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Characterization of the Murine SIRT3 Mitochondrial Localization Sequence and Comparison of Mitochondrial Enrichment and Deacetylase Activity of Long and Short SIRT3 Isoforms

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ABSTRACT

SIRT3 is identified as the major mitochondrial deacetylase. Two distinct isoforms of the murine SIRT3 have been identified with the short isoform having no recognizable mitochondrial localization sequence (MLS) and the long isoform having a putative MLS. A recent study questions the mitochondrial deacetylase activity of this short isoform. In contrast, the long isoform has been shown to be predominantly mitochondrial with robust deacetylase activity. In this study, we investigate whether the amino-terminus of the long SIRT3 isoform is a legitimate MLS and evaluate in-situ mitochondrial deacetylase activity of both isoforms. We confirm the presence of long and short isoforms in murine liver and kidney. The long isoform is generated via intra-exon splicing creating a frame-shift to expose a novel upstream translation start site. Mitochondrial localization is significantly more robust following transfection of the long compared with the short isoform. Insertion of this alternatively spliced novel 5′ sequence upstream of a GFP-reporter plasmid shows greater than 80% enrichment in mitochondria, confirming this region as a legitimate mitochondrial localization sequence. Despite lower mitochondrial expression of the short isoform, the capacity to deacetylate mitochondrial proteins and to restore mitochondrial respiration is equally robust following transient transfection of either isoform into SIRT3 knockout embryonic fibroblasts. How these alternative transcripts are regulated and whether they modulate distinct targets is unknown. Furthermore, in contrast to exclusive mitochondrial enrichment of endogenous SIRT3, overexpression of both isoforms shows nuclear localization. This overexpression effect, may partially account for previously observed divergent phenotypes attributed to SIRT3. J. Cell. Biochem. 110: 238–247, 2010. Published 2010 Wiley-Liss, Inc.[†]

KEY WORDS: SIRT3; MITOCHONDRIAL PROTEIN DEACETYLATION; MITOCHONDRIAL LOCALIZATION SEQUENCE; NUCLEAR LOCALIZATION

The Sir2 or sirtuin family of class III deacetylases is distinguished from the class I and II histone deacetylases (HDACs), by distinct protein sequences and in that they are NAD^+-dependent deacetylases [Haigis and Guarente, 2006]. The yeast sir2 orchestrates replicative-senescence and is considered a pivotal mediator of longevity [Haigis and Guarente, 2006]. Seven mammalian homologues of the sir2 family have been identified and are designated as SIRT1 through SIRT7 [Frye, 2000]. These sirtuins have distinct subcellular locations and are proposed to modulate specific biological functions unique to their subcellular locations including transcriptional silencing, diverse effects on cell growth, aging, stress tolerance, and metabolism [Schwer and Verdin, 2008].

Protein acetylation/deacetylation is now recognized as an important post-translational modification and recent proteomic profiling implicates that approximately 20% of mitochondrial

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proteins are modified by acetylation/deacetylation of their lysine residues [Kim et al., 2006]. Mitochondrial protein lysine residue acetylation is additionally conserved from prokaryotes to eukaryotes and investigations into the functional consequences of these post-translational modifications are being actively pursued [Zhang et al., 2009]. Three sirtuins, namely SIRT3, 4 and 5 are mitochondria-enriched [Schwer and Verdin, 2008]. Analysis of mitochondrial protein acetylation modifications in SIRT3, 4 and 5 knockout mice show that SIRT3 is the primary mitochondrial deacetylase [Lombard et al., 2007]. Numerous mitochondrial target proteins have been shown to be substrates for SIRT3 deacetylation [Hallows et al., 2006; Schwer et al., 2006; Lombard et al., 2007; Ahn et al., 2008; Jacobs et al., 2008; Schlicker et al., 2008] and in all instances this deacetylation of protein lysine residues enhances biological activity/function. Interestingly nuclear and cytosolic targets of SIRT3 deacetylation have also been identified [Shi et al., 2005; Sundaresan et al., 2008, 2009]. The biological roles of SIRT3 have begun to be explored and these include: a potential role in modulating adipocyte thermogenesis [Shi et al., 2005]; a cytoprotective function in mediating tolerance to cellular genotoxic and oxidative stress [Jacobs et al., 2008; Sundaresan et al., 2008], a cardiac negative regulator of hypertrophic growth [Sundaresan et al., 2009], and a direct role in the modulation ATP generation through facilitating mitochondrial oxidative phosphorylation [Ahn et al., 2008].

The full-length human SIRT3 (hSIRT3) is a 44 kDa protein, including a 142 residue amino-terminal mitochondrial localization sequence (MLS) [Onyango et al., 2002; Schwer et al., 2002]. Following mitochondrial import, this 142 amino acid presequence is proteolytically cleaved to generate a 28 kDa active enzyme [Schwer et al., 2002]. The initial sequence of mouse Sirt3 (mSirt3) encoded for a 28 kDa protein lacking a putative amino-terminal MLS. How this isoform of mSIRT3 is imported into mitochondria remains unclear and this discrepancy compared with the human protein was recently questioned [Cooper and Spelbrink, 2008; Hallows et al., 2008]. Two recent studies now identify two longer isoforms of the murine SIRT3 and dispute whether the short isoform has biological significance [Cooper et al., 2009; Jin et al., 2009].

The purpose of this study was to confirm the transcribed identity of the DNA sequence encoding for the long isoform of murine SIRT3 (mSIRT3-L), to evaluate whether the additional 5′ sequence functions as a legitimate MLS, to compare relative mitochondrial enrichment comparing the long and short isoforms and to compare their respective in-vitro mitochondrial deacetylase activities. Furthermore, in light of the conflicting data with respect to SIRT3 nuclear localization we used sucrose gradient centrifugation to investigate whether these isoforms show nuclear localization and compared this with endogenous protein levels in mouse embryonic fibroblasts (MEFs).

In this report, we describe the identification of an alternatively spliced transcript of the mouse Sirt3 gene, which encodes an mSIRT3-L isoform. This novel amino-terminus 63 residue sequence is shown to specifically enhance mitochondrial targeting and functions as a legitimate MLS. Furthermore, we show that the mSIRT3-L is cleaved to generate shorter mitochondrial proteins. The shortest protein product has a similar molecular weight to the less abundant short isoform of SIRT3 (mSIRT3-S). Despite the significantly greater relative mitochondrial expression of mSIRT3-L, both isoforms show similar mitochondrial deacetylase activity when transiently expressed in SIRT3 knockout MEFs. This similar capacity to promote deacetylation of mitochondrial proteins extends to their ability to restore mitochondrial respiration in SIRT3 knockout MEFs. In addition, overexpression of FLAG-tagged constructs of both isoforms is evident in the nuclear fraction of cardiac-derived transformed H9c2 cells and MEFs. However, employing sucrose gradient electrophoresis, we identify that the endogenous SIRT3 is exclusively expressed in the mitochondria of MEF cells.

MATERIALS AND METHODS

5′-RACE (RAPID AMPLIFICATION OF CDNA ENDS)

To identify the 5′ end of mouse SIRT3, 5′-RACE was performed using 5′/3′-RACE kit following the manufacturer's instruction (Roche). Briefly, 2 μg total RNA extracted from mouse liver and kidney (RNeasy Mini Kit; Qiagen) was used as template to synthesize the first strand cDNA. The reverse transcription reaction was performed at 55°C for 1 h using the gene-specific primer 10R (5′-AGCCTTTC-CACACCATGAAC-3′). The synthesized cDNA was tailed by addition of poly A to the 3′ end of the cDNA using recombinant terminal transferase and dATP at 37°C for 20 min. dA-Tailed cDNA was amplified using oligo dT-anchor primer (5′-GACCACGCGTAT-CGATGTCGACTTTTTTTTTTTTTTV, V = A, C or G) and gene-specific primer 9R (5′-TCGACAGACCGTGCATGTAG-3′). The PCR product was further amplified with PCR anchor primer (5′-GACCACGCGTATCGATGTCGAC-3′) and 9R and the PCR products were subcloned into pCR4 TOPO vector (Invitrogen) for sequencing.

PLASMID CONSTRUCTS

Plasmid expressing short isoform mouse Sirt3 (mSirt3-S) was obtained from Open Biosystems (Clone-5253854, accession number—BC025878). Long isoform mSirt3 cDNA was amplified by PCR using both 5′-RACE fragment and short mSirt3 cDNA fused with 3× FLAG as templates. Both isoforms were further subcloned into pcDNA3.1 (+) (Invitrogen) vector at EcoRI and XbaI sites.

To generate the 5′-mSirt3-GFP chimera, an 189 bp fragment of the 5′-mSirt3-L cDNA starting from the first ATG (Fig. 1A) was amplified by PCR, and inserted into pEGFP-N3 vector (Clontech) at EcoRI and BamHI sites. The primers used were: forward, 5′-CGG-AATTCCGTCGTCCTGCAGAGCATCAT-3′; reverse, 5′-TCGGGCTCC-GACCACCACCC TACTGCAGGCTCT-3′. All the constructs described above were confirmed by sequencing.

CELL CULTURE STUDIES

HEK293 cells and H9c2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS). Transformed wildtype (WT) and SIRT3 knockout (SIRT3 KO) mouse embryonic fibroblasts (MEF) were generated from 12.5 day embryos cultured in DMEM supplemented with 15% FBS. HEK293 cells and H9c2 cells were transfected with appropriate plasmids using Lipofectamine 2000 (Invitrogen) according to the protocol.

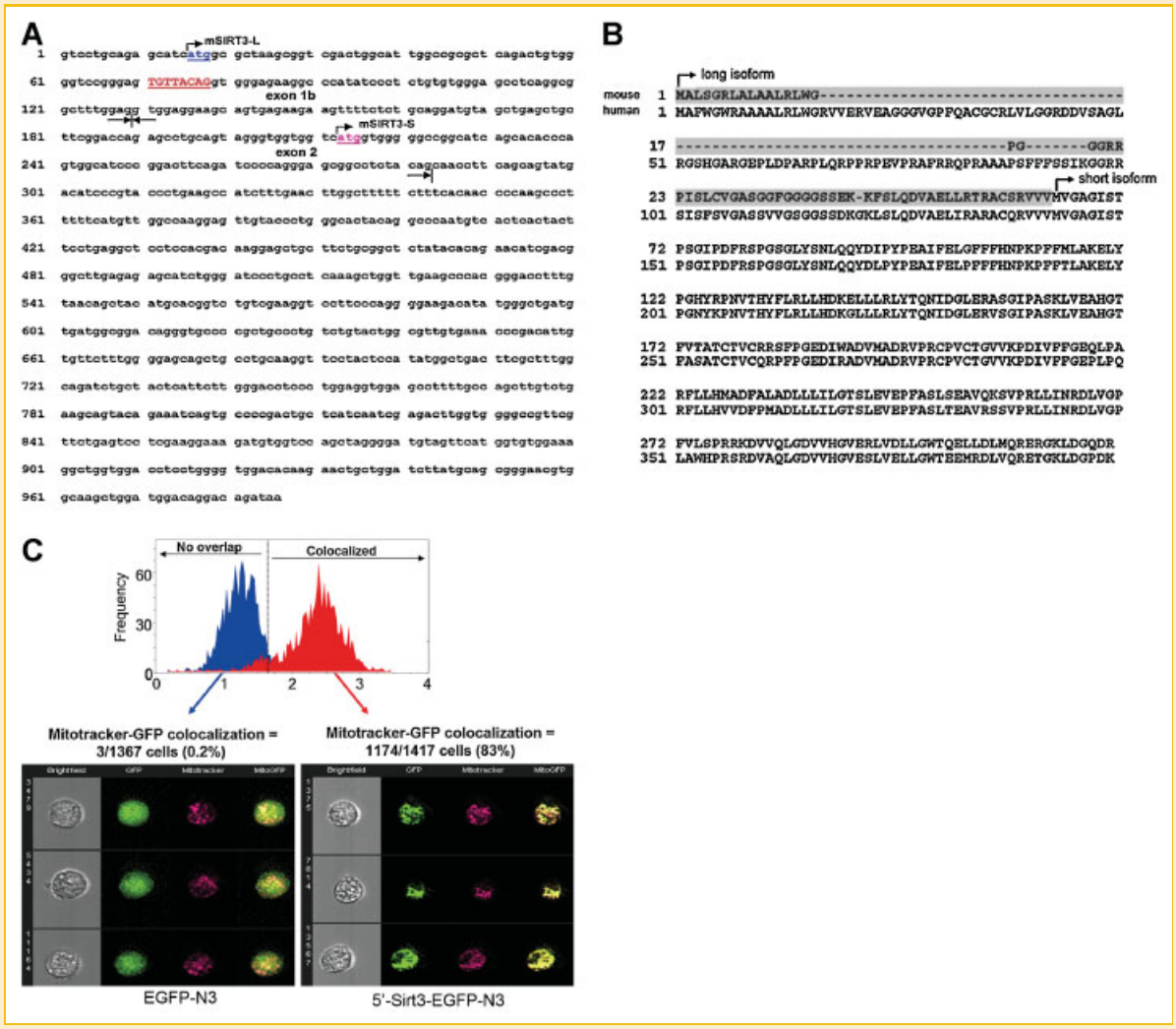

Fig. 1. Gene and amino acid sequences comparing the long and short isoforms of murine SIRT3 and verification of the murine SIRT3 mitochondrial localization sequence. A: Alternative splicing of mouse SIRT3 gene. 5′ RACE of cDNA from mouse liver and kidney tissues shows two isoforms of the mouse SIRT3 gene as a result of eight nucleotide deletion (highlighted upper case letters) within its exon 1b region. This leads to two different 5′ start sites for mouse SIRT3 gene transcription: one starts from exon1b, the other starts from exon2. B: Comparison of mouse SIRT3 amino acid sequence with human SIRT3. The mSIRT3-L isoform has a 63 amino acid extension at its N-terminus, which shows high fidelity with the human sequence at the amino-terminus with 'dropout' of amino acids in the mid-region of this putative mitochondrial localization sequence. C: Targeting of GFP into mitochondria by 5′- cDNA fragment of long mSIRT3. H9c2 cells were transfected with either 5′-mSIRT3-EGFP chimera (right image) or EGFP-N3 (left image), stained with Mitotracker deep red 633, then analyzed by ImageStream for cytometry imaging. The histogram represents the colocalization index of the two colored fluorescent stains with the right box showing overlap. The percentage colocalization of the constructs is shown below the histogram. A representative image of the colocalization of GFP signal (green) and mitochondria (red) is represented by the yellow staining comparing the EGFP-N3 and the 5′-mSIRT3-EGFP chimera-transfected cells.

MEFs were electroporated with pcDNA3-Sirt3 plasmids using Nucleofector (Amaxa) according to the manufacturer's instructions.

SUBCELLULAR FRACTIONATION AND WESTERN BLOT ANALYSIS

Forty-eight hours after plasmid transfection, cytoplasmic and mitochondrial fractions from cultured cells were prepared using Q-proteome Mitochondria Isolation Kit (Qiagen) and nuclear isolation employing the NE-PER isolation kit (Pierce). Five or 10 μg of cytosolic, mitochondrial proteins or nuclear protein were used for Western blot analyses. Additional isolation of nuclear fractions was performed by sucrose gradient centrifugation as described previously [Blobel and Potter, 1966]. Purified nuclei were obtained from H9c2 and MEF cells following centrifugation and extraction of the pellet from below the 2.0 M sucrose cushion. To exclude ex-vivo deacetylation, protein extraction was performed in the presence of the deacetylase inhibitors tricostatin A (TSA; class I/II HDAC inhibitor—1 μM) and nicotinamide (sirtuin inhibitor—5 mM). Antibodies used included monoclonal anti-FLAG M2 antibody (Sigma), monoclonal anti-porin (VDAC) antibody (Invitrogen), polycloncal anti-aldose reductase and anti-Sp1 antibodies (Santa Cruz Biotechnology), polycloncal catalase antibody (Abcam) and the polyclonal acetylated lysine antibody (Cell Signal). Mouse SIRT3 antisera were raised against a COOH-terminal peptide [Lombard et al., 2007]. Western blot signals were visualized using the Odyssey infrared imaging system (Li-Cor) or by standard chemiluminescence (Thermo-Scientific).

IMMUNOSTAINING AND CYTOMETRY IMAGING

Immunostaining of the H9c2 cells was performed as described previously [Lynn et al., 2008]. Briefly, 24 h after transfection, cells were fixed with 4% paraformaldehyde, permealized with 0.1% Triton X-100 and blocked with 4% normal goat serum in PBS for 30 min at room temperature. The cells were then incubated with polyclonal anti-FLAG antibody (1:100; Sigma) and monoclonal anti-cytochrome oxidase I (COXI) antibody (1:100; MitoSciences) for 1 h at room temperature followed by incubation with Alexa Fluor 488-conjugated goat anti-rabbit IgG and 594-conjugated goat anti-mouse IgG (1:250; Invitrogen) at room temperature. Nuclei were counterstained with 4′6′-diamidino-2 phenylindole (DAPI; Invitrogen). The images were collected using a Zeiss LSM 510 confocal microscope.

For cytometry imaging, 24 h after transfected with EGFP-N3 or 5′-mSIRT3-EGFP, H9c2 cells were harvested and stained with 100 nM MitoTracker Deep Red 633 (Invitrogen) in serum-free DMEM for 20 min at 37°C. After three washes with ice-cold PBS, the stained cells were suspended with 250 μl of PBS. The imaging was performed on an ImageStream (Amnis Corporation, Seattle, WA) utilizing 488 and 658 lasers and an Extended Depth of Field (EDF) element. Data were collected using INSPIRE 3.0, with subsequent compensation and analysis in IDEAS 3.0 software (Amnis Corporation). Dual-positive, focused single cells were used to generate a Similarity Bright Detail R3 score (the log-transformed Pearson's correlation coefficient of the localized bright spots with a radius of 3 pixels or less within the two input images), which is a measure of the degree to which two images are linearly correlated within a masked region. Colocalization of 5′-mSIRT3-EGFP to mitochondria was defined as cells having a Bright Detail Similarity R3 score of 2 or greater, and was based on comparison with the EGFP-N3 sample, which was not targeted to the mitochondria.

CELLULAR RESPIRATION ASSAY

The oxygen consumption of intact MEFs was performed by using the XF24 analyzer [Wu et al., 2007] (Seahorse Bioscience) according to the manual. Briefly, 24 h post-transfected MEFs were seeded at 40,000 cells/well in assay plate and cultured over night. The oxygen consumption was measured in non-buffered DMEM (without $NaHCO_3$) containing 25 mM glucose, 1 mM pyruvate, and 2 mM GlutaMax, pH 7.4.

STATISTICAL ANALYSIS

Differences between respirations of the different MEF groups were evaluated for significance using ANOVA. $P < 0.05$ was considered statistically significant. Data are expressed as mean ± SEM.

RESULTS

SIRT3 GENE TRANSCRIPTION SHOWS ALTERNATIVE SPLICING IN MOUSE LIVER AND KIDNEY

To confirm that mSirt3 mRNA has a longer nucleotide sequence at its 5′-end, we performed 5′-RACE on total RNA extracted from both mouse liver and kidney. We sequenced nine clones from liver and seven clones from kidney. We found that in addition to the short isoform, a single longer isoform was identified. The short isoform has its putative translation start site in exon 2 (Schematic shown in Fig. 1A). Four of the nine liver clones and two of the seven kidney clones showed an alternative splice variant that includes an eight-nucleotide deletion in exon 1b, resulting in a frame shift with an alternate 5′-start site (this sequence encodes for a longer isoform of Sirt3, Fig. 1A). The amino acid sequence translated from the 5′-race cDNA sequence suggests that the mSIRT3-L isoform has a 63 amino acid extension compared with the short isoform at its amino-terminal (schematized in Fig. 1B and compared with the human amino acid sequence). Incorporation of this 63 amino acid sequence into the mitochondrial targeting analysis program—MITOPROT assigns a possibility of 0.9488 of this sequence will facilitate import into the mitochondria.

THE 63 AMINO ACID REGION OF mSIRT3-L IS SUFFICIENT FOR MITOCHONDRIAL TARGETING

To functionally prove that this novel amino-terminus of mSIRT3-L is a legitimate MLS, we generated a chimera incorporating this cDNA fragment upstream of the reporter gene, GFP (5′-mSirt3-EGFP-N3), and employed ImageStream cytometry for GFP subcellular-localization. EGFP-N3 transfected H9c2 cells diffusely expressed GFP with <1% mitochondrial localization. In contrast, 5′-mSirt3-EGFP-N3 expression shows >80% mitochondrial localization (Fig. 1C).

THE LONG ISOFORM SHOWS GREATER MITOCHONDRIAL LOCALIZATION THAN THE SHORT mSIRT3 ISOFORM

Mitochondrial localization was compared by transfecting HEK293 cells with FLAG-tagged plasmids harboring both isoforms. Their relative subcellular localization was quantified by subcellular fractionation and subsequent immunoblot analyses. As shown in Figure 2A, mSIRT3-L exhibits increasing mitochondrial expression in a plasmid dose-dependent manner as detected by both anti-FLAG and anti-mSIRT3 antibodies. In contrast, in HEK293 cells the majority of the mSIRT3-S is cytoplasmic, with no evidence of mitochondrial enrichment even at the highest concentration of cDNA transfection (Fig. 2A). Of note, presence of mSIRT3-L in the cytoplasmic fraction is only evident at higher concentrations of plasmid transfection and is at a molecular weight of about 40 kDa, which would correlate to the unprocessed human SIRT3, taking into account the shorter murine amino-terminal. The presence of this longer construct in the cytoplasm following high concentration transfection may reflect this overexpression as opposed to the endogenous levels in the cytoplasm. In mitochondria, the majority of the mSIRT3-L shows molecular weight of 34 kDa with evidence of a shorter protein with an approximate molecular weight of 31 kDa. This higher molecular weight compared with the endogenous protein reflects that incorporation of a 3× FLAG sequence at the carboxyl terminus. This small product is similar to the cytosolic protein evident following cellular transfection with the plasmid encoding for the mSirt3-S-FLAG construct. These results suggest multiple mSIRT3-L cleavage products in the mitochondria and if the mSIRT3-S shows mitochondrial deacetylase activity would implicate that the 31 kDa fragment possesses deacetylase activity.

As prior studies suggest that the mSIRT3-S localizes to the mitochondria, we evaluated whether subcellular localization may be

tissue-specific. Transient transfection of the long and short forms into cardiac-derived H9c2 cells show a relatively greater abundance of the mSIRT-L versus mSIRT-S in the mitochondria, although both isoforms do show mitochondrial localization (Fig. 2B). It is of interest to note that both isoforms showed nuclear localization that parallels the finding of endogenous SIRT3 in the murine heart [Sundaresan et al., 2008]. Additionally, we introduced the SIRT3 constructs into SIRT3 knockout MEF cells via electroporation. Here, the mitochondrial distribution of FLAG-tagged transient transfection isoform constructs show a similar distribution to that seen in H9c2 cells with more robust mitochondrial enrichment of the long versus short isoforms (Fig. 2C).

Immunostaining analysis was then performed to visualize the different subcellular localizations of mSIRT3 isoforms using transient transfection in H9c2 cells. Most of the H9c2 cells transfected with mSirt3-L showed expression as detected by anti-FLAG antibody (green), and showed robust colocalization with COXI (red), a mitochondrial marker protein (Fig. 2D, columns D–F). In contrast, the transfection efficiency is much lower in mSIRT3-S transfected H9c2 cells. Following mSirt3-S transfection, we only observed a few populations of FLAG-positive expressing cells, and these show cytosolic expression with a lesser degree of mitochondrial enrichment (Fig. 2D, columns G–I). In parallel, immunostaining in SIRT3 knockout MEF cells similarly showed more robust mitochondrial enrichment following transfection with the mSirt3-L versus mSirt3-S constructs (data not shown).

BOTH LONG AND SHORT ISOFORM OF mSIRT3 ARE ACTIVE MITOCHONDRIAL DEACETYLASES

To compare the mitochondrial phenotype of the mSIRT3-S and mSIRT3-L isoforms, we employed MEF's from wildtype and knockout SIRT3 mice. The expression of SIRT3 is absent in the knockout MEF's (Fig. 3A). To functionally assess mitochondrial activity of these two isoforms, we evaluated total mitochondrial protein lysine acetylation. As would be expected, we see that mitochondrial protein acetylation is markedly higher in the knockout versus wildtype MEF's (Fig. 3B). Mitochondrial protein deacetylation was restored to wildtype control levels following transfection of either SIRT3 isoform (Fig. 3B). As SIRT3 enhances oxidative phosphorylation, we evaluated the isoform-specific capacity to restore oxygen consumption. Again both isoforms completely rescued SIRT3 knockout MEF respiration (Fig. 3C).

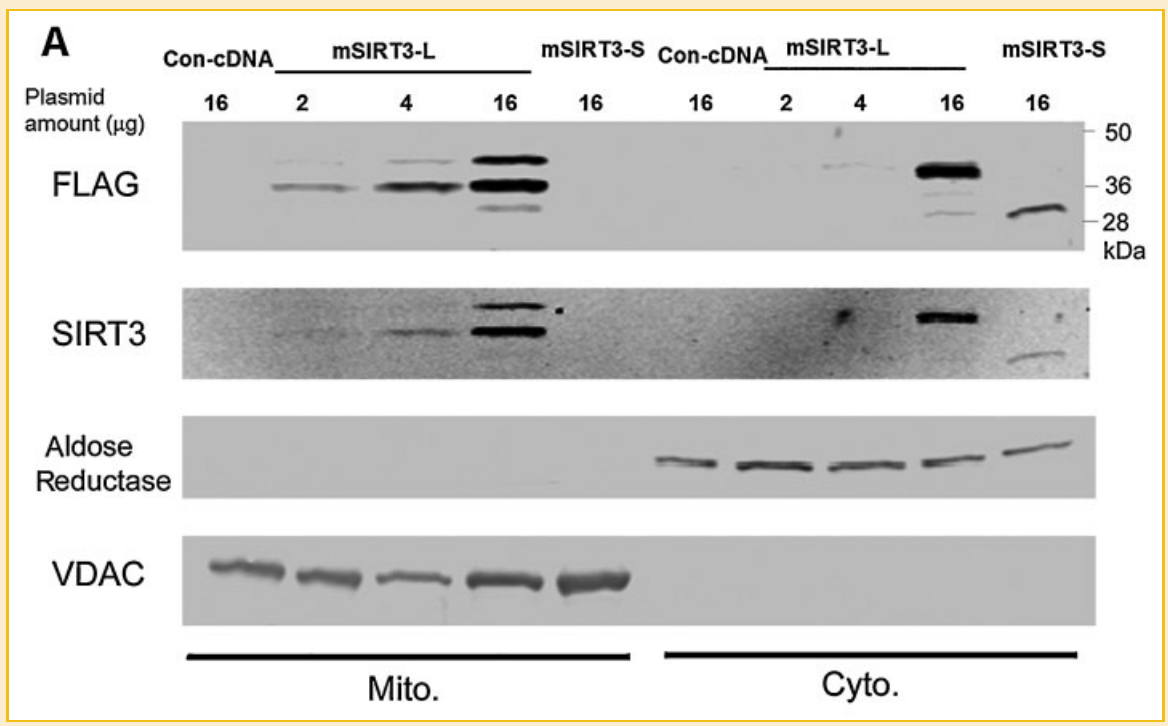

Fig. 2. Different subcellular localizations of mouse SIRT3 isoforms. A: HEK293 cells were transfected with pcDNA3.1 (Control cDNA), or pcDNA3.1-mSirt3-L at various amounts or pcDNA3.1-mSirt3-S at the highest amount for 48 h, then subjected to subcellular fractionation and immunoblot analysis. Overexpressed SIRT3 isoforms were detected with anti-FLAG and anti-mouse SIRT3 antibodies. The various putative cleavage products are confirmed by correlation between the FLAG and SIRT3 antibodies. Subcellular fractionation is characterized by antibodies directed against aldose reductase (cytoplasmic enzyme) and [voltage dependent anion channel (VDAC)—mitochondrial membrane] respectively. Mitochondrial fractions (Mito.) and cytoplasmic fractions (Cyto.). B: Subcellular localization was investigated in H9c2 cells comparing the short and long isoforms of SIRT3. Subcellular localization was confirmed with antibodies directed against aldose reductase, VDAC and against Sp1 as a nuclear target. C: Mitochondrial targeting of both isoforms is also evident in SIRT3 knockout MEF cells following electroporation-induced overexpression of FLAG-tagged expression vectors. Tubulin represents an additional cytoplasmic marker. D: Immunostaining of H9c2 cells transfected with pcDNA3.1 (A–C), pcDNA3.1-mSirt3-L (D–F), and pcDNA3.1-mSirt3-S (G–I). SIRT3 was detected by anti-FLAG antibody (green, A, D, and G), and mitochondrial localization was detected by COXI (red, B, E, and H). The colocalization of SIRT3 isoforms and mitochondria is shown in the overlay images with DAPI counterstaining of the nuclei (C, F, and I). Scale bar, 20 μm.

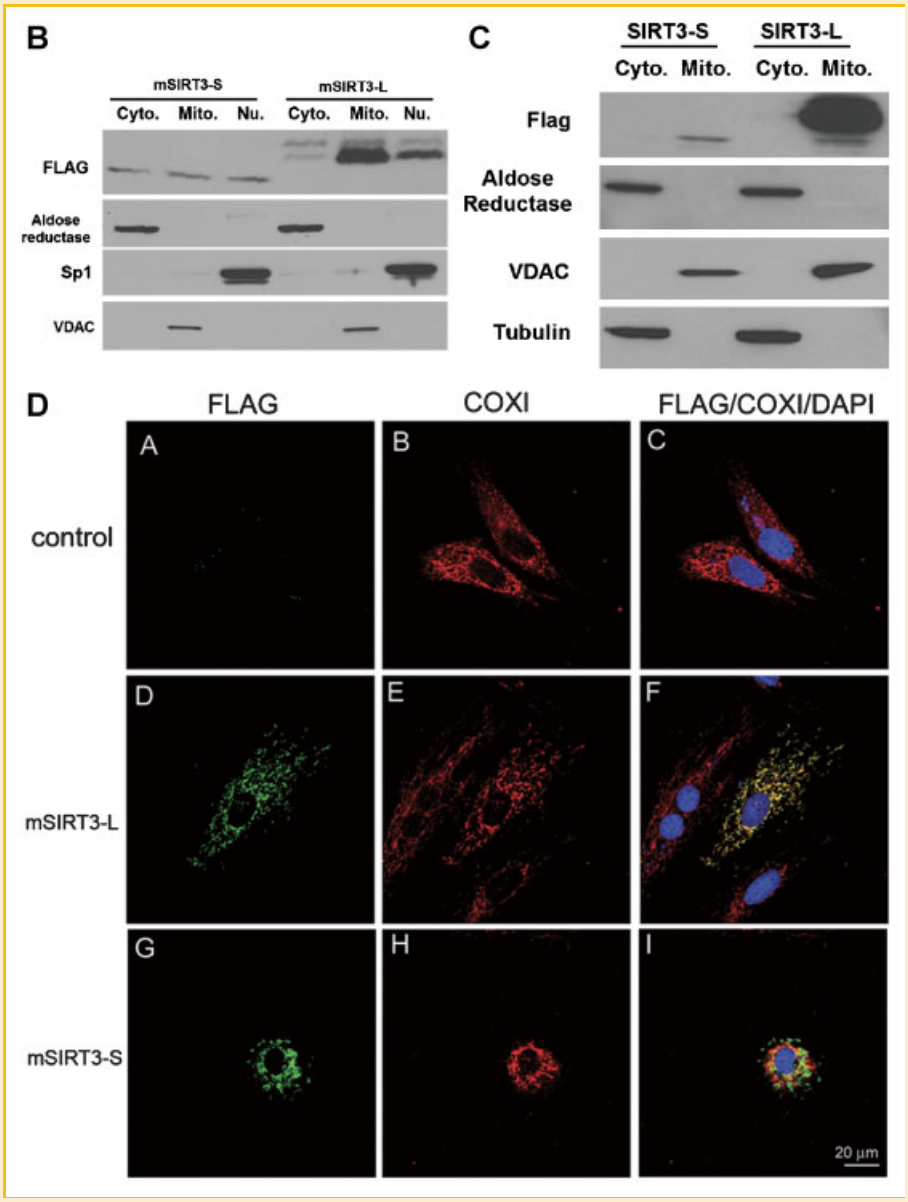

Fig. 2. (*Continued*)

THE QUESTION OF NUCLEAR LOCALIZATION OF MURINE SIRT3 ISOFORMS

In light of the discordant data concerning nuclear localization of SIRT3, it was interesting to note the suggestion of the presence of both SIRT3 isoforms in the nuclear extract in H9c2 cells shown in Figure 2B, despite no obvious nuclear localization in the immunostained cells. To further address this, we investigated nuclear localization following overexpression of the SIRT3 constructs via electroporation into the null MEF cells using the NE-PER Pierce isolation kit. Although, it appears that both SIRT3 constructs are enriched in the nucleus here (Fig. 4A), mitochondrial contamination of the nuclear fraction is evident by the concurrent expression of VDAC in the nuclear extracts (Fig. 4A). As sucrose gradient electrophoresis enables more pure nuclear extracts [Blobel and Potter, 1966], we employed this additional technique to extract nuclear extracts from MEF cells following transient transfection with the long and short SIRT3 constructs. Using purified nuclear fractions, VDAC was not identified by immunoblot analysis (Fig. 4B); however, both the Flag-tagged proteins isoforms were shown in parallel with overexpression of the nuclear transcription factor Sp1. Sucrose gradient electrophoresis to separate the nuclear fraction similarly showed the FLAG-tagged SIRT3 isoforms in the nuclear extract of H9c2 cells (data not shown). We then explored the endogenous protein levels of SIRT3. We were unable to detect endogenous levels in the rat H9c2 cells using our antibody designed to recognize the murine SIRT3 carboxyl terminus. However, using

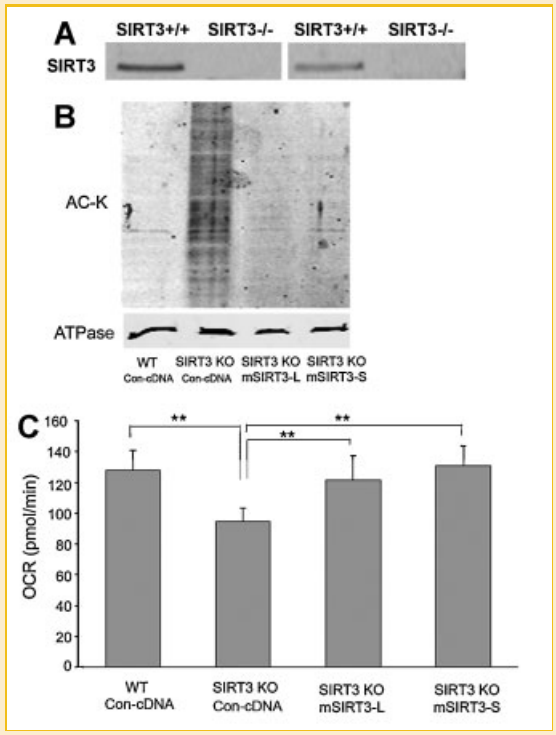

Fig. 3. Comparison of subcellular localization, mitochondrial deacetylase capacity and respiratory function of murine short and long SIRT3 isoforms. A: Western blot analysis showing the steady-state protein levels of SIRT3 in wildtype and SIRT3 -/ - MEF's. B: Wildtype and SIRT3 KO MEFs transfected with control empty pcDNA vectors (Con-cDNA) or the SIRT3 isoforms by electroporation followed by mitochondrial extraction. Lysine acetylation examined with anti-acetylated lysine antibody (Ac-K). F_1F_o-ATP synthase used as a loading control. C: Basal oxygen consumption rates (OCR) of intact wildtype and SIRT3 KO MEFs transfected with each isoform or control cDNA were measured by Seahorse XF24 analyzer. Each data represent mean ± SD from five wells, repeated thrice. $^{**}P < 0.01$.

sucrose gradient subcellular extraction we demonstrate exclusive expression of endogenous SIRT3 in wildtype MEF mitochondria and no nuclear expression (Fig. 4C).

DISCUSSION

The murine SIRT3 sequence was identified almost a decade ago, and although two isoforms were initially isolated, the longer form was thought unlikely to be functional because of its distinct amino-terminus sequence compared with the human protein and because of the inability to amplify this isoform by reverse transcription PCR [Yang et al., 2000]. However, uncertainty as to whether the short isoform is the "complete" SIRT3 arose as studies demonstrated that its subcellular localization did not mirror that of the human SIRT3 and furthermore that a putative MLS was not evident in the short murine SIRT3 amino-terminal [Hallows et al., 2008; Nakamura et al., 2008; Cooper et al., 2009]. Two recent studies now show that one or two longer murine Sirt3 cDNAs exist and furthermore question the deacetylase activity of the initial shorter SIRT3 product [Cooper

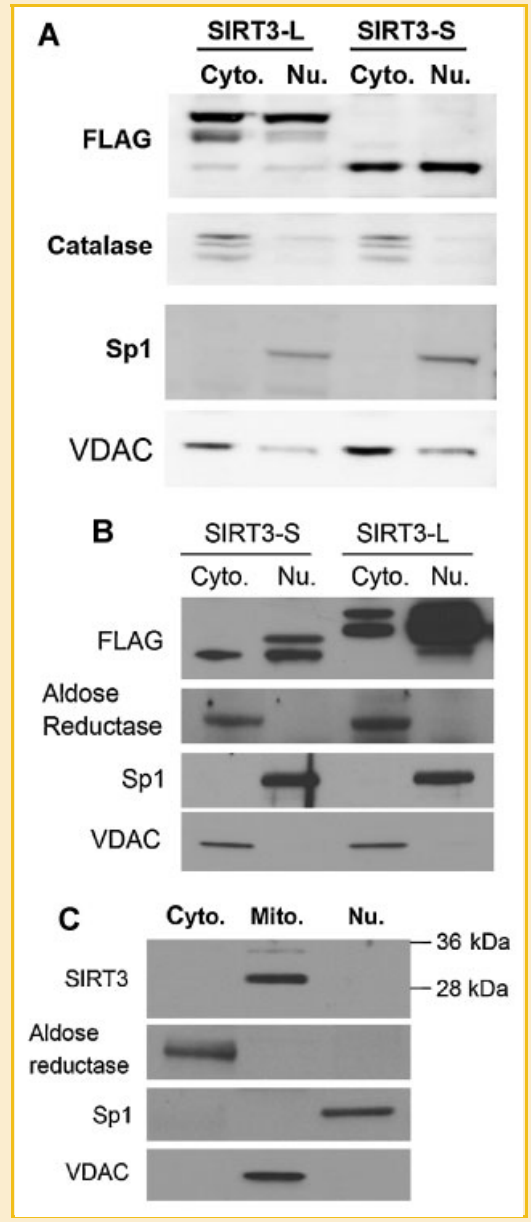

Fig. 4. Distinct nuclear localization of SIRT3 isoform overexpression compared with endogenous expression in MEF cells. A: Crude nuclear extraction from SIRT3 knockout MEF cells with overexpression of both constructs by electroporation, using the Pierce commercial nuclear isolation kit. Nuclear localization is suggested by the expression of the FLAG constructs of both isoforms. However, mitochondrial contamination of the nuclear extract is shown by the expression of VDAC in the nuclear extracts. Sp1 confirms nuclear contents in the nuclear fraction and catalase, representing the non-nuclear fraction. B: Following the same, electroporation regimen described in this figure. A: the nuclear fraction was separated by sucrose gradient centrifugation. Here, VDAC is not present in the nuclear fraction although the FLAG-tagged constructs are still evident in the nuclear fraction. The upper FLAG band noted in the nuclear extracts following overexpression of mSIR3-S is routinely seen following sucrose gradient nuclear separation. The significance of this band is currently unexplained. C: Sucrose gradient centrifugation to identify the subcellular localization of endogenous SIRT3 extracted from wildtype MEFs. Aldose reductase is a marker of the cytoplasmic fraction, VDAC—the mitochondrial fraction and Sp1—the nuclear fraction.

et al., 2009; Jin et al., 2009]. In this study, we confirm the existence of a long and short isoform of SIRT3 and isolate and demonstrate that the long isoform amino-terminus is indeed a functional MLS. Additionally, these data show that the capacity for mitochondrial localization of the short isoform, appears to be cell-type specific. In SIRT3 knockout mouse embryonic fibroblasts, where SIRT3-S does exhibit mitochondrial localization, we show that both the long and short isoforms are functionally active and similarly promote mitochondrial protein deacetylation and, similarly rescue the depressed respiratory phenotype of the SIRT3 knockout MEFs. Furthermore, in H9c2 and MEF cells, the overexpression of FLAG-tagged SIRT3 constructs do show expression in sucrose gradient purified nuclear protein extracts. However, the endogenous SIRT3 protein is only evident in mitochondria in MEF cells and not in the nuclear compartment.

The long isoform of murine SIRT3 is transcribed via internal splicing of eight nucleotides within exon 1b, with a concomitant frame shift and the resultant creation of an alternate translational start codon. Although, internal exon splicing events with the generation of alternate mRNA variants is an uncommon post-transcriptional regulatory mechanism, this program has been previously described in the translation of multiple isoforms from a single gene [Lokeshwar et al., 2002]. The putative regulatory control of and relative abundance of the long and short isoforms of the mSIRT3 is unknown and whether this is orchestrated to modulate mitochondrial versus non-mitochondrial localization and deacetylase activity would be an intriguing regulatory control point that requires investigation. Although we did not identify the additional longer isoform found by other investigators [Cooper et al., 2009; Jin et al., 2009], it is of interest to note that the long isoform we identified aligns to the full length sequences identified in both rat and rabbit [Jin et al., 2009].

Mitochondrial proteins are usually synthesized as preproteins in the cytosol and usually include an amino-terminus mitochondrial targeting/localization signal. This amino-terminus extension is termed the presequence and usually ranges from 10 to 80 amino acid residues [Pfanner, 2000]. In contrast to nuclear localization sequences, the mitochondrial localization presequence is not readily identified by amino-acid homology but rather by distinct physicochemical properties including charge, hydrophobocity and the potential to form amphiphilic α helixes [Roise and Schatz, 1988; von Heijne et al., 1989]. In light of these properties, computer modeling has been employed to predict mitochondrial localization sequences. A modeling system created by Claros and Vincens [1996] employing 47 parameters has been interrogated and validated using known mitochondrial protein sequences from the SwissProt database. Using this modeling system to interrogate confirmed mitochondrial proteins between 75% and 97% of mitochondrial protein, presequences were identified and this specificity was confirmed in proteins with characterized mitochondrial localization sequences [Claros and Vincens, 1996]. Additional databases to identify MLS sequences have been derived and numerous of these assign a strong probability that the amino-terminal of the long isoform of SIRT3 is a MLS [Jin et al., 2009]. In this study, we functionally validate the mitochondrial localization potential of this sequence by showing that its incorporation upstream of a GFP-reporter vector localizes in excess of 80% with the mitochondrial potentiometric fluorescent agent mitotracker red.

As the long isoform of murine SIRT3 has only recently been confirmed, the characterization of the role of short isoform of murine SIRT3 has been more actively pursued. Overexpression of the short isoform of murine SIRT3 in H1B1B adipocyte-derived cells is associated with upregulation of gene transcripts encoding for the mitochondrial biogenesis master regulator PGC-1α and for mitochondrial proteins including UCP-1. These changes are associated with the phosphorylation and putative activation of the CREB transcription factor known to upregulate PGC-1α [Shi et al., 2005]. Interestingly, these regulatory changes were associated with increased basal, induced and uncoupled mitochondrial respiration and implicated a role of SIRT3 in modulating thermogenesis [Shi et al., 2005]. With respect to the extra-mitochondrial functioning of the short SIRT3, deacetylase and ADP-ribosyltransferase mutant constructs have been shown to abolish the PGC-1α and UCP1 gene transcript inducing effects and to inhibit the phosphorylation of CREB [Shi et al., 2005]. Taken together these data suggest that if the short SIRT3 is functional in-vivo that its regulatory effects may operate both within mitochondria and the nucleus [Shi et al., 2005]. The complexity of the regulatory role of this short isoform is illustrated in that its role in thermogenesis is questioned by the lack of effect on adaptive thermogenesis to cold exposure comparing the SIRT3 knockout and wildtype control mice [Lombard et al., 2007]. However, a regulatory role of the short SIRT3 isoform has additionally been shown by resistance to angiotensin II induced cardiac hypertrophy in transgenic mice overexpressing mSIRT3-S in the heart [Sundaresan et al., 2009]. Another study that employed overexpression shows partitioning of the short form of SIRT3 between the nuclear and mitochondrial compartments where SIRT5 is implicated in modulating this subcellular localization [Nakamura et al., 2008]. The potential for modulation of the subcellular localization of SIRT3 isoforms, especially in the context of redox stress, has also been shown in the investigation of human SIRT3 [Scher et al., 2007]. Although, the controversy of mitochondrial localization of the mSIRT-S is not fully resolved, our data show that localization of exogenous mSIRT3-S in the mitochondria may be tissue-specific comparing the expression in HEK293 to H9c2 and MEF cells. Furthermore, and importantly from a functional perspective, we show that low molar concentration transient transfection of mSirt3-S into SIRT3 knockout MEF cells supports that this isoform modulates mitochondrial deacetylase and mitochondrial respiratory activity.

The exclusive mitochondrial localization of the SIRT3 is similarly not completely resolved [Cooper and Spelbrink, 2008; Scher et al., 2007] in that nuclear identification of endogenous human SIRT3 by immuno-localization has been shown using an antibody directed against the amino-terminal of this protein [Scher et al., 2007]. Moreover, electron microscopic immunostaining shows that endogenous SIRT3 is present in the murine heart nucleus [Sundaresan et al., 2008] but not in the mouse liver [Lombard et al., 2007]. Furthermore, the overexpression of the full-length human SIRT3 in primary neonatal cardiomyocytes does show transcriptional activation of nuclear genes via the deacetylation and activation of Foxo3a [Sundaresan et al., 2009]. Our data show that

overexpression of both isoforms of SIRT3 are evident in nuclear fractions of H9c2 and MEF cells by western blot analysis, although not evident by indirect immunostaining of individual cells. This discrepancy probably reflects low nuclear expression that becomes evident following the concentration of multitudes of nuclei during lysis and subcellular separation procedures employed for immunoblot analysis. However, the endogenous protein is confined to the mitochondrial and not nuclear fraction. Although, the full functional characterization of both SIRT3 isoforms remains incomplete, the data are now convincing that the mouse does have at least one functional longer SIRT3 protein with a legitimate mitochondrial localization sequence. Another emerging concept that overexpression of long and short forms of SIRT3 control extra-mitochondrial regulatory programs. These data raise interesting questions as to whether potential distinct roles of these lysine-deacetylases in different subcellular locations are operational, or whether the overexpression constructs alternatively evoke biological effects from artifactual localization of robustly overexpressed protein.

In conclusion, this study demonstrates that: (i) both SIRT3 isoforms are transcribed; (ii) the mSIRT3-L is generated by intra-exon splicing; (iii) the amino-terminus of the mSIRT3-L is a legitimate mitochondrial localization sequence; (iv) that both isoforms function as mitochondrial deacetylases in-situ and the activity and the subcellular location of the short-isoform may be cell-type specific; and (v) finally that although the over-expression of both constructs do show nuclear localization, the expression of the endogenous murine SIRT3 is exclusively restricted to the mitochondrial in mouse embryonic fibroblasts. Whether the regulation and function of these alternatively spliced isoforms orchestrate distinct subcellular and biological functioning requires further investigation.

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