors (Complete, EDTA Free; Roche) with potter homogenizer, followed by centrifuged at 800*g* at 4 °C for 10 min to obtain post-nuclear supernatant (PNS). PNS was centrifuged at 10,000*g* at 4 °C for 10 min to obtain the mitochondria-enriched precipitate fraction. The supernatant was centrifuged at 100,000*g* at 4 °C for 30 min to separate the microsome-enriched precipitate and supernatant fractions. The subcellular fractions were separated by SDS–PAGE and then analyzed by Western blotting.

Alkaline treatment of mitochondria. Mitochondria were prepared from the COS7 cells expressing each SIRT protein, and treated with 100 mM Na_2CO_3 in 10 times volume of mitochondria suspension for 1 h on ice. The reaction mixtures were centrifuged at 100,000*g* at 4 °C for 30 min to separate the precipitate and supernatant fractions. The fractions were subjected to SDS–PAGE followed by Western blot analysis.

Submitochondrial fractionation. The mitochondria were treated with either H_2O or 2% TX-100 in 10 times volume of mitochondria suspension on ice for 1 h, and then treated with 50 μg/ml trypsin on ice for 1 h. The reaction mixtures were separated by SDS–PAGE and then analyzed by Western blotting.

Subcellular fractionation using digitonin. The transfected COS7 cells were harvested and lysed with PBS containing 2% digitonin. The cell lysate was centrifuged at 800*g* at 4 °C for 10 min to obtain nucleus-enriched insoluble and soluble fractions. The fractions were separated by SDS–PAGE and then analyzed by Western blotting.

Results and discussion

Distinct localizations of SIRT3, SIRT4, and SIRT5 in mitochondria

To determine the intracellular localization of mouse SIRT3, SIRT4, and SIRT5 proteins, expression plasmid encoding each of these SIRT proteins fused with myc tag or FLAG tag at the C terminus was transfected into COS7 cells. The SIRT proteins were stained with anti-myc antibody or anti-FLAG antibody and its intracellular localization was examined using confocal microscopy (Fig. 1A). All three images of SIRT3, SIRT4, and SIRT5 proteins merged well with that of MitoTracker Red, a marker of mitochondria, indicating that all of these SIRT proteins are localized in mitochondria. Cell fractionation was then performed using cells transfected with the SIRT expression plasmids. PNS of the cells was centrifuged and fractionated into the mitochondria-enriched low-speed precipitate (P1), the microsome-enriched high-speed precipitate (P2), and the supernatant (S) fractions (Fig. 1B). All of the three SIRT proteins were found in the P1 fraction as was hsp60 protein, a marker of mitochondria, affirming their localization in mitochondria.

To clarify localization of the three SIRT proteins in mitochondria, mitochondrial fraction prepared from COS7 cells expressing each of the SIRT proteins was treated with Na_2CO_3 and centrifuged. SIRT3 protein was detected in the precipitate fraction, while SIRT4 and SIRT5 proteins were detected in the supernatant fraction, indicating that SIRT3 protein is integrated into either mitochondrial outer or inner membrane and that SIRT4 and SIRT5 are soluble and not membrane proteins (Fig. 1C). After treating the mitochondrial fractions with either H_2O or TX-100, the fractions were treated with trypsin. When mitochondria are treated with H_2O, the mitoplast can be obtained. SIRT3 and SIRT5 proteins were digested with trypsin in both H_2O- and TX-100-treated mitochondria but were not digested in untreated mitochondria (Fig. 1D), indicating that these proteins are localized either in intermembrane space or in inner membrane. In contrast, SIRT4 was digested only in the TX-100-treated mitochondria. Taken together, these results indicate that SIRT3, SIRT4, and SIRT5 proteins are localized in inner membrane, matrix, and intermembrane space, respectively, in mitochondria. In human, SIRT3 protein was reported to localize in mitochondrial matrix [14]. Since mouse SIRT3 protein lacks a region corresponding to the N-terminal 142-amino acid residues of human SIRT3 protein, the region could be critical in determining localization in mitochondria. In addition, the function of SIRT3 might differ in humans and mice.

SIRT3 is localized in nucleus when co-expressed with SIRT5

We then examined localization of these three mitochondrial SIRT proteins when two of them were co-expressed in

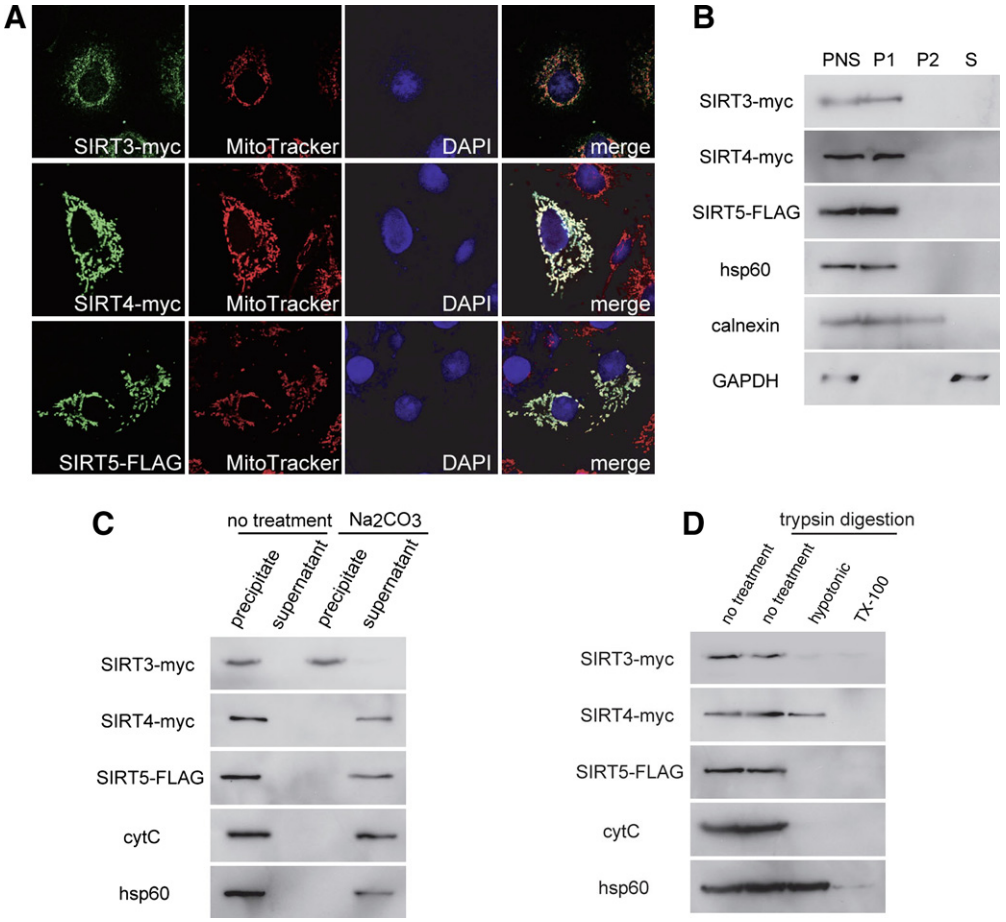

Fig. 1. Localization of SIRT3, SIRT4, and SIRT5 in mitochondria. (A) Confocal microscopy. SIRT3-myc (upper panels), SIRT4-myc (middle panels), and SIRT5-FLAG (lower panels) were expressed in COS7 cells and immunostained with anti-myc antibody or anti-FLAG antibody. Mitochondria and nuclei were stained by MitoTracker Red and DAPI, respectively, and fluorescent images were obtained using a confocal microscope. (B) Fractionation of post-nuclear supernatant. SIRT3-myc, SIRT4-myc, and SIRT5-FLAG proteins each was expressed in COS7 cells, and the obtained PNS was fractionated into mitochondria-enriched precipitate (P1), microsome-enriched precipitate (P2), and supernatant (S) fractions. The three fractions were separated by SDS–PAGE and then analyzed by Western blotting using anti-myc antibody for SIRT3-myc and SIRT4-myc or anti-FLAG antibody for SIRT5-FLAG. Hsp60, calnexin, and GAPDH were used as endogenous markers for mitochondria, microsome, and cytosol, respectively. (C) Alkaline treatment of mitochondria. Mitochondria prepared from the COS7 cells expressing each of the SIRT3-myc, SIRT4-myc, and SIRT5-FLAG proteins were treated with Na_2CO_3. The reaction mixture was centrifuged to separate the precipitate and supernatant fractions, containing membrane-integrated proteins and soluble proteins, respectively. The two fractions were analyzed by Western blotting. Cytochrome *c* (cyt*c*) and hsp60 were used as endogenous protein markers for mitochondrial soluble protein. (D) Submitochondrial fractionation. The mitochondria from COS7 cells expressing one of three SIRT proteins were treated with either H_2O (hypotonic) or TX-100, and then treated with trypsin. The reaction mixtures were analyzed by Western blotting. Cytochrome *c* and hsp60 were used as endogenous markers for mitochondrial intermembrane space protein and matrix protein, respectively.

COS7 cells (Fig. 2A). When SIRT3 and SIRT5 proteins were co-expressed, the localization of SIRT3 protein but not SIRT5 protein changed from mitochondria to nucleus (upper panels). However, SIRT proteins remained in mitochondria with the other combinations of co-expression (SIRT3 and SIRT4, middle panels, and SIRT4 and SIRT5, lower panels). To confirm the localization shift of SIRT3 protein by co-expression with SIRT5, we performed fractionation of PNS (Fig. 2B). SIRT5 protein appears in P1 fraction, indicating its localization in mitochondria. In contrast, SIRT3 protein is not found in PNS but appears in whole cell lysate, indicating that SIRT3 is localized in nucleus. These results are consistent with confocal microscopic study. In addition, we performed another cell fractionation to separate nucleus and the remaining portion of the cell using digitonin (Fig. 2C). The nuclear proteins lamin A/C appeared in insoluble (INS) fraction and the mitochondrial protein hsp60 occurred in both insoluble and soluble (SOL) fractions to a similar extent. SIRT3 protein also was detected in both insoluble and soluble fractions to a similar extent when expressed alone. However, the majority of SIRT3 protein was detected in the insoluble fraction when co-expressed with SIRT5, further confirming the localization shift of SIRT3 protein from mitochondria to nucleus. Taken together, we clearly show here for the first time that the intracellular localization of SIRT3 protein changes from mitochondria to nucleus in the presence of SIRT5, suggesting that SIRT3 plays a role as a regulator of nuclear proteins through its deacetylase or other, unknown activity.

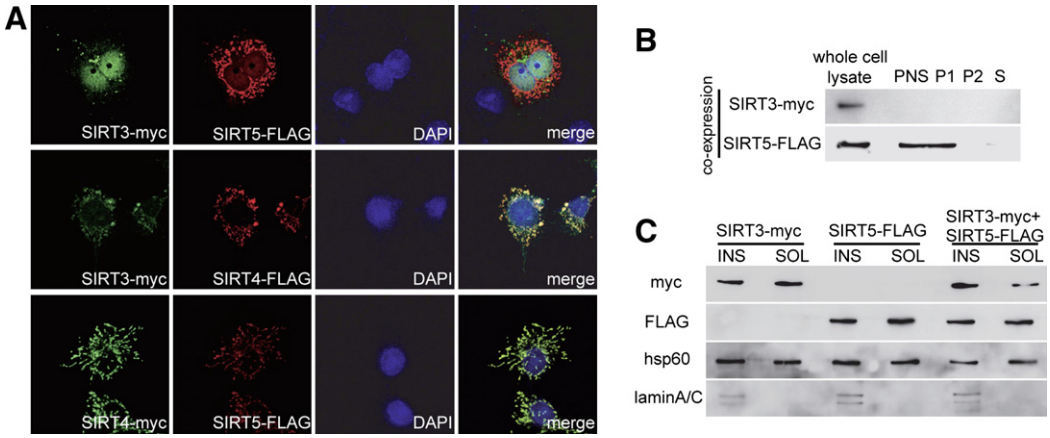

Fig. 2. Localization of SIRT3 when co-expressed with SIRT5. (A) Confocal microscopic analysis of COS7 cells expressing two of the three mitochondrial SIRT proteins. SIRT3-myc and SIRT5-FLAG (upper panels), SIRT3-myc and SIRT4-FLAG (middle panels), and SIRT4-myc and SIRT5-FLAG (lower panels) were co-expressed in COS7 cells, and immunostained using antibodies against myc tag and FLAG tag. Nuclei were stained by DAPI. (B) Subcellular fractionation of PNS. PNS of COS7 cells co-expressing SIRT3-myc and SIRT5-FLAG was fractionated into mitochondria-enriched precipitate (P1), microsome-enriched precipitate (P2), and supernatant (S) fractions, and these fractions along with whole cell lysate were analyzed by Western blotting. (C) Subcellular fractionation using digitonin. COS7 cells expressing either SIRT3-myc (left) or SIRT5-FLAG (middle) or both (right) were solubilized by digitonin, and the obtained lysate was centrifuged and fractionated into nuclear-enriched insoluble (INS), and soluble (SOL) fractions. Hsp60 and laminA/C were used as endogenous markers for mitochondria protein and nucleus protein, respectively.

Disruption of putative mitochondrial targeting signal of SIRT3

Because the segment containing amino acid residues 66–88 potentially forms a basic amphiphilic α-helical structure, it could serve as a MTS. To examine the role of this segment, SIRT3 mutant SIRT3mt, in which the four amino acid residues 72–75 were replaced by four alanine residues, was constructed (Fig. 3A). When SIRT3mt alone was expressed in COS7 cells, SIRT3mt protein was not detected in mitochondria but was widely distributed in the cell in confocal microscopic analysis (Fig. 3B, upper panels). In addition, when SIRT3mt and SIRT5 were co-expressed, the distribution of SIRT3mt protein was not changed compared to that expressed alone (Fig. 3B, lower panels). In fractionation of PNS, SIRT3mt protein was fractionated into S fraction both when SIRT3mt was expressed alone and when SIRT3mt and SIRT5 were co-expressed. SIRT5 protein was localized in mitochondria when SIRT3mt and SIRT5 were co-expressed (Fig. 3C). These results indicate that the MTS is necessary not only for targeting SIRT3 to mitochondria in the absence of SIRT5 but also for targeting SIRT3 to nucleus in the presence of SIRT5.

Disruption of putative nuclear localization signal of SIRT3

We found that the sequence containing amino acid residues 213–219 of the SIRT3 protein closely resembles the

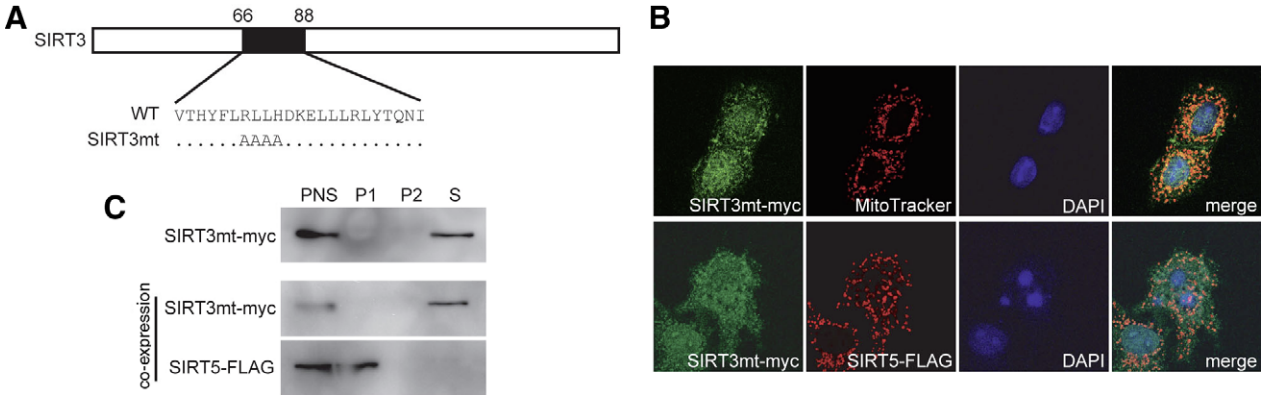

Fig. 3. Effect of disruption of putative mitochondrial targeting signal of SIRT3. (A) Alanine replacement of putative MTS of SIRT3. Four residues of the putative MTS of SIRT3 (amino acid residues 72–75) were replaced with four alanine residues. In the SIRT3mt sequence, amino acid residues identical with wild-type SIRT3 protein are indicated with dots. (B) Confocal microscopy. Immunofluorescent images of COS7 cells expressing SIRT3mt-myc alone (upper panels) or both SIRT3mt-myc and SIRT5-FLAG (lower panels) are shown. Mitochondria and nuclei were stained by MitoTracker Red and DAPI, respectively. (C) Subcellular fractionation of PNS. PNSs of COS7 cells expressing SIRT3mt-myc alone (an upper panel) or co-expressing SIRT3mt-myc and SIRT5-FLAG (middle and lower panels) were centrifuged and fractionated into mitochondria-enriched precipitate (P1), microsome-enriched precipitate (P2), and supernatant (S) fractions. The fractions were analyzed by Western blotting.

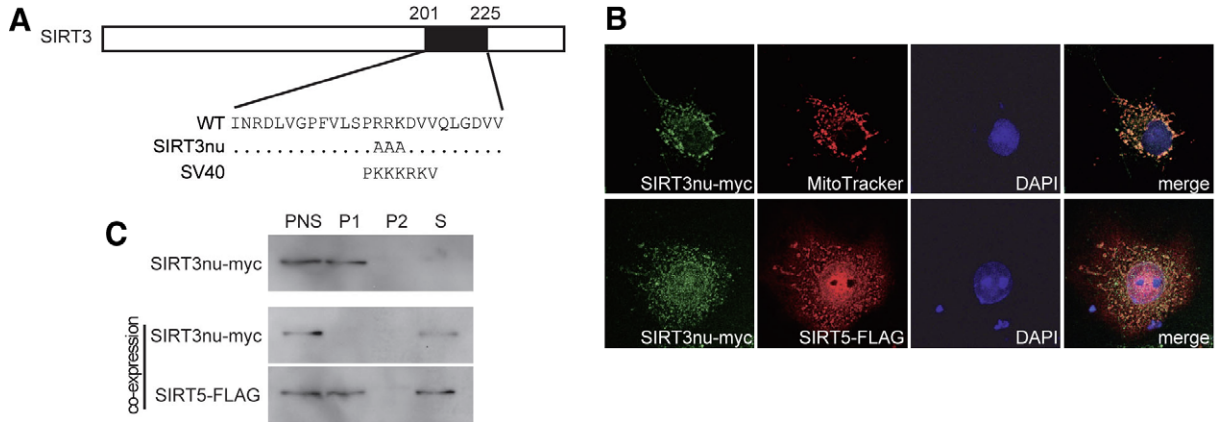

Fig. 4. Effect of disruption of putative nuclear localization signal of SIRT3. (A) Comparison of the amino acid sequences of putative NLS of SIRT3, SIRT3nu, and SV40 large T antigen. Three basic amino acid residues of the putative NLS of SIRT3 (amino acid residues 214–216) were replaced with three alanine residues. In the SIRT3nu sequence, amino acid residues identical with wild-type SIRT3 protein are indicated with dots. The classical NLS of SV40 large T antigen also is shown (SV40). (B) Confocal microscopy. Immunofluorescent images of COS7 cells expressing SIRT3nu-myc alone (upper panels) or both SIRT3nu-myc and SIRT5-FLAG (lower panels) are shown. Mitochondria and nuclei were stained by MitoTracker Red and DAPI, respectively. (C) Subcellular fractionation of PNS. PNSs of the COS7 cells expressing SIRT3nu-myc alone (an upper panel) or co-expressing SIRT3nu-myc and SIRT5-FLAG (middle and lower panels) were fractionated into mitochondria-enriched precipitate (P1), microsome-enriched precipitate (P2), and supernatant (S) fractions. The fractions were analyzed by Western blotting.

classical NLS of the SV40 T antigen (Fig. 4A). To examine whether this sequence functions as a NLS, the mutant SIRT3 protein SIRT3nu, in which the three basic amino acid residues (214–216) in the putative NLS of SIRT3 were replaced by three alanine residues (Fig. 4A), was constructed. When SIRT3nu alone was expressed in COS7 cells, it was localized in mitochondria (Fig. 4B, upper panels). In the cells co-expressing SIRT3nu and SIRT5, a shift of SIRT3nu protein to the nucleus was not observed, and SIRT3nu protein and a part of SIRT5 protein were scattered widely in the cell in confocal microscopic analysis (Fig. 4B, lower panels). In fractionation of PNS, all of the SIRT3nu protein and nearly half of the SIRT5 protein were shifted from P1 fraction to S fraction by co-expression (Figs. 1B and 4C). These results suggest that the segment containing amino acid residues 213–219 of SIRT3 plays an important role in the localization shift of SIRT3 protein to nucleus when co-expressed with SIRT5. Furthermore, SIRT5 may well hamper SIRT3nu localization in mitochondria through interaction with SIRT3nu. However, further study is required to elucidate the mechanism of the localization shift of SIRT3 protein.

Interestingly, recent study has reported that human prohibitin 2 (PHB2), known as a repressor of estrogen receptor (ER) activity, is localized in the mitochondrial inner membrane, and translocates to the nucleus in the presence of ER and estradiol [18]. Although the mechanism of regulation of the expression level of SIRT5 remains unknown, SIRT3 might play a role in communication between nucleus and mitochondria in a SIRT5-dependent manner.

The function of mitochondrial SIRT proteins is still not well known. In the present study, we determined the exact localization of mouse SIRT3, SIRT4, and SIRT5 proteins in mitochondria. In addition, we demonstrated that SIRT3 can be present in nucleus in the presence of SIRT5. It has been reported that SIRT3 deacetylates proteins that are not localized in mitochondria *in vitro* such as histone-4 peptide and tubulin [14]. Thus, if SIRT3 is present in nucleus *in vivo,* SIRT3 protein might well deacetylate nuclear proteins. These results provide useful information for the investigation of the function of these proteins.

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