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Murine Sirt3 Protein Isoforms Have Variable Half-lives

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Abstract

Sirt3 is a NAD⁺-dependent protein deacetylase mainly localized in mitochondria. Recent studies indicate that the murine *Sirt3* gene expresses different transcript variants resulting in three possible Sirt3 protein isoforms with variable lengths at the N-terminus: M1 (aa 1–334), M2 (aa 15–334), and M3 (aa 78–334). In this study, we stably expressed these variants in several cell lines. We found that Sirt3 M1 or M2 could be stably expressed with predominant mitochondrial localization. However, stable expression of Sirt3 M3 protein was consistently at very low levels. Fast proteasomal degradation contributed to the low expression of Sirt3 M3 protein, as proteasome inhibitor treatment increased Sirt3 M3 protein levels in these cells. Sirt3 M3 protein is ubiquitinated and the E3 ubiquitin ligase subunit Skp2 is involved in Sirt3 M3 protein degradation. Additionally, we found Sirt3 M3 protein could be produced from Sirt3 transcripts encoding longer M1 and M2 isoforms. To explore the mechanism underlying the instability of Sirt3 M3 protein, we found that Sirt3 M1 and M2 proteins, but not M3, specifically interacted with HSP60. This suggests that heat shock proteins might play a role in the maintenance of Sirt3 protein stability.

Keywords

sirtuin; Sirt3; mouse; mitochondria; proteasome; protein half-life; HSP60

1. Introduction

The NAD⁺-dependent deacetylase Sirt3 is one of the seven mammalian sirtuin family members (Guarente, 2005). Sirt3 expression is elevated by caloric restriction or fasting (Shi et al., 2005; Palacios et al., 2009). Sirt3 can deacetylate many mitochondrial proteins to regulate mitochondrial functions (Ozden et al.,; Someya et al.,; Tao et al.,; Lombard et al., 2007; Ahn et al., 2008; Hirschey et al., 2010; Yang et al., 2010a). Human *SIRT3* gene encodes a 399 aa protein with a 25 amino acid mitochondrial localization sequence at its N-terminus (Onyango et al., 2002; Schwer et al., 2002). The mouse Sirt3 gene expresses alternative transcript variants (Cooper and Spelbrink, 2008; Cooper et al., 2009; Jin et al., 2009; Bao et al., 2010; Yang et al., 2010b) to produce three different protein isoforms Sirt3 M1 (aa 1–334), M2 (aa 15–334), and M3 (aa 78–334) with variations at the N-terminus (Cooper et al., 2009; Jin et al., 2009; Bao et al., 2010; Yang et al., 2010b). Sirt3 M1 or M2 proteins, which contain mitochondrial localization sequence at the N-terminus and were shown to be mitochondrial proteins (Cooper et al., 2009; Jin et al., 2009; Bao et al.,; Yang et

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al., 2010b), Sirt3 M3 proteins do not exhibit a significant pattern of mitochondrial localization (Cooper et al., 2009; Jin et al., 2009; Yang et al., 2010b).

All the above-mentioned studies of murine Sirt3 protein isoforms were based on transient expression. In this study, we investigated the function of murine Sirt3 isoforms by stable expression in various cell lines. We found that stably expressed Sirt3 M1 or M2 proteins could be easily detected in mitochondria. In contrast, Sirt3 M3 protein expression was always at a very low level. We went on to identify that ubiquitination and proteasomal degradation is responsible for the short half-life of Sirt3 M3 protein.

2. Materials and Methods

2.1. Plasmid construction

Sirt3-M1(1–334), Sirt3-M2(15–334) and Sirt3-M3(78–334) were generated by PCR with the corresponding primers: 5'-ATAGAATTCATGGCGCTTGACCCTC-3', 5'-ATAGAATTCATGGCGCTAAGCGGTCG-3' and 5'-ATAGAATTCATGGTGGGGGCCGGC-3', paired with a common reverse primer 5'-ATAGAATTCTCTGTCCTGTCCATCC-3'. To generate the M1 mutant with Met15 changed to alanine, the following primer 5'-AGAATTCATGGCGCTTGACCCTCTAGGCGCCGTCGTCCTGCAGAGCATCGCGGC GCT AAG-3' was paired with the above mentioned reverse primer for PCR. The M1 and M2 mutants with Met78 changed to alanine were generated by PCR (Giebel and Spritz, 1990) using the following primer 5'-TGGTCGCGGTGGGGGCCGGCATC-3' and 5'-CCCACCGCGACCAACCACCTAC-3', together with the full-length primer pairs (for M1 or M2) mentioned above (underlined). All the PCR products were then sub-cloned into the EcoR I site of pBabe-puro or pBabe-puro-Flag vector (in which the Flag tag was inserted between EcoR I and Sal I of pBabe-puro). The pcDNA3.1-Sirt3 M1, M2 and M3 were previously described (Yang et al., 2010b). Mouse Sirt5 was PCR amplified from the mouse liver cDNA using the following primers 5'-GAATGATCAATGCGACCTCTCCTGATTGC-3' and 5'-ATAGAATTCAGAAGTCCTTTCAGTTTCATG-3'. The PCR product was then sub-cloned into pcDNA3.1-Flag vector (Yang et al., 2010b) at the BamHI and EcoR I sites. The GST-Sirt3(38–334) and GST-Sirt3(78–334) constructs were previously described (Yang et al., 2010b). Skp2-targeting shRNA in the pSUPER-puro construct was described previously (Wang et al., 2011), and was used for the knockdown of Skp2 in 293T cells by transient transfection. All expression constructs were verified by DNA sequencing.

2.2. Cell culture

BOSC23, 293T, C2C12, HIB1B and NIH3T3 cells were cultured in DMEM supplemented with 10% bovine calf serum (Hyclone, Logan, Utah), 100 IU/ml penicillin and 100 µg/ml streptomycin, at 37°C and 5% CO₂. 293T cells were transfected as previously described (Yang et al., 2010a) and the transfected cells were lysed in cell lysis buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 20 mM NaF, 1 mM Na₃VO₄, 5 mM EDTA and 0.5% NP-40) supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). For retroviral infection and stable cell establishment, retroviral constructs with various murine Sirt3 isoforms in pBabe-puro vector were used to transfect BOSC23 cells (Shi et al., 2005). Two days after transfection, supernatants containing viral particles were harvested and used to infect HIB1B, C2C12 or NIH3T3 cells. The cells were then selected with 2–4 µg/ml puromycin.

2.3. Immunoprecipitation and Immunoblot assay

For immunoprecipitation, cells were lysed and incubated with anti-Flag antibody conjugated agarose beads (Sigma, St. Louis, MO) at 4 °C overnight. Beads were washed three times, and proteins were released from beads in SDS-sample buffer and analyzed by immune blot analysis. For immunoblotting, protein samples were resolved by SDS-PAGE and transferred to nitrocellulose membranes. The blots were probed with the following primary antibodies: mouse monoclonal anti-Flag M2-Peroxidase at 1:3000 (Sigma, St. Louis, MO), rabbit polyclonal anti-Sirt3 antibody at a 1:3000 (Palacios et al., 2009), mouse anti-COX IV antibody at 1:2000 (Cayman Chemical), rabbit anti-ubiquitin antibody at 1:500 and rabbit polyclonal anti-Actin at 1:3000 (Santa Cruz Biotechnology), rabbit polyclonal anti-Skp2 (Santa Cruz Biotechnology), mouse monoclonal anti-GAPDH antibody at 1:500000 (Ambion), rabbit anti-HSP60 (Epitomics). The secondary antibodies were Affinipure Rabbit Anti-Mouse IgG, or Goat Anti-Rabbit IgG (Jackson ImmunoResearch, West Grove, PA) all at 1:10,000 dilution. The blots were developed with the SuperSignal West Pico Chemiluminescent Substrate (Pierce Biochemicals, Rockford, IL) according to the protocol provided by the manufacturer.

2.4. Immunofluorescence

For immunofluorescence detection, cells were grown on coverslips in 6-well plates and fixed using 4% paraformaldehyde in DMEM medium for 30 min. This was followed by three washes in PBS and 30 min incubation with 0.05% Triton X-100 in PBS. Cells were then blocked with 15% BSA for 1–2h at room temperature and incubated with primary antibody (anti-Flag, 1:200, Sigma; anti-MnSOD, 1:500, Cayman Chemical) and secondary antibodies (Donkey anti-mouse-Dylight 488 or donkey anti-rabbit-Dylight 649, 1:500, Jackson ImmunoResearch) in PBS with 3% BSA for overnight or 1 h, respectively. Slides were then mounted using Fluoromount-G (SouthernBiotech, Birmingham, AL). Image acquisition was carried out using an Olympus IX-70 confocal microscope.

2.5. Sub-cellular Fractionation

Kidney tissues obtained from Sirt3^{+/-} and Sirt3^{-/-} mice were homogenized with Dounce homogenizer (Wheaton) as previously described (Palacios et al., 2009). Mitochondria were isolated from cultured cells in isotonic mitochondrial isolation buffer (250 mM sucrose, 1 mM EDTA, 10 mM HEPES-KOH, pH 7.5, 1 mM phenylmethylsulfonyl fluoride, 50 µg/ml leupeptin) on ice. The suspension was centrifuged twice at 800 × g for 10 min at 4 °C to remove nuclei and cell debris. Mitochondria were pelleted by centrifugation at 10,000 g for 10 min at 4°C and the supernatant was the cytosolic fraction. The crude mitochondrial fraction was washed once with isolation buffer. Protein concentration of the mitochondrial fractions was measured by BCA Protein Assay Kit (Pierce).

3. Results

3.1. Stable expression of murine Sirt3 protein isoforms in cells

To study murine Sirt3 protein isoforms in stably expressed cells, murine HIB1B brown preadipocytes, C2C12 myoblasts or NIH3T3 fibroblasts were infected with retroviruses expressing Sirt3 M1, M2 or M3 with or without Flag-tag at the C-terminus. Sirt3 proteins expressed in these cells were detected by Western blot analysis. As shown in Fig. 1A, in HIB1B cells stably expressing Sirt3 M1 or Sirt3 M2, a single Sirt3 band corresponding to the mature Sirt3 was specifically detected, which had the same size as the endogenous Sirt3 in the kidney of a mouse heterozygous for the Sirt3 gene, but absent from kidney of a Sirt3 deficient mouse. However, no Sirt3 protein could be detected in Sirt3 M3 stable cells. Similar results were obtained from C2C12 (Fig. S1A) and NIH3T3 stable cells (data not

shown). In HIB1B cells stably expressing Flag-tagged Sirt3 M1, M2 or M3, either anti-Flag or anti-Sirt3 antibodies could recognize the mature form of Flag-tagged Sirt3 M1 or M2, with a 2 kD molecular weight shift compared to the endogenous Sirt3 (Fig. 1B). However, neither antibody could detect the expression of Flag-tagged Sirt3 M3 protein. Similar observations were made in C2C12 and NIH3T3 stable cells (data not shown).

To detect Sirt3 M3 protein expression, we employed immunoprecipitation using anti-Flag antibody-conjugated beads to enrich Flag-tagged Sirt3 proteins. The mature form for the Flag-tagged Sirt3 M1 or M2 could be readily detected in anti-Flag immunoprecipitates from HIB1B (Fig. 1C) or C2C12 cells (Fig. S1B). In addition, band(s) with higher molecular weights, likely the precursor or processing intermediates, were observed. When enriched by immunoprecipitation, Sirt3 M3 proteins could be detected at a much lower level than M1 or M2 proteins in HIB1B (Fig. 1C) or C2C12 stable cells (Fig. S1B).

To explore the sub-cellular localization of the stably expressed Sirt3 protein isoforms, HIB1B cells with stably expressed Flag-tagged Sirt3 M1, M2 or M3 were detected using immunofluorescence with anti-Flag antibody. Consistent with the results of the transiently expressed Sirt3 (Cooper et al., 2009; Jin et al., 2009; Bao et al., 2010; Yang et al., 2010b), stably expressed M1 and M2 Sirt3 proteins exhibited mitochondrial localization (Fig. 1D). However, the Sirt3 M3 proteins expression was too low to be detected by immunofluorescence (data not shown). Similar observations were made in C2C12 and NIH-3T3 cells with stable expression of these three murine Sirt3 isoforms (data not shown).

To further confirm the mitochondrial localization of murine Sirt3 proteins, we isolated the mitochondrial fraction from these stable cells. The mature form for either Sirt3 M1 or M2 could be readily detected in the mitochondrial fractions isolated from corresponding stable HIB1B cells (Fig. 1E) or C2C12 cells (Fig. S1C). In addition, Sirt3 M1 precursor and processing intermediates could also be observed. However, Sirt3 M3 protein level was too low to be detected by direct Western blot analysis even in partially enriched mitochondrial fractions.

Previously, we have reported that when the initiation codon for Sirt3 M2 in the Sirt3 M1 coding region was mutated to alanine (Sirt3 M1-M15A), the expression pattern were not significantly altered (Yang et al., 2010b). We also stably expressed this Sirt3 M1 mutant in HIB1B cells. As shown in Fig. 1F, Sirt3 M1-M15A mutant exhibited a similar expression pattern as the wild type Sirt3 M1, indicating that when stably expressed, the Sirt3 M1 coding transcript mainly produces M1 protein isoform which could be processed to the mature form without M2 protein translation from an internal in-frame ATG site.

3.2. Sirt3 M3 protein has short half-life through Skp2 mediated proteasome degradation

The low expression of Sirt3 M3 proteins in stable cells was intriguing. Quantitative RT-PCR revealed that the Sirt3 M3 mRNA was abundantly expressed in stable cells (data not shown). We investigated the stability of Sirt3 M3 protein by detecting its half-life. For this purpose, HIB1B cells with stable expression of Flag-tagged Sirt3 M3 were treated with cycloheximide. The level of Sirt3 M3 protein was detected by immunoprecipitation followed by Western blot analysis. As shown in Fig. 2A, the Sirt3 M3 protein level was quickly decreased when protein synthesis was blocked with a half-life less than 5 minutes. On the contrary, Sirt3 M1 or M2 proteins possessed significant longer half-lives (Fig. 2B). To investigate the pathway that involved in the degradation of M3, we treated cells with various protease or proteasome inhibitors, such as proteasome inhibitor MG132, cysteine proteases inhibitor E-64, or serine protease inhibitor, AEBSF. We found that only MG132 and another specific proteasome inhibitor, epoxomicin, were able to block Sirt3 M3 protein degradation in these cells (Fig. 2C). We further detected poly-ubiquitination of Sirt3 M3 in

HIB1B stable cells treated with MG132 (Fig. 2D), suggesting Sirt3 M3 proteins expressed in stable cells are quickly ubiquitinated and degraded through the proteasome system. In addition, we investigated whether the E3 ubiquitin ligase component Skp2 is involved in Sirt3 M3 degradation. We found that when Skp2 was knocked down using shRNA in 293T cells (Fig. 2E), Sirt3 M3 protein level was significantly elevated with extended half-life (Fig. 2F). These results suggest that Skp2 plays a role in Sirt3 M3 degradation.

3.3. Sirt3 M3 can be expressed from Sirt3 M1 or Sirt3 M2 coding regions

Since the coding region of Sirt3 M1 or M2 contains in frame ATG site for Sirt3 M3 expression, there is a possibility that Sirt3 M3 could be produced from M1 or M2. As Sirt3 M3 protein is unstable, the level of this protein could be very low. We noticed a protein with similar molecular size as Sirt3 M3 could be detected in cells stably expressing Flag-tagged Sirt3 M1 or Sirt3 M2, when the expressed Sirt3 proteins were concentrated by immunoprecipitation, as shown in Fig. 1C and Fig. S1B. To facilitate the detection of Sirt3 M3 protein, we treated HIB1B cells stably expressing Sirt3 M2 with MG132. As shown in Fig. 3A, after treatment of MG132, the level of mature form of Sirt3 M2 was decreased and the precursor form started to accumulate, suggesting that MG132 might affect the processing and maturation of Sirt3 M2 protein. Interestingly, a band corresponding to the size of Sirt3 M3 appeared after MG132 treatment (Fig. 3A). To confirm that this protein is actually Sirt3 M3, the ATG site for Sirt3 M3 in Sirt3 M2 coding region was mutated to GCG (Sirt3 M2–M78A). HIB1B cells stably expressing this mutant were established. In these cells, no Sirt3 M3 protein was detected after MG-132 treatment (Fig. 3B). Similar results were obtained from C2C12 stable cells (Fig. S2A). Similarly, Sirt3 M3 could be detected only from cells stably expressing Sirt3 M1 but not Sirt3 M1–M78A mutant, which lacked the initiation codon for Sirt3 M3 (Fig. S2B).

3.4. Sirt3 M1 and M2, but not M3, protein isoforms interact with HSP60

Sirt3 protein isoforms only differ at N-terminal length. We reasoned that the extended N-termini of Sirt3 M1 or M2 may bind protective proteins against degradation. To explore this possibility, we expressed Flag-tagged Sirt3 M1, M2 or M3 in 293T cells and immunoprecipitated these proteins with anti-Flag beads. The immunoprecipitated protein complexes were resolved by SDS-PAGE and visualized by Coomassie Blue staining. A 60kD protein band that specifically associated with Sirt3 M1 and M2 but not M3 was observed (Fig. S3) and identified by mass spectrometry to be heat shock protein 60 (HSP60). To confirm this interaction, Flag-tagged mouse Sirt3 M2 or M3, human Sirt3, or mouse Sirt5 were transiently expressed in 293T cells and immunoprecipitated with anti-Flag beads. The co-purification of endogenous HSP60 was detected by anti-HSP60 antibody. As shown in Fig. 4A, murine Sirt3 M2 and human Sirt3, but not murine Sirt3 M3 or Sirt5, interacted with HSP60. To further confirm the direct interaction between Sirt3 and HSP60, we incubated GST-fused Sirt3 recombinant proteins with mouse liver lysates. Since both Sirt3 M1 and M2 proteins are processed in the mitochondria to the mature form (aa 38–334), we compared GST-Sirt3(38–334) and GST-Sirt3M3(78–334). As shown in Fig. 4B, only the recombinant GST-Sirt3 mature form but not GST-Sirt3M3 interacted with HSP60.

4. Discussion

In this study, we investigated murine Sirt3 protein isoforms when they were stably expressed in cells. We found that Sirt3 M1 and M2 proteins had longer half-life with predominantly mitochondrial localization. In contrast, Sirt3 M3 protein was unstable with quick degradation through the ubiquitin-proteasome system. Similarly, when transiently transfected into 293T cells, Sirt3 M3 was also unstable when compared with Sirt3 M2 (data not shown). Furthermore, we found that the E3 ubiquitin ligase subunit Skp2 mediated Sirt3

M3 protein degradation. The underlying molecular mechanism for the differences of these Sirt3 protein isoforms was explored and we found that Sirt3 M1, M2 or the mature form derived from them (aa 38–334), but not M3 (aa 78–334), bind to HSP60. Therefore, it is likely that the region aa 38–77 is mediating the interaction. HSP60 is a mitochondrial chaperonin participating in the maintenance of protein folding in mitochondrial matrix, although extra-mitochondrial localization of HSP60 has also been reported (Deocaris et al., 2006). The differential interaction with HSP60 and possibly other heat shock proteins might be the reason that the proteins of Sirt3 M1 and M2, but not M3, are stable. The inability of Sirt3 M3 protein to interact with HSP60 may lead to the access of E3 ubiquitin ligase Skp2, and trigger subsequent ubiquitination and proteasomal degradation. The actual role of HSP60 in the maintenance of the half-life of Sirt3 protein needs to be confirmed by further experiments.

The short murine Sirt3 M3 protein exhibits several other differences to the longer murine Sirt3 isoforms. First of all, the shorter Sirt3 M3 protein lacks an N-terminal mitochondrial targeting signal, although an internal mitochondrial targeting sequence was reported (Nakamura et al., 2008). As a consequence, Sirt3 M3 protein does not show significantly mitochondrial localization as Sirt3 M1 or M2, which contains N-terminal mitochondrial targeting signal (Cooper et al., 2009; Jin et al., 2009; Bao et al., 2010; Yang et al., 2010b). In addition, it was shown that only the long Sirt3 M1 or M2 isoform, but not the shorter M3 protein, possesses deacetylase activity against peptide substrates (Jin et al., 2009; Yang et al., 2010b). However, we have found that Sirt3 M3 possesses comparable enzymatic activity with Sirt3 M1 and M2, when full-length native protein substrate was used (Yang et al., 2010b).

There are three transcript variants for mouse Sirt3 and the transcript variants encode exclusively for Sirt3 M3 are abundantly expressed in mouse tissues (Yang et al., 2010b). Our result showed that Sirt3 M3 protein can also be translated from Sirt3 M1 and M2 coding regions. Therefore, Sirt3 M3 protein can be produced from all transcript variants. However, only the mature Sirt3 protein derived from M1 or M2 proteins, but not the shorter Sirt3 M3 proteins, can be detected in mouse tissues (Fig. 1 and (Cooper et al., 2009; Jin et al., 2009; Bao et al., 2010; Yang et al., 2010b)). In light of the result of the current study, it is possible that the reason that no Sirt3 M3 protein has been detected in mouse tissues is that it has a very short half-life and is maintained at a very low level *in vivo*. Whether the short-lived Sirt3 M3 protein play any physiological role needs to be addressed in future studies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1A

Fig. 1B

Fig. 1C

Fig. 1D

Fig. 1F

Fig. 1.

Expression of Sirt3 M1, M2 and M3 in stable cells. (A) HIB1B cells with retroviral mediated stable expression of vector control, Sirt3-M1, M2, or M3 were examined for the expression of Sirt3 protein isoforms by Western blot analysis using anti-Sirt3 (top panel) or anti-actin (lower panel) antibodies. The kidney mitochondria from *Sirt3*^{-/-} or *Sirt3*^{+/-} mice were included as controls. (B) HIB1B cells with stable expression of vector control, Flag-tagged Sirt3 M1, M2, or M3 were examined for the expression of Sirt3 protein isoforms by Western blot analysis using anti-Sirt3 (top panel), anti-Flag (middle panel) or anti-actin (lower panel) antibodies. (C) HIB1B cells with stable expression of vector control, Flag-

tagged Sirt3 M1, M2, or M3 were lysed and immunoprecipitated with anti-Flag beads. The immunoprecipitated Sirt3-Flag proteins were detected by Western blot analysis with anti-Sirt3 antibody. (D) HIB1B cells with stable expression of Flag-tagged Sirt3 M1 or M2 were detected by immunofluorescence with anti-Flag antibody (green). Mitochondria were labeled with anti-MnSOD antibody (red). (E) Mitochondria were isolated from HIB1B cells with stable expression of vector control or Sirt3 M1, M2 or M3. Sirt3 proteins were detected by Western blot analysis using antibodies against Sirt3 (top panel) for Sirt3 and COX IV (lower panel) for loading control. The kidney mitochondria from *Sirt3*^{-/-} or *Sirt3*^{+/-} mice were included as controls. (F) Mitochondria were isolated from HIB1B cells with stable expression of vector control, Flag-tagged Sirt3-M1, M1-M15A, or M2. Flag-tagged Sirt3 proteins were detected by Western blot analysis using anti-Flag antibody (top panel) for Sirt3 and COX IV (lower panel) for loading control.

Fig. 2A

Fig.2B

Fig.2C

Fig.2D

Fig.2E

Fig.2F

Fig. 2.

Sirt3 M3 protein has a short half-life. (A) HIB1B cells with stable expression of Sirt3 M3-Flag were treated with 100 μ g/ml cycloheximide for indicated lengths of time. Cells were lysed and Sirt3 M3-Flag proteins were immunoprecipitated with anti-Flag beads and detected by Western blot analysis using anti-Flag antibody (top panel). Two nonspecific bands were used as loading control (lower panel). (B) HIB1B cells with stable expression of Flag-tagged Sirt3 M1 or M2 were treated with cycloheximide for indicated lengths of time. The cells were lysed and Flag-tagged Sirt3 proteins were detected by Western blot analysis using anti-Flag antibody (top panel). GAPDH was evaluated as loading control (lower panel). (C) HIB1B cells with stable expression of Sirt3 M3-Flag were treated with proteasome inhibitors MG132 (10 μ M) and/or epoxomicin (1 μ g/ml) (Meng et al., 1999) for overnight. Cells were lysed and detected by Western blot analysis using anti-Flag (top panel) and anti-actin (lower panel) antibodies. (D) HIB1B cells with stable expression of vector or Flag-tagged Sirt3 M3 were treated overnight with MG132. The cells were lysed and the ubiquitination of Flag-tagged Sirt3 proteins was immunoprecipitated (IP) with anti-Flag beads and detected by immunoblot analysis (IB) using anti-ubiquitin (top panel) and anti-Flag (lower panel) antibodies. (E) Skp2 expression in 293T cells was knocked down by transient transfection with a plasmid containing Skp2 targeting shRNA. The expressions of Skp2 and actin were measured using immunoblot analysis. (F) 293T cells were transiently transfected with Sirt3 M3-Flag combined with a plasmid containing Skp2 targeting shRNA or control vector, and treated with 100 μ g/ml cycloheximide for indicated lengths of time. Immunoblot analysis was performed using anti-Flag (top panel) and anti-actin (lower panel) antibodies.

Fig.3A

Fig.3B

Fig. 3. Sirt3 M3 can be produced from Sirt3 M1 or M2. (A) HIB1B cells with stable expression of vector, Sirt3 M3 or Sirt3 M2 were treated with MG132 for indicated lengths of time. Cells were then lysed and Sirt3-Flag proteins were detected by Western blot analysis using anti-Flag (top panel) or anti-actin (lower panel) antibodies. (B) Mitochondria were isolated from the HIB1B cells with stable expression of Sirt3 M2, M2M78A or M3 with or without MG132 treatment overnight. Sirt3 proteins were detected using anti-Flag (top panel) or anti-COX IV antibodies (lower panel).

Fig. 4A

Fig. 4B

Fig. 4.

HSP60 specifically interacts with Sirt3 M1 or M2, but not M3. (A) 293T cells were transfected with Flag-tagged Sirt3 M2, M3, human Sirt3 or murine Sirt5. Cells were lysed and proteins were immunoprecipitated with anti-Flag beads. The co-precipitated endogenous HSP60 proteins were detected with anti-HSP60 antibody. (B) GST, GST-Sirt3(38–334) and GST-M3(78–334) were incubated with 500 μ g mouse liver lysates. The proteins associated with the GST-fused proteins were pulled down using glutathione-conjugated beads and detected with anti-HSP60 (top panel). The GST-fusion proteins were stained with Ponceau S (lower panel).